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Contents

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Contents

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Volume 13 **Number** 4 **January** 28, 2007

Contents

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REVIEW

Physiological and clinical significance of enterochromaffin-like cell activation in the regulation of gastric acid secretion

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Abstract

Gastric acid plays an important role in digesting food (especially protein), iron absorption, and destroying swallowed micro-organisms. H+ is secreted by the oxyntic parietal cells and its secretion is regulated by endocrine, neurocrine and paracrine mechanisms. Gastrin released from the antral G cell is the principal physiological stimulus of gastric acid secretion. Activation of the enterochromaffin-like (ECL) cell is accepted as the main source of histamine participating in the regulation of acid secretion and is functionally and trophically controlled by gastrin, which is mediated by gastrin/CCK-2 receptors expressed on the ECL cell. However, long-term hypergastrinemia will induce ECL cell hyperplasia and probably carcinoids. Clinically, potent inhibitors of acid secretion have been prescribed widely to patients with acid-related disorders. Long-term potent acid inhibition evokes a marked increase in plasma gastrin levels, leading to enlargement of oxyntic mucosa with ECL cell hyperplasia. Accordingly, the induction of ECL cell hyperplasia and carcinoids remains a topic of considerable concern, especially in long-term use. In addition, the activation of ECL cells also induces another clinical concern, i.e., rebound acid hypersecretion after acid inhibition. Recent experimental and clinical findings indicate that the activation of ECL cells plays a critical role both physiologically and clinically in the regulation of gastric acid secretion.

Key words: Enterochromaffin-like cell; Gastrin; Gastric acid; Gastric carcinoid; Rebound acid hypersecretion

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INTRODUCTION

One of the main functions of the stomach is to produce hydrochloric acid, which plays an important role in protein digestion, iron absorption and particularly in destroying swallowed micro-organisms[1,2]. The stomach is rich in neuroendocrine cells[3-5]. At present, at least six endocrine cells have been described in the stomach: G cells, D cells, enterochromaffin-like (ECL) cells, A-like cells, D1/P cells, and enterochromaffin (EC) cells. In the stomach, G cells are found only in the antral mucosa, while A-like and ECL cells are confined to the oxyntic mucosa[4]. D and D1/P cells are found in both the antral and oxyntic mucosa. These endocrine cells constitute approximately 2% of the oxyntic mucosal cells in rodents. The ECL cell was originally described by Hakanson *et al*[6] and Capella *et al*[7] respectively. However, its physiological function was also long disputed, except for in rat where it was initially recognised as the major histamine producing cell of the stomach[8]. It is now recognized that the ECL cell is the dominant endocrine cell in the oxyntic mucosa of all mammals studied so far. Localization within the glands differs from one species to another. In rodents, they are mainly located in the basal third of the oxyntic mucosa. Gastric acid is produced by the parietal cell in the oxyntic mucosa[9], and the production of acid is regulated by neurons, hormones and paracrine substances[10,11].

Gastrin released from the antral G cells, histamine from the oxyntic ECL cells and acetylcholine (Ach) from postganglionic cholinergic neurons are the main stimuli of acid secretion[9]. The ECL cell is under the control of gastrin. Gastrin-ECL cell axis activation has been found to be important physiologically and pathophysiologically. In this mini-review, we will summarize the physiological and clinical significance of ECL cell activation in regulating gastric acid secretion.

THE PHYSIOLOGICAL SIGNIFICANCE OF ECL CELL ACTIVATION IN REGULATING GASTRIC ACID SECRETION

Gastrin is a potent stimulus of gastric acid secretion by stimulating the release of histamine from ECL cells[12-16]. The gastrin-ECL cell axis plays a critical role in regulating acid secretion from parietal cells. In the totally isolated vascularly perfused rat stomach model, gastrin induces an immediate and concentration-dependent histamine release from the ECL cell[12]. With concomitant administration of the histamine-2 (H2) receptor antagonist, ranitidine, together with gastrin, the acid secretion in the isolated stomach model is reduced to baseline level. Thus, the stimulation of acid secretion by gastrin occurs most likely *via* histamine release from the ECL cells *via* gastrin/CCK-2 receptors[17-20]. This finding was supported by studies using isolated ECL cells *in vivo*[21-23]. Not only histamine release but also the synthesis of histamine in the ECL cell is regulated by gastrin[24-26]. Administration of exogenous gastrin, at a dose giving concentration in the physiological range, can evoke a significant increase in histidine decarboxylase (HDC) activity[13], as well as an increase in HDC mRNA abundance[24-26]. HDC catalyses the formation of histamine from histidine. Endogeneous hypergastrinemia after potent acid inhibition can induce a similar increase in HDC activity[13,27]. Histamine release from ECL cells is considered to be a limiting step in gastrin-stimulated maximal gastric acid secretion[12,28]. Now it is generally accepted that the gastrin-histamine sequence is the main pathway for gastrin stimulation of gastric acid secretion. Recently, the role of gastrin precursors (glycine-extended gastrin and progastrin) in stimulating acid secretion was also postulated[29,30]. It was found that a high dose infusion of glycine-extended gastrin into isolated stomach can activate histamine release from ECL cells and acid secretion, which could be blocked by antagonists of H2 receptors and gastrin/CCK-2 receptors[31,32]. This supported the activation of ECL cells as mediating the main pathway of glycine-extended gastrin acid secretion stimulation. Moreover, the role of glycine-extended gastrin in preserving parietal cell density was found. Coexpression of glycine-extended gastrin with gastrin in transgenic mice reduced long-term hypergastrinemia induced parietal cell loss. Thus, an important physiological role of gastrin precursors was postulated[30].

Furthermore, the stomach is innervated by different nerves[33] and peptides produced by intrinsic neurons influence stomach functions, including acid secretion[34]. The vagal efferent fibers are preganglionic, and do not directly innervate stomach endocrine or exocrine cells[33]. The targets of these vagal preganglionic neurons are the intrinsic neurons that are located in the myenteric ganglion cells. The intrinsic neurons contain Ach and different peptides[33], such as GRP, VIP, galanin, and PACAP. They innervate the G, D, ECL and parietal cells. The effect of vagal nerves on gastric acid secretion is complex. Ach mainly has a direct effect on acid secretion by acting on a M3 receptor on the parietal cell. *In vivo*, galanin and PYY, for example, have been shown to inhibit histamine release from ECL cells *via* their own receptors[35-39]. VIP induces somatostatin release from D cells, but stimulates histamine release from ECL cells probably *via* a PACAP receptor[35,36,39,40]. PACAP is a potent stimulus of histamine release from ECL cells *via* PACAP-1 receptors[36,39,41,42]. Gastric acid secretion, besides being regulated by the hormonal and neural routes, is also regulated by paracrine factors[11]. Somatostatin, which is a principal paracrine inhibitory factor, can exert its inhibitory effect on gastric acid secretion[17]. The reciprocal paracrine pathway between D and G cells is well known[43], and ECL cells are also in close contact with oxyntic D cells[4]. The antral somatostatin acts on the antral G cells, while the oxyntic somatostatin affects both ECL cells and parietal cells. Thus, somatostatin inhibits acid secretion *via* actions on different cells of the gastrin-ECL cell axis.

THE CLINICAL SIGNIFICANCE OF ECL CELL ACTIVATION IN LONG-TERM GASTRIC ACID INHIBITION

Potent acid inhibitors, such as proton pump inhibitors (PPIs), are highly effective gastric antisecretory agents with long duration[44]. They are intensively used to treat acid related disorders, and are nowadays prescribed even for children[45]. ECL cells are activated during the use of potent acid inhibitors. From a clinical viewpoint, safety concerns for such long-term activation by acid inhibitors have to be considered.

Rebound acid hypersecretion was first described in rats more than 20 years ago after treatment with omeprazole[46]. In humans, rebound acid hypersecretion was found in patients who received long-term acid inhibitors, such as H2 receptor antagonists and PPIs[47-51]. It has been observed that a 3-mo omeprazole treatment, at a dose of 40 mg daily in patients with reflux esophagitis, resulted in a significant (over 50%) increased maximal acid secretion accompanied by remarkable elevated gastrin and histamine levels[48]. This finding was confirmed in our subsequent studies[52,53] and others[50,51], and is due to the fact that gastrin is the most important trophic factor for ECL cell self-replication and that histamine released from ECLs cell is the main stimulator for gastric acid secretion. Long-term acid inhibition induces hypergastrinemia and ECL cell hyperplasia in patients treated with PPIs for various diseases with dyspepsia. The mechanism of rebound acid hypersecretion is likely related to the activation of the gastrin-ECL cell axis caused by drug-induced hypoacidity.

Apart from a stimulatory action on gastric acid secretion, gastrin also has a trophic effect on the oxyntic mucosa[54-56], particularly on ECL cells, which are stimulated to replicate *via* gastrin/CCK-2 receptors expressed in ECL cells[13,19,20]. It has become apparent that rat ECL cells, in response to hypergastrinemia, whether endogenous or exogenous, show hypertrophy within days, hyperplasia within weeks and carcinoids after months through a sequence of diffuse-linear-micronodular hyperplasia to ECL carcinoids[13]. Therefore, there is a causal connection between hypergastrinemia and ECL cell carcinogenesis[13,57-59]. Thus, in patients received long-term

acid inhibiton treatment, another concern is the increased gastric carcinoid risk. In fact, sporadic gastric carcinoid cases have been reported in patients exposed to long-term PPI treatments[60,61]. Long-term safety is still of high concern.

Finally, gastrin was also connected with other types of human cancers; i.e. gastric and colonic adenocarcinoma, and more recently, studying the important role of precursors for gastrin progastrin and glycine-extended gastrin in the carcinogeneiss of gastrointestinal mucosa has been one of the developing research fields. Several outstanding reviews have summarised this topic[62-65]. Thus, whether long-term activation of ECL cells by potent acid inhibition can contribute to increased risk of gastrointestinal adenocarcinoma in humans is still unknown and needs to be studied in the future. In addition, one of the growth factors that regenerates gene proteins, i.e., (Reg)-1, that is mainly released from ECL cells has been found to be a unique growth factor of gastric mucosal cells[66] and may play an important trophic role in the development of gastric cancer[67].

CONCLUSION

It is now generally accepted that ECL cell activation is the most important physiological pathway in the regulation of gastric acid secretion, which is being influenced by both activating and inhibiting stimuli. Furthermore, it has become apparent that the gastrin-ECL cell axis also plays a role in gastric acid disorder, such as rebound acid hypersecretion, and increased risk for gastric tumorigenesis, especially in chronic hypergastrinemic conditions. Therefore, clinicians should be aware that there are important clinical safety issues related to the dose and duration of potent inhibitors of acid secretion.

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REVIEW

Impact of tiny miRNAs on cancers

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Abstract

miRNAs are a class of small, ~22nt, non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. They play profound and pervasive roles in manipulating gene expression involved in cell development, proliferation and apoptosis in various eukaryotes, which, in theory, could provide an access to many human diseases in theory. Recent evidence demonstrates that aberrant miRNA expression is a hallmark of tumor development, revealing that miRNA genes could function as potential oncogenes and repressors in the human body. miRNAs can affect tumorigenesis mainly by interrupting the cell cycle at the cellular level and by interacting with signaling, oncogenes and with the response to environmental factors at the molecular level. The established miRNA expression signature could be a potent tool to diagnose and treat human cancers in the future.

Key words: miRNAs; Cancers; Oncogenes and repressors; Tumorigenesis; miRNA expression signature

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INTRODUCTION

miRNAs (microRNAs) are a subset of small, typically 21-23 nt in length, non-coding RNAs evolutionarily conserved in many organisms as disparate as yeast, fruit flies, human and plants[1]. This growing family of small RNAs was first discovered in *Caenorhabditis elegans* in 1993[2] and newly honored as a milestone in the process of the gene concept[3]. Until now hundreds of miRNAs have been identified in many organisms by using experimental and bioinformatic prediction approaches. It is well known that, unlike its cousin signal interfering RNAs (siRNAs), miRNAs have the unique ability to negatively regulate gene expression involved in cell development, proliferation, apoptosis and the stress response[4]. Consequently, it is proposed that these biological properties of miRNAs could offer an access to many human diseases including cancers[5]. Recent findings have demonstrated that miRNAs play critical roles in human cancer, revealing that miRNAs could act as potential oncogenes and repressors[6,7]. Thus, this class of miRNAs are now dubbed 'oncomirs'-miRNAs which is closely related to tumor[8].

Cancer is characterized by uncontrolled proliferation and the inappropriate survival of damaged cells. Although cells have evolutionarily developed several safeguards to prevent malignant transformation during development and adulthood, this normal process can be disrupted in cancer cells. Cancer cells can take advantage of their unique strategy to escape scrutiny during cell division. The oncogenesis conventionally refers to tumor suppressors and oncogenes, such as APC, κ-RAS, Myc, P53 and P21. Although these regulatory molecules do play critical roles in tumor development, recent intense interest is being attached to miRNAs.

We are just beginning to appreciate the novel involvement of miRNAs in human cancers but much more remain obscure. Few investigations to date have converged to support the concrete links between miRNAs and each cancer species. However, most cancer species share the same mechanisms that give rise to tumorigenesis even in different tissues, so the general mechanism may be applied extensively to each cancer species. The potential link between miRNAs and tumors discussed in this review will be limited to only a few of the elucidated cancers.

miRNA BIOGENESIS AND MECHANISMS OF GENE EXPRESSION CONTROL

The biogenesis of miRNAs has recently been elucidated (Figure 1)[1]. RNA Pol Ⅱ generally transcribes miRNA genes in the nucleus and gives rise to large primary miRNA (pri-miRNA) transcripts that, like mRNA, are capped at 5' terminus and polyadenylated at 3' terminus. The initial pri-miRNAs are then processed by RNase Ⅲ,

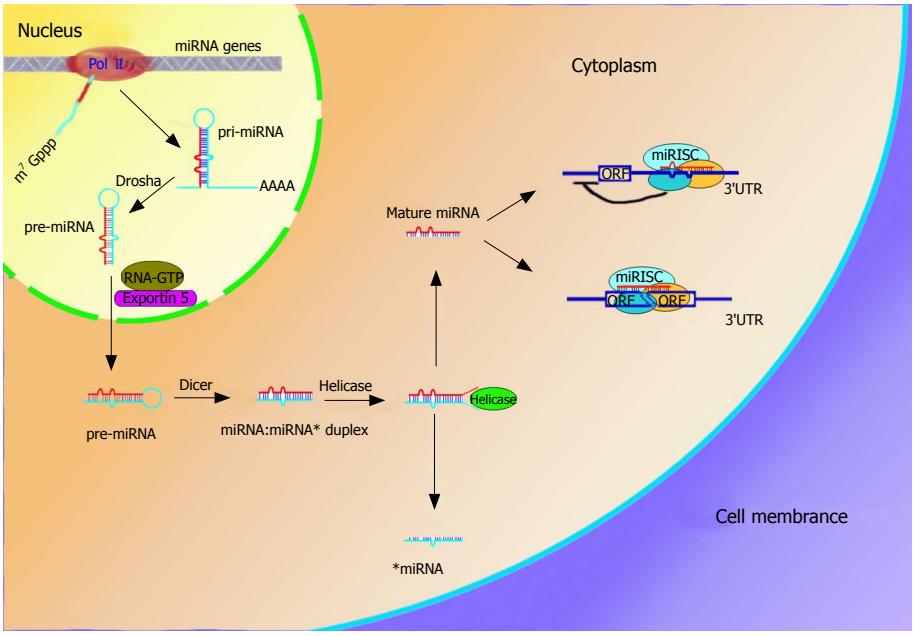

Figure 1 miRNAs biogenesis and two mechanisms involved in gene expression. MiRNAs genes often cluster on the chromosome and are transcribed by RNA Pol Ⅱ to form pri-miRNAs in the nucleus. The pri-miRNAs then undergo procession by RNase Ⅲ, Drasha, and are exported to cytoplasm by Exportin 5. Another RNase Ⅲ, Dicer, further processes the pre-miRNA to generate a ~22nt miRNA:miRNA* duplex, where miRNA* is complementary to miRNA. Helicase can divide the duplex into two separate ones. Whereas miRNA* is degraded, mature miRNA can enter the the miRNA-induced silence complex (miRISC). The miRISC complex block protein synthesis by imperfectly binding to the 3'UTR of the mRNA (upper right), the other one is to endonucleolytically cleave the target mRNA by perfect or nearly perfect base pairing (lower right).

Drasha, to form 70-bp pre-miRNAs in the nucleus. The pre-miRNAs are exported into the cytoplasm by the RNA GTP-dependent transporter Exportin 5 and undergo an additional processing step to produce a miRNA:miRNA* duplex with the aid of another RNase Ⅲ, Dicer. The RNA duplex is subsequently unwound by Helicase and the mature miRNA finally enter the RNA-induced silence complex (RISC)[9,10].

Two mechanisms of miRNA repression have been suggested depending on the degree of complementarities between the miRNAs and the target mRNAs (Figure 1)[4]. First, is interference with protein synthesis by binding to imperfect complementary sites within the 3' untranslated region of the target mRNA. The 3'UTR of target mRNA usually contains multiple complementary sites for distinct miRNAs, but the precise mechanism is still poorly understood. Second, is endonucleolytic cleavage of the target mRNA by perfect base pairing. The latter was previously thought to function solely in flowering plants, but the paradigm that animal miRNAs do not affect th stability of imperfectly base-paired miRNA has been challenged recently. Lim *et al*[11] used microarray analysis to investigate changes in global mRNA level in HeLa cells in response to miRNAs that are normally undetectable in those malignant cells and observed the reduction of 100-200 genes at the mRNA level. This observed reduction in mRNA level was attributed to AU-rich elements (AREs) that induced mRNA turnover by degrading mRNAs in exosome[12]. AREs are often found in transcripts that encode cell proliferation factors (e.g., TNF-α, GM-CSF, c-Fos, IL-6 and IL-8), and therefore stability of these transcripts in the absence of specific miRNAs contributes to the cell proliferation that accelerate tumor formation and development[13]. Nevertheless, one recent finding indicated that the reverse step also exists in the latter mechanism, where the mRNA can be relieved from the miRNA-induced inhibition in cells subject to different stress conditions[14]. This repression and derepression of mRNA expression cooperatively contribute to the dynamic balance of mRNA in cells, but the detailed mechanism is still not clear.

miRNAs could provide a convenient and efficient pathway to manipulate gene expression at posttranscriptional level. Natural miRNAs exert their effects by base pairing with the target mRNAs in a much more compact and energy-efficient manner than protein encoding regulatory molecules like enzymes and hormones, which show a necessary adaptation to regulate gene expression in eukaryotes[15].

miRNA GENE CLUSTERS AND LOCI

The human genome contains up to 1000 miRNA genes, which constitute approximately 1-5% of the expressed genes[16]. miRNAs are endogenetically conserved with evolutionary plasticity in eukaryotic genomes and are often organized in tandem and closely clustered on the chromosome[17]. This arrangement can have particular significance in the control of gene expression. When clustered miRNAs have a similar sequence, miRNAs gene products may synchronize to regulate a set of mRNA targets. However, clusters can also contain miRNAs with different sequences that extensively deploy toward their specific targets. These closely related characteristics may allow miRNAs to function as pleiotropic regulators at the cellular level in many organisms.

Over half of miRNA genes (52.5%) are located in or near fragile sites or cancer-associated genomic regions[18]. These sites are preferential sites of sister chromatin exchange, translocation, deletion, amplification or integration of plasmid DNA and tumor-associated virus, which frequently cause the aberrant miRNA expression during pathogenesis. For instance, *miR-15a* and *miR-16a* genes, frequently deleted and/or underexpressed in patients with B cell chronic lymphocytic leukemia, map to 13q14 that is deleted in many cases[19]. This result highlights that aberrant miRNA expression is possibly geared towards the intrinsic defect that gives rise to tumors.

miRNA IN CANCER STEM CELLS

Most tissues contain rare cells that follow the norm of stem cell biology to tissue self-renewal and repair[20]. The unprecedented self-renewal rate of robust tissues (like intestine, skin, blood and breast) often parallels a high susceptibility to malignant transformation, because the molecular mechanisms that control homeostatic self-renewal and those underlie tumors are evidently symmetric[21]. Currently the emerging notion is that tumor might contain stem cell-like 'cancer stem cells'-rare cells with indefinite proliferative potential that trigger tumor formation and growth, and with the presumed ability to transport new tumor seeds to distant sites[22]. Despite this controversial notion, one cancer (leukaemias) of the haematopoietic system provides the strong evidence that cancer cell proliferation is driven by cancer stem cells[23]. Given that cancer cells and normal stem cells share the similar potential to indefinite self-renew, it seems reasonable to propose that newly arising cancer cells appropriate the machinery for self-renewing cell division which is normally used in stem cells[24].

It is well known that miRNAs function as critical regulators of gene expression in the control of stem cells during development[5]. If miRNAs play a similar role in cancer stem cells then, in theory, it is possible support the hypothesis that several miRNAs appropriate the miRNA-mediated machinery for self-renewal in stem cells to develop tumors. Indeed, it has been validated that tumor tissues are constantly characterized with altered miRNA expressions.

A HALLMARK OF TUMOR

Currently the potential connection between miRNAs and cancers is just beginning to be appreciated. Cancer cells tend to undergo the distinct expression of miRNA, distinguishing them from the normal ones. Calin *et al*[25] first found that specific miRNA expression is abnormal in B-leukemia, suggesting that altered miRNA expression correlate with specific tumor development. In accordance with this, Michael *et al*[26] investigated possible changes at the miRNA level during tumorigenesis and showed the reduced accumulation of two specific miRNAs: miR-143 and miR-145, but consistent levels of the 70-bp precursor pre-miRNA in colorectal neoplasia as well as breast carcinoma, prostate carcinoma, chronic myelogenous leukemia and cervical carcinoma, implying that many tumors share aberrant specific miRNA expression. Recently Lu *et al*[27] found that cancer cell lines showed low miRNA expression profile when they used a new, bead-based flow cytometric miRNA expression profiling method to analyze a large-scale expression of 217 mammalian miRNA from 334 samples including multiple human cancers. Their study further demonstrated that tumors originating from tissue with a common embryonic source share the similar miRNA, but not mRNA, expression signature, and that distinct patterns of miRNA expression are consistent with the developmental history of human cancer, revealing that miRNAs could be used to classify different cancers and validate the developmental history of cancer. Volinia *et al*[7] further found that global miRNA expression signatures from solid tumors show a good separation between the different tissues, but not all miRNA expressions are underexpressed compared with their respective normal tissues. The studies support the hypothesis that the global change in miRNA expression is a hallmark of all human cancers, providing a hint that miRNAs correlate with various tumor development.

Moreover, impaired components of machinery mediating miRNA processing and miRNA-mediating gene repression give rise to tumorigenesis[28,29], demonstrating that altered specific miRNAs expression might play a causal role in the generation or/and maintenance of tumors. This description of cancers in molecular terms is likely to improve the way in which human cancers are diagnosed, classified, monitored, and (specially) treated, which will promise the emergency of the new era in cancer research in the future[30].

miRNA AND CELL CYCLE

Normal cells can tightly control cell proliferation and death by means of the cell cycle, thereby preventing malignant transformation during development and adulthood. The 3'UTR of mRNAs encoding many cell cycle-associated cytokines often contain binding sites to miRNAs, indicating that normal miRNAs are essential for cell cycle control. Hatfield *et al*[31] reported that *Drosophila melanogaster* germline stem cells subject to constitutively eliminated miRNA expression exhibited normal identity but were defective in cell cycle control, showing that miRNAs are essential in the control of cell cycle. Thus constitutive miRNAs are necessary to control cell cycle and maintain the balance of cell proliferation, differentiation and apoptosis, which play essential roles in preventing normal tissues from malignancy[32,33].

However, cancer cells are insensitive to cell division stop signals in an environment where most of the cells are quiescent. It is tempting to speculate that miRNAs could have a similar role in cancer cells and particularly rare cancer stem cells, where the disrupted miRNAs expression makes cells insensitive to environmental signals that normally stop the cell cycle. Brennecke *et al*[34] assigned a novel role to miRNA encoded by the *bantam* gene in control of cell proliferation and apoptosis during Drosophila development. *Bantam* miRNA could stimulate cell proliferation and simultaneously suppress apoptosis by manipulating the proapoptosis gene *hid* expression. It controls both cell growth and cycle progression in a coordinated manner, revealing that the putative vertebrate homologs of *bantam* miRNA genes may be oncogenes, whereas there are no homologues of *bantam* in human, other oncogenic miRNAs could play a similar role in control of cell proliferation and apoptosis. When the miRNA expression is impaired, normal tissues could have a high risk to develop tumor.

miRNA AND CANCER-ASSOCIATED SIGNALING PATHWAYS

The signaling transductions (Wnt, Notch, SHH and BMP) play essential roles in the processes of cell life at the

molecular level, but these pathways controlling cell growth and differentiation in normal cell are almost invariably changed in cancer. Consistent with the altered miRNA expression in cancer cells, it is reasonable that the two can work together to control cell fate.

Several cancer-associated signaling pathways directly regulate expression of specific miRNA genes. Yoo *et al*[35] found that LIN-12/Notch signaling pathway directly binds to one miRNA gene, *mir-61*, and promote its expression in vulval precursor cells during *C. elegans* development. Stimulated expression of *mir-61* gene subsequently represses the translation of Vav-1, the ortholog of the vav oncogene, who negatively regulates the *lin-2* gene activity. These cyclic regulations form a positive feedback loop that helps maximize *lin-12* activity and continually stimulate Notch signaling pathway (Figure 2). It has been known that Notch signaling pathway plays an important role in many cancer species. If the similar mechanisms exist in cancer cell, this positive loop could trigger and accelerate tumorigenesis.

miRNAs can influence the signaling pathway by repressing several secreted signaling proteins. RAS is a signaling protein in many significant signaling pathways and its overexpression usually results in oncogenic transformation. The 3'UTL of the human RAS genes contains multiple *let-7* complementary sites, allowing *let-7* to regulate RAS expression. Johnson *et al*[36] found that *let*-7 expression is lower in lung tumors than in the normal lung tissue, providing a possible strategy to treat lung cancer by repairing mutated *let-7* gene. It was reported that miR-143 and miR-145, lower in colorectal cancer than in normal tissues, are predicted to regulate several target mRNAs encoding components of signal transduction pathway (Raf, Rho, GTPase activating protein, G-protein γ, NF-κB and HGK)[26]. Hence, the direct and indirect interaction between miRNAs and secreted signaling protein can influence tumorigenesis.

Thus, it is supposed that miRNAs and signal as well as other regulatory molecules constitute a network where normal cells follow a rule to divide, differentiate, and die. When the regulatory network is impaired, cell cycle will be out of control, giving rise to tumor development. However, the networks of miRNAs and signals are largely elusive to date.

INTERACTION BETWEEN miRNA AND ONCOGENES

Due to mutation, many human proto-oncogenes can convert to oncogenes. These oncogenes often encode common regulatory molecules that can stimulate the tumor development in the body. For example, the proto-oncogene *C-MYC* encodes a helix-loop-helix leucine zipper transcriptional factor that regulates cell proliferation, growth and apoptosis. Recent findings revealed that c-Myc directly binds to the locus of a cluster of six miRNAs and stimulates their expression[37]. Overexpression of *miR-17-5p* and *miR-20a*, two miRNAs in this cluster, reduced the expression of E2F1 (one transcriptional factor). c-Myc and E2F1 are reciprocally induced in normal cell to form a putative positive feedback loop, like the one between *mir-16* and LIN-12. So these two miRNAs provide a potent tool to dampen this reciprocal activation and tightly regulate c-Myc-mediated cellular proliferation in normal cells. When this cluster of miRNA genes is deleted or underexpressed, the cell cycle would be out of control and have a risk of tumorigenesis.

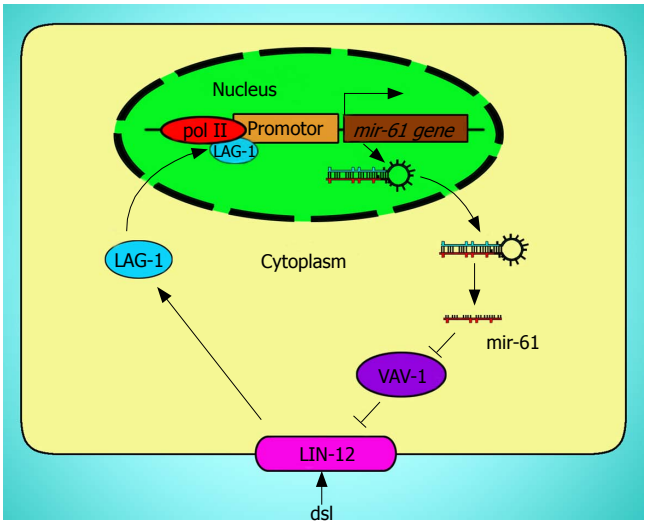

Figure 2 The positive loop between *mir-16* and LIN-12. Exogenous signal activates LIN-12/Notch signaling pathway, which promotes the transcription of *mir-61*. Overexpression of *mir-61* inhibits the activity of VAV-1 (purple) that reduces the activity of LIN-12 (pink), where LIN-12, mir-61, and VAV-1 form a feedback loop that helps maximize LIN-12 activity.

Notably, not all miRNAs function as tumor repressors in the body. For example, enforced expression of the *mir-17-92* cluster positively cooperates with c-Myc expression to accelerate tumor development in a mouse B-cell lymphoma model, implicating that the *mir-17-92* cluster as a potential oncogene[2]. The oncogenic miRNAs can be upregulated in cancer cells, which are consistent with the conclusion drawn by Volinia that not all miRNAs are underexpressed in cancer cells[7]. In fact, specific miRNA expression influencing cell fate is dependant on the milieu of miRNAs and their target mRNAs expressed in individual cell.

RULERS OF miRNA

As the pleiotropic regulators in cells, who regulate the expression of miRNA genes? As LIN-12/Notch signaling pathway directly regulates the expression of *mir-16* gene discussed above[35], it seems that the regulation of miRNA expression follows the classic model widely used to control the mRNA expression. Taganov *et al*[38] recently reported that three putative NF-κB consensus binding sites locate upstream of the predicted *miRNA-146* gene, so *miRNA-146* is a NF-κB-dependent gene. Is this general model the common one or just an exceptional one with respect to diverse miRNAs and cytokines in cells? Our understanding of this knowledge awaits further investigations.

NOT THE END OF STORY

We have discussed the potential roles of miRNAs in cancers by means of cell cycle, signaling and oncogene, but they are just the tip of emerging iceberg, because exploding data show that miRNAs have a potentially much more widely influence over diverse developmental and physiological pathways than imagined. Recent evidence revealed that miRNAs could participate in genomic stability and epigenetic modification[2,39], in metabolic changes compatible with tumor formation, growth and metastasis[40], and in the immune response to virus-mediated infection[41-45]. Many more aspects of miRNAs are available for exploration, so this is not the whole story.

More intriguingly, the other cousin of miRNAs, piwi-interacting RNAs (piRNAs), have just been discovered in rat germline cells and are also shown to control gene expression involved in sperm development at posttranscriptional level[46]. The world of three small RNAs (siRNAs, miRNAs, and piRNAs) is undoubtedly yielding newly provocative insights and revolutionizing our thinking about genome control[47]. Although transcriptional control is the most prevalent form of gene expression control, it is by no means the only way in complex eukaryotes. For example, it is important for mature blood red cells to control the stability of expressed mRNAs accounting for no extra mRNA transcription any more or is for immune cells to make a rapid response to stress without mRNAs transcription initiation. Posttranscriptional control has been ignored, but many novel small RNAs are changing our thinking.

PROSPEROUS OUTLOOK IN miRNA

Evaluating the novel roles of miRNAs as repressors and oncogenes enriches our knowledge, which addresses the precise mechanism leading to tumorigenesis. The investigation will certainly bring about a potent tool to diagnose and treat human cancers, but a detailed, mechanistic understanding of miRNAs functions as oncogenes and tumor repressors is now retarded by lacking a valid and efficient biochemical technique to precisely identify miRNAs and their corresponding targets. It is estimated that many more miRNAs are still waiting to be discovered in the human genome and functions of most known miRNAs have not been elucidated. The other challenge is to accurately identify targets that are manipulated by miRNAs, because miRNAs can bind to their imperfect targets, even with no canonical complementarities that allow short stretch of mismatched base-pairs and G-U base pair. Other outstanding questions about miRNAs remain unresolved. What regulates the expression of miRNAs? Do distinct miRNAs have a direct function in cancer progression, or just simply differentially modulate in tumor? Who determines the opposite roles of miRNAs as both oncogenes and repressors? Do miRNAs act mainly to 'fine-tune' gene expression or more often as binary on/off switch? What factors affect the accessibility and efficacy of a miRNA at a 3'UTR? Another key one is how to apply this novel technique to cancer therapy. However, more sophisticated experimental approaches, in combination with computational prediction strategies, will shed light on these challenges.

It has been shown that miRNA expression profile is a more accurate signature than protein expression one, and several patients got better prognosis after repairing the abrogated miRNAs. We enthusiastically expect that traditional and bioinformatics technique will generate a tremendous amount of excitement and inspiration about miRNAs in future.

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Chromosome 11 aneusomy in esophageal cancers and precancerous lesions- an early event in neoplastic transformation: An interphase fluorescence *in situ* hybridization study from south India

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Abstract

AIM: To detect aneusomic changes with respect to chromosome 11 copy number in esophageal precancers and cancers wherein the generation of cancer-specific phenotypes is believed to be associated with specific chromosomal aneuploidies.

METHODS: We performed fluorescence *in situ* hybridization (FISH) on esophageal tissue paraffin sections to analyze changes in chromosome 11 copy number using apotome-generated images by optical sectioning microscopy. Sections were prepared from esophageal tumor tissue, tissues showing preneoplastic changes and histologically normal tissues (control) obtained from patients referred to the clinic for endoscopic evaluation.

RESULTS: Our results demonstrated that aneusomy was seen in all the cancers and preneoplastic tissues, while none of the controls showed aneusomic cells. There was no increase in aneusomy from precancers to cancers.

CONCLUSION: Our results suggest that evaluation of chromosome 11 aneusomy in esophageal tissue using FISH with an appropriate signal capture-analysis system, can be used as an ancillary molecular marker predictive of early neoplastic changes. Future studies can be directed towards the genes on chromosome 11, which may play a role in the neoplastic transformation of esophageal precancerous lesions to cancers.

Key words: Esophageal cancer; Aneusomy; Chromosome 11; Fluorescence *in situ* hybridization; Early detection

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INTRODUCTION

More than 30% of the adult population exhibits upper gastrointestinal tract disorders associated with symptoms such as regurgitation, heartburn and dysphagia, warranting an endoscopic evaluation. Some of these esophageal pathologies require medication while others can be managed by altering life-style and dietary habits. A certain percentage of these, however, progress into esophageal malignancies. In India, esophageal cancer is the second leading cancer in men and fourth leading cancer in women[1].

Epithelial tumors of the esophagus [squamous cell carcinoma (SCC) and adenocarcinoma (ADC)] are responsible for more than 95% of all esophageal carcinomas. This malignancy presents generally as a locally advanced disease, hence leading to poor prognosis with an average 5-year survival of < 12% in India[2]. Abnormal proliferation of the esophageal epithelial cells with hyperplasia and dysplasia in the normal squamous lining are regarded as premalignant lesions[3,4]. Another common premalignant condition is Barrett's esophagus (BE), where patients have a forty-fold increased risk for developing adenocarcinoma as compared to normal individuals. Although significant advances have been made in the diagnosis and treatment of esophageal carcinomas, not many studies have evaluated markers in the target tissue, in association with increased risk for malignant

transformation. Hence, there is a need to identify the genetic and molecular factors responsible for the progression of these esophageal lesions into malignancy.

Neoplastic progression is a complex multistep process associated with gross chromosomal alterations and mutations in regulatory genes, culminating in tumorigenesis. Genomic instability is a prominent feature of most cancers, wherein aneuploidy, a change in chromosomal number caused by unequal partitioning of chromosomes during cell division, occurs frequently in many solid tumors[5,6]. Aneuploidy then generates specific aneusomies autocatalytically, due to errors in chromosome segregation and repair processes[7]. Aneusomies have been reported in non-cancerous conditions such as Down's syndrome, wherein the extra chromosome 21 is considered to be associated with an increased risk for leukemias. The study of human cancers shows evidence for cancer-specific aneusomies, despite a plethora of unspecific aneuploidies[8,9]. Recently, it has been demonstrated cytogenetically that chromosome 11 may be important in the etiology of SCC of the esophagus[10,11].

The present study was a hospital-based, unmatched, case-control study in 25 cases referred to our clinic from different hospitals in Hyderabad, South India. Chromosome 11 aneusomy was investigated in esophageal biopsies taken from controls, premalignant and malignant lesions, using fluorescence *in situ* hybridisation (FISH). Our results demonstrated that aneusomy was seen in all the cancers and preneoplastic tissues, while none of the controls showed aneusomic cells. There was no increase in aneusomy from precancers to cancers. Evaluation of chromosome 11 aneusomy in esophageal tissue can be used as an ancillary molecular marker predictive of early neoplastic changes.

MATERIALS AND METHODS

Tissue specimens

Esophageal biopsy specimens were endoscopically resected from patients referred for histopathological evaluation by a qualified gastroenterologist. The control samples were taken from those patients undergoing an endoscopy, which showed normal tissue histology. All samples were included in the study after informed consent was obtained from the patients. The study was approved by our Institutional Ethical Committee. Formalin-fixed, paraffin-embedded tissue sections from 25 selected cases were subjected to FISH analysis subsequent to confirmation by histology.

Histopathological analysis

Paraffin-embedded tissue sections (4 μm thick) were first subject to deparaffinization. Slides were placed in xylene (3 min × 3 min), 100% alcohol (3 min × 3 min), rinsed in running water and stained with haematoxylin (Harries, Merck) for 5-10 min. They were then placed in running water, dipped in 1% hydrochloric acid and subsequently transferred to Eosin yellow staining (Merck) for 30 s. After that the slides were passed through graded alcohol series for dehydration, placed in xylene and mounted in DPX (Ref: Histopathology Laboratory, Armed Forces Institute of Pathology, Washington DC, 20 305, USA). Suitable images of the required areas from representative tissue sections were taken using a CCD camera (Figure 1).

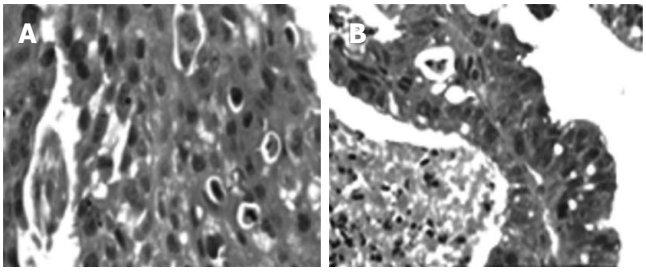

Figure 1 Representative HE stained sections at × 400 magnification selected for FISH analysis. **A**: Neoplastic streaks with eosin stained keratin pearls indicating a well-differentiated squamous cell carcinoma; **B**: Adenocarcinomatous tissue showing columnar epithelial replacement of the normal squamous lining.

Based on the endoscopic and histopathological evaluation, 25 tissue biopsies were selected for FISH analysis using a Spectrum Green labeled, centromere enumeration probe (CEP) for chromosome 11 [Vysis India Ltd] (This was used instead of the LSI probes that would help evaluate gene amplification).

FISH on esophageal tissue sections

Paraffin sections of 4 μm thick were deparaffinized in an oven at 95℃ for 20 min, then immediately placed in xylene (3 min × 3 min) and transferred into 100% ethanol (3 min × 5 min). Dried slides were incubated in 2 × SSC solution at 75℃ for 10 min followed by treatment with proteinase K solution (2 mg/mL) at 37℃ for 15 min. The slides were rinsed in 2 × SSC solution at room temperature. They were then immersed in a 75℃ denaturant bath (70% formamide/2 × SSC) for 5 min and dehydrated in gradient ethanol. Dry slides were placed on a 45-50℃ slide warmer. Probe mixture was simultaneously prepared at room temperature (7 μL of hybridization buffer + 1 μL Spectrum Green labeled CEP 11 DNA probe + 2 μL purified double distilled H_2O) and then denatured at 95℃. Ten microliters of the probe mix were applied to the slide, and a coverslip was placed on it immediately. The slides were hybridized in a pre-warmed humidified chamber overnight (12-16 h) at 37℃. Post-hybridization washes were done with freshly prepared 0.4 × SSC/0.3% NP-40 solution at 55℃ followed by 2 × SSC/0.1% NP-40 at room temperature. The slides were then air-dried in the dark. Ten microliter DAPI counterstain was applied to the target area and a coverslip was placed on it carefully to avoid formation of air bubbles.

The slides were viewed under a fluorescence microscope (Olympus, Optical sectioning microscope attached to an Axioplan imaging Apotome apparatus, Zeiss, Germany) using a suitable filter set (DAPI Exc: 367nm; Emi: 452 nm and Spectrum Green Exc: 509 nm; Emi: 538 nm). The optical sectioning microscope provided a high-quality image enhancement required for proper signal visualization. The sections were visualized through Optical sectioning mode using the Apotome for the best probe signals in a 3D mode. Ten to fifteen areas per slide were taken for analysis based on the density of the nuclei. Each area allowed optical sectioning of about

Table 1 Details of cases analyzed for chromosome 11 aneusomy using FISH in different esophageal pathologies and controls

Case, No.	Case type	Details	Chromosome 11 % aneusomy		
			Trisomy (%)	Tetrasomy (%)	Pentasomy (%)
1	Controls	Normal	-	-	-
2		Normal	-	-	-
3		Normal	-	-	-
4		Normal	-	-	-
5		Normal	-	-	-
6	Precancers	Sq. dysplasia (High grade)	4.0	-	-
7		Sq. dysplasia (High grade)	10.0	0.4	-
8		Barrett's dysplasia (High grade)	15.2	2.4	0.8
9		Barrett's dysplasia (High grade)	5.6	-	-
10	Cancers	ADC	12.8	2.0	-
11		ADC	10.0	2.0	-
12		WDSCC	11.2	-	-
13		WDSCC	12.8	0.4	-
14		WDSCC	8.8	2.4	-
15		MDSCC	10.0	0.8	-
16		MDSCC	12.8	0.4	0.4
17		MDSCC	5.2	0.8	-
18		PDSCC	1.6	-	-
19		PDSCC	6.0	-	-
20		PDSCC	3.2	-	-

Percentage of aneusomy in different cases from the first 250 cells scored. (Monosomies and normal disomies are not shown in the table). ADC: Adenocarcinoma; WDSCC: Well differentiated SCC; MDSCC: Moderately differentiated SCC; PDSCC: Poorly differentiated SCC; Sq. : Squamous.

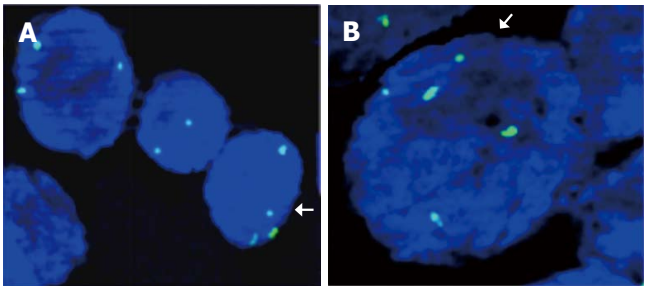

Figure 2 Images showing aneusomy with regard to chromosome 11 in esophageal cancer cells. **A**: Trisomy and tetrasomy chromosome 11 in the same area; **B**: Pentasomy chromosome 11 with normal disomy.

8-10 consecutive sections using the apotome. The images were captured at 1500 × magnification with oil immersion. Analysis was done using the 3D analysis of the Axiovision Apotome 1 imaging and Adobe Photoshop 7.0 version softwares.

Criteria for evaluating FISH signals

Data were scored from areas showing uniform fluorescence intensity. We screened a large number of nuclei per sample in order that we did not miss any aneusomic cells, especially with respect to precancer tissues. In each case, clear, distinct FISH signals were evaluated by counting 250 non-overlapping nuclei. Only those samples in which the artifactual nullisomy (negative nuclei) did not exceed the prescribed 25% were chosen for evaluation. A baseline frequency of monosomic population was established to control 'truncation' artifacts resulting from cut nuclei during microtomy.

RESULTS

From the patients with upper GI tract disorders referred for endoscopic evaluation, 25 cases were selected into the FISH study based on endoscopic and histopathological categorization. Of these 25 cases, 20 gave analyzable results; they included five normal tissues, four precancers (two squamous dysplasias and two BE with high-grade dysplasia) and eleven esophageal cancers as shown in Table 1.

FISH was performed on a few metaphase cells and, in conjunction with G-banded chromosome analysis, we confirmed the probe hybridization. Subsequently the method was applied to esophageal tissue sections obtained from paraffin blocks. Optical sectioning generated 3D images were evaluated and screened for chromosome 11 probe signals (Figure 2).

The results in each sample were tabulated as nullisomy (absence of signals), monosomy (single signal), normal disomic condition (two signals), trisomy (three signals), tetrasomy (four signals) and pentasomy (five signals). Esophageal tumor cells with trisomic, tetrasomic and pentasomic signals indicating the presence of extra chromosome 11 are shown in Figure 3.

Artifactual nullisomy was found to be less than 15% in all 20 cases studied. Monosomy was seen in < 35% of the cells in all three groups of controls, precancers and cancers; this was not included in the analysis as it was considered as a technical artifact. The controls showed normal disomy in all the remaining cells analyzed. Premalignant tissues showed an increase in the copy number of chromosome 11; all the four precancer cases showed trisomy of chromosome 11 (4%, 10%, 15.2%, 5.6%); of these, one case with severe squamous dysplasia exhibited tetrasomy (0.4%), and a case of BE with high

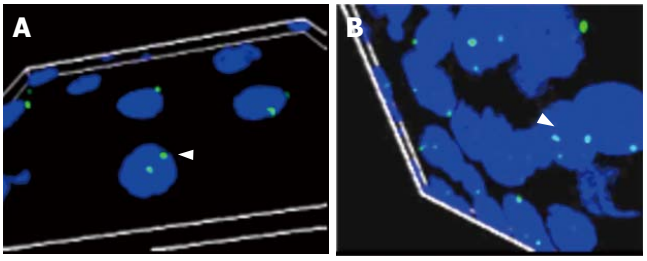

Figure 3 Axiovision Apotome software generated 3D-image from overlay of 10 consecutive images captured by the Optical sectioning microscopy. **A**: A portion of the esophageal tissue section from the control showing normal disomy of chromosome 11 (indicated by an arrow); **B**: Tissues from patients with well-differentiated squamous cell carcinoma (arrow indicating cell with trisomy for chromosome 11).

grade dysplasia showed tetrasomy and pentasomy of chromosome 11 (2.4% and 0.8%), respectively (Table 1).

All eleven cancer cases showed varying degrees of aneusomy of chromosome 11 in the affected tissues. Both cases of adenocarcinoma showed trisomy and tetrasomy. Of the remaining SCC cases, 4/9 showed only trisomy, 5/9 also showed tetrasomy and 1/9 also exhibited pentasomy of chromosome 11 (Table 1).

DISCUSSION

Chromosomal instability leading to aneuploidy and subsequently, specific aneusomies is characteristic of cancers[7,12]. In spite of the somatic gene-mutation hypothesis supporting the role of oncogenes and tumor suppressor genes in carcinogenesis, it has not been possible to disprove the century-old aneuploidy hypothesis even today. Cancer independent evidence suggests that specific aneusomies encode the phenotypes of irreversible precancerous lesions and are sufficient to alter the phenotype of eukaryotic cells to trigger cellular transformation[13-16]. Given this, it follows that chromosome number mutation, not gene mutation alone, is a probable cause of many dominant cancer cell phenotypes. Aneusomy can alter the dosage and thus the relevant activities of thousands of genes on the chromosome involved. Dividing aneuploid cells become increasingly unstable and most of these cells die eventually; rarely, these generate a specific aneusomy which then promotes the cell toward neoplastic transformation.

Specific chromosomal aneuploidies are characteristic of solid tumors unlike the random aneuploidies exhibited in hematological malignancies[9,17]. Chromosome 11 has a number of oncogenes and tumor suppressors including Cyclin D1, which is overexpressed in a wide variety of human neoplasms[18,19]. Reports suggest that overexpression of cyclin D1 is not due to gene amplification but due to chromosome number changes[5]. Other studies show the partial segmental aneusomy involving chromosome 11 in esophageal cancers, indicating its probable role in the disease etiology[10,11]. In the present work, FISH was used to assess change in chromosome 11 copy number in esophageal tumors using centromeric DNA probes.

Interestingly, our results showed that 100% of the cancerous lesions of the esophagus were aneusomic for chromosome 11. These ranged from trisomies to pentasomies in the same sample, indicating heterogeneity in the tumor tissue (Table 1). The baseline monosomy established (due to experimental artifacts) was similar in all controls, precancers and cancers. This is the first study to report that chromosome 11 copy number is altered in both esophageal SCC and ADC tumors. Because the number of ADC cases was small, there was no significant difference in the levels of aneusomy between SCC and ADC tumors. None of the controls showed any aneusomy suggesting that this chromosomal alteration is associated only with the neoplastic changes.

In addition, we also made a detailed investigation regarding the esophageal pathologies in each sub-group (though small in number) and the aneusomy in them.

There have been very few reports of specific aneusomies in esophageal premalignant lesions; using FISH, some studies showed hyperdiploidy involving chromosomes 4 and 8 in BE, aneuploidy in chromosome 11 in esophageal tumors and Barrett's dysplasias[20-23]. In the present work, all the precancerous lesions including BE with high-grade dysplasia exhibited aneusomy of chromosome 11. Our data indicate that levels of aneusomy of chromosome 11 seemed to occur increasingly in preneoplastic tissues suggesting that there may be genes on chromosome 11 that have a role in the initiation and neoplastic transformation of esophageal lesions.

Cytogenetic analysis by conventional chromosomal banding is labor-intensive and time-consuming. Besides, it is difficult to analyze some of the complex karyotypes characteristic of many human tumors. On the other hand, FISH is a relatively more convenient and quick method for evaluating tissue cell chromosomal changes[24,25]. Moreover, we have observed that by using adequate fluorescent signal capture (optical sectioning microscopic method) and analysis systems, an accurate estimate of the changes in chromosome copy number could be obtained from tumor tissues, based on which we could make satisfactory interpretations. Our study supports the finding that solid tumor cancers are often aneuploid for specific chromosomes and this may be used as an ancillary marker for detecting early changes associated with cancer.

Epigenetic changes like DNA methylation may lead to chromosomal instability, activation of endogenous parasitic sequences and mutations[26]. Cancer cells undergo methylation changes resulting in overexpression/silencing of several genes that are responsible for maintaining chromosomal integrity. Our study also revealed that 4/11 cancers and 2/5 precancers with increased copies of chromosome 11 had a hypermethylated hMLH1 repair gene promoter known to be responsible for altered repair efficiency (data not shown).

We did not perform any studies to assess whether this aneusomy correlates with the DNA content aneuploidy (i.e. gross chromosomal instability) of the tissues. The sample number (with a mix of ADCs and SCCs, which might have different etiologies), though, was not sufficient to make conclusions about predictive values, the results showed the differences in the frequencies of aneusomy in the two types of tumor. The present work clearly indicates that aneusomy is observed in early esophageal

lesions and may be involved in neoplastic transformation. Aneusomy of other chromosomes cannot be ruled out, however here, chromosome 11 is clearly demonstrated in all the esophageal pathologies studied. This marker merits investigation in a larger number of cases to determine its potential as a predictive molecular marker for an increased risk for malignant transformation.

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COMMENTS

Background

More than 30% of the adult population exhibits esophageal tract disorders, where a certain proportion of chronic cases even develop malignancy. Markers for early detection, which will help improve survival and treatment response are still lacking. This study investigates specific aneusomies which are believed to be associated with solid cancer phenotypes.

Research frontiers

Current biopsy surveillance programs are based on histopathological assessment of the tissue. However, molecular changes precede visible histopathological changes in cancer. FISH-based assay using brush cytology specimens (more easily accessible than biopsy) are increasingly used for investigation of chromosomal alterations and after validation these molecular markers can be used for routine surveillance in order to aid in early detection.

Innovations and breakthroughs

FISH, when compared to conventional cytogenetic analysis, is a more convenient and quick method for evaluating tissue cell chromosomal changes. An apotome-attached optical sectioning microscope to capture fluorescent probe signals has shown maximum efficiency for signal analysis in tissue FISH. This will aid in satisfactory data collection and interpretation.

Applications

Chromosome 11 aneusomy has been demonstrated in all the cases of cancer and precancer studied by us indicating its potential as an early marker for neoplastic transformation. Our results call for evaluating this marker in a larger cohort of patients.

Terminology

Regurgitation: bringing back undigested food from the stomach; Dysphagia: difficulty in swallowing; Dysplasia: Nuclear atypia, loss of normal cell polarity, and abnormal tissue maturation.

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Effect of 2-(8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid in combination with carboplatin on gastric carcinoma growth *in vivo*

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Abstract

AIM: To investigate the effects of 2-(8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid (NM-3) alone and in combination with carboplatin on tumor growth and apoptosis in mouse models of human gastric cancer constructed by subcutaneous implantation of histologically intact tumor tissue.

METHODS: Human gastric cancer SGC-7901 tissues were implanted into the dorsal subcutis of nude mice. One week after tumors reached to a volume of 50-100 mm^3 for around 1 wk, these mice were randomly divided into 8 groups (n = 10). NM-3 was injected peritoneally at the dose of 10 mg/kg, 20 mg/kg or 40 mg/kg every other day for 5 wk, combined with carboplatin (5 mg/kg) every third day for 4 wk. As controls of combined treatment, another 4 groups of mice were injected with either NM-3 at 10 mg/kg, 20 mg/kg or 40 mg/kg, or with carboplatin alone (5 mg/kg). The control mice received normal saline. Tumor weight, tumor growth inhibition (TGI), and intratumoral microvessel density (MVD) were evaluated. Apoptosis of human gastric cancer was detected by TUNEL method and flow cytometry analysis, respectively.

RESULTS: The mean tumor volume (692.40 ± 58.43 mm^3, 548.30 ± 66.02 mm^3, 382.13 ± 43.52 mm^3) after treatment with carboplatin combined NM-3 at the dose of 10 mg/kg, 20 mg/kg or 40 mg/kg was lower than that after treatment with either NM-3 at the dose of 10 mg/kg, 20 mg/kg or 40 mg/kg or with carboplatin alone. Compared with the normal saline group, NM-3 administered at 10 mg/kg, 20 mg/kg or 40 mg/kg significantly reduced the tumor weight in these groups (P < 0.05). Carboplatin used alone at 5 mg/kg showed minimal effects. But NM-3 in combination with carboplatin had greater effects of tumor weight than either NM-3 or carboplatin alone. NM-3 alone at the dose 10 mg/kg or in combination with carboplatin had no obvious effects on body changes. Two mice died of diarrhea in each of the two groups treated with 40 mg/kg NM-3 or with 40 mg/kg NM-3 in combination with carboplatin. A significant increase in apoptosis was observed in the NM-3 treated groups, and the effect was more significant in the groups treated with carboplatin in combination with NM-3 at 10 mg/kg, 20 mg/kg and 40 mg/kg, than in the control group. The induction of apoptosis was positively associated with the dose of NM-3. NM-3 significantly reduced the neo-microvascular formation of gastric cancer. The MVD was lower in the groups treated with NM-3 or with NM-3 in combination with carboplatin than in the group treated with carboplatin or in the normal saline group (P < 0.05).

CONCLUSION: The results suggest that the inhibitory effect of NM-3 on gastric cancer growth is mediated through decreased angiogenesis and the increased induction of apoptosis. Furthermore, NM-3 alone at the dose of 10 mg/kg or in combination with carboplatin has no obvious effects on body changes, indicating that NM-3 in combination with carboplatin may be effective in the treatment of gastric cancer. The toxicity of NM-3 needs further studies.

Key words: NM-3; Carboplatin; Gastric carcinoma; Angiogenesis; Apoptosis

Chen JL, Zhu JS, Hong J, Chen MX, Lu JL, Chen WX, Shen B, Zhu ZM, Chen NW. Effect of 2-(8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid in combination with carboplatin on gastric carcinoma growth *in vivo*. *World J Gastroenterol* 2007; 13(4): 509-514

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INTRODUCTION

Gastric carcinoma is one of the most frequent malignancies and one of the major causes of cancer deaths

in China[1]. Up to now, the prognosis of patients with gastric cancer is very poor because gastric cancer is usually diagnosed at its advanced stage throughout the world. Even after curative resection, it remains at a high risk of relapse. Chemotherapy is one of the most important treatment modalities for gastric cancer. However, its effect is limited due to its adverse reactions and resistance of tumor cells to chemotherapeutic agents[2].

Apoptosis plays an important role in the growth of malignant tumor cells. It has been shown that apoptosis can be induced in gastric cancer by some chemotherapeutic drugs, such as 5-fluorouracil, cisplatin and paclitaxel[3]. Recent studies have demonstrated that angiogenesis plays a crucial role in tumor growth and metastasis. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are the main factors promoting angiogenesis[4-6]. It has been shown that radiation-induced tumor regression is enhanced by angiogenesis inhibitors. Angiostatin or antibody to VEGF in combination with chemotherapy produces greater antitumor effects than either treatment alone[7,8].

Recently, induction of apoptosis by antiangiogenic therapy has been suggested as a new anticancer strategy[9]. 2- (8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid (NM-3), is a synthetic derivative of cytogenin. *In vitro* studies have demonstrated that the inhibitory effects of NM-3 at lower concentrations are stronger on the growth of human umbilical vein endothelial cells than on the growth of normal fibroblasts or tumor cells[10]. NM-3 alone inhibits endothelial sprouting and tube formation *in vitro*. It has been shown that NM-3 can enhance 5-fluorouracil-induced tumor growth inhibition in breast carcinoma xenografts with no effects on body changes[11]. In this study, we investigated the effects of NM-3 alone or in combination with carboplatin on tumor growth and apoptosis in mouse models of human gastric cancer constructed by subcutaneous implantation of histologically intact tumor tissue.

MATERIALS AND METHODS

Materials

Male BALB/c/nu/nu nude mice were obtained from Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Animals used were 6-wk old and weighed 20-25 g. Human gastric cancer SGC-7901 was obtained from Shanghai Cancer Institute. NM-3 was provided by professor Robert (National Cancer Research Center of America), concentration of NM-3 was 20 mg/mL.

Animal experimental method

Animal models were made using subcutaneous implantation of histologically intact tissue of human gastric carcinoma. Tumors were resected aseptically. Necrotic tissue was removed and the remaining tumor tissues were minced into pieces about 2 mm in diameter and implanted into the dorsal subcutis of mice.

One week after tumors reached a volume of 50-100 mm^3 for around 1 wk, these mice were randomly divided into 8 groups (n = 10). NM-3 was injected peritoneally at the dose of 10 mg/kg, 20 mg/kg or 40 mg/kg every other day for 5 wk, in combination with carboplatin (5 mg/kg) every third day for 4 wk. As controls of combined treatment, another four groups of mice were injected NM-3 or carboplatin alone. The control mice received normal saline as indicated. The mice were weighed twice weekly, and tumor measurements were taken by calipers twice weekly. Tumor volume was measured using the formula ($V = ab^2/2$), where a is the largest diameter and b the smallest diameter. Tumor growth inhibition (TGI) in each group was (mean control tumor weight - mean treated tumor weight)/mean control tumor weight × 100%.

Sample collection and pathological examination

All animals were sacrificed 7 wk after the implantation. Tumors were biopsied, fixed in 10% formalin, and processed for routine paraffin embedding. Tumors were evaluated histologically under microscope.

Mean microvascular density (MVD of tumor)

Four-micron-thick sections were deparaffined in xylene and rehydrated in graded alcohol. Immunostaining was performed using a labeled streptavidin biotin method. Immunohistochemical staining was carried out to detect CD34 expression following the manufacturer's protocol (Santa Cruz Biotech Company). The modified Weidner's method was used for the evaluation of MVD according to CD34 endothelial cell immunostaining. For microvessel counting, positive staining for MVD in five most highly vascularized areas in each section was counted in 200 × fields. MVD was expressed as the average of the microvessel count in the 5 areas. Any endothelial cell or endothelial cluster positive for CD34 (brown-yellow staining) was a single countable microvessel.

Detection of cell apoptosis idex (AI)

Apoptosis of human gastric cancer was detected by terminal deoxynuclotidyl transferase- mediated deoxyuridine triphosphate-fluorescene nick end labeling (TUNEL) method and flow cytometry analysis, respectively. TUNEL method was performed as indicated. Flow cytometry analysis was conducted as follows. In brief, propidium iodide (PI) staining was used for flow cytometric detection of apoptosis, 1×10^6 cells from each of the samples were treated with RNase and stained with PI. DNA strand-labeled apoptotic cells were calculated with a flow cytometer (FACS Calibur, Becton Dickinson, USA.). Data were collected from 1×10^6 cells/sample, stored and analyzed using CELLQUES'T and MODFITLT for macV 1.01 software.

Statistical analysis

All data were expressed as mean ± SD. Student's t test was used to determine changes in different groups. $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory effect of NM-3 on growth of xenografted human gastric cancer in nude mice

The mean tumor volume (MTV) in the groups treated

Table 1 Inhibitory effect of NM-3 on gastric cancer growth (mean ± SD)

Groups	*n*	MTV (mm^3)	Body weight (g)	Tumor weight (mg)	TGI (%)
Normal saline	10	81.24 ± 12.63	25.8 ± 1.04	1754.0 ± 144.2	
NM-3 (10 mg/kg)	10	79.68 ± 13.72	24.4 ± 0.76	1351.0 ± 116.9	23.0[a]
NM-3 (20 mg/kg)	10	81.08 ± 12.90	23.1 ± 0.82	1041.1 ± 143.5	40.6[a]
NM-3 (40 mg/kg)	8	81.36 ± 11.20	22.9 ± 1.06	765.5 ± 140.1	56.2[a,c]
NM-3 (10 mg/kg) + carplatin	10	80.29 ± 14.26	24.2 ± 0.88	1002.0 ± 101.4	42.7[a,c]
NM-3 (20 mg/kg) + carplatin	10	82.30 ± 14.53	24.4 ± 0.78	919.0 ± 149.8	47.6[a,c]
NM-3 (40 mg/kg) + carplatin	8	81.97 ± 12.77	22.5 ± 1.13	645.7 ± 135.1	63.2[a,c]
Carplatin	10	80.01 ± 13.67	23.0 ± 1.03	1655.0 ± 157.4	5.6

[a]$P < 0.05$ *vs* normal saline control group and carboplatin group, [c]$P < 0.05$ *vs* NM-3 (10 mg/kg) group.

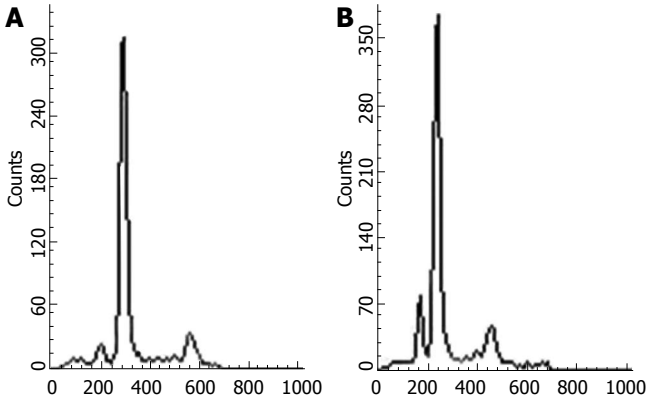

Figure 1 Propidium iodide (PI) staining for flow cytometric detection of apoptosis in control group (**A**) and NM-3- treated group (**B**).

with 10 mg/kg, 20 mg/kg or 40 mg/kg NM-3 was 989.50 ± 102.17 mm^3, 826.20 ± 76.52 mm^3, and 709.75 ± 89.30 mm^3, respectively, which was significantly smaller than that in the control group receiving normal saline (1609.60 ± 122.11 mm^3, $P < 0.05$). Carboplatin at the dose of 5 mg/kg had no significant inhibitory action on gastric carcinoma with MTV being 1532.14 ± 110.12 mm^3. However, the mean tumor volume in groups treated with carboplatin in combination with NM-3 was 692.40 ± 58.43 mm^3, 548.30 ± 66.02 mm^3, and 382.13 ± 43.52 mm^3, respectively, which was lower than that in the groups treated either with NM-3 or with carboplatin alone. Compared with the normal saline group, NM-3 administered at 10 mg/kg, 20 mg/kg or 40 mg/kg significantly reduced the tumor weight in these groups, and the effect was more significant when NM-3 was given at a dose of 40 mg/kg ($P < 0.05$). Carboplatin used alone at the dose of 5 mg/kg showed minimal effects. But NM-3 in combination with carboplatin, however, had a more significant effect on tumor weight than NM-3 or carboplatin alone (Table 1).The mean tumor volume did not differ among groups before treatment (Table 1).

During the experiment, diarrhea occurred in some mice when NM-3 was given at the dose of 20 mg/kg or 40 mg/kg. Two mice died of diarrhea in each of the two groups treated with 40 mg/kg NM-3 or with 40 mg/kg NM-3 in combination with carboplatin. The remaining mice recovered at the latter stage of the experiment. NM-3 alone at the dose of 10 mg/kg or in combination with carboplatin had no obvious effects on body changes.

Table 2 Effect of NM-3 on cell apoptosis of gastric cancer (mean ± SD, %)

Groups	*n*	AI (TUNEL)	AI (FAScan)
Normal saline	10	2.12 ± 2.19	1.59 ± 0.24
NM-3 (10 mg/kg)	10	6.02 ± 1.63[a]	3.84 ± 0.68[a]
NM-3 (20 mg/kg)	10	10.43 ± 3.15[a,c]	8.21 ± 1.01[a,c]
NM-3 (40 mg/kg)	8	22.06 ± 5.68[a,c]	18.26 ± 4.46[a,c]
NM-3 (10 mg/kg) + carplatin	10	8.66 ± 2.35[a,c]	6.96 ± 0.65[a,c]
NM-3 (20 mg/kg) + carplatin	10	12.63 ± 3.75[a,c]	10.65 ± 1.43[a,c]
NM-3 (40 mg/kg) + carplatin	8	24.63 ± 3.67[a,c]	21.66 ± 2.96[a,c]
Carplatin	10	2.47 ± 0.31	1.85 ± 0.34

[a]$P < 0.05$ *vs* normal saline control group and carboplatin group, [c]$P < 0.05$ *vs* NM-3 (10 mg/kg) group.

Effect of NM-3 on cell apoptosis of human gastric cancer cells

Apoptosis of gastric cancer cells was observed microscopically. Manifestations of apoptosis could be found more frequently in NM-3-treated groups, such as cell shrinkage, nuclear condensation, DNA fragmentation and formation of apoptotic bodies. Apoptosis was detected with flow cytometry (Figure 1).

The apoptosis index of tumors treated with carboplatin alone was not significantly different from that of the control group ($P > 0.05$). However, a significant increase in apoptosis was observed in the NM-3-treated groups, and the effect was more significant in the groups treated with carplatin in combination with NM-3 at 10 mg/kg, 20 mg/kg and 40 mg/kg than in the control group (Table 2). The induction of apoptosis was positively associated with the dose of NM-3.

Effect of NM-3 on MVD

NM-3 significantly reduced the neo-microvascular formation of gastric cancer implanted into nude mice. The MVD was lower in groups treated with NM-3 or with NM-3 in combination with carboplatin than in carboplatin group or normal saline group ($P < 0.05$). More significant inhibitory effects on MVD of tumor were observed in NM-3-treated groups at the dose of 40 mg/kg or in combination with carboplatin than in control group ($P < 0.05$, Table 3, Figure 2A-H).

DISCUSSION

Recent studies have shown that angiogenesis plays a

Table 3 Effect of NM-3 on MVD (mean ± SD)

Groups	*n*	MVD
Normal saline	10	7.30 ± 0.53
NM-3 (10 mg/kg)	10	5.10 ± 0.40[a]
NM-3 (20 mg/kg)	10	4.72 ± 0.51[a]
NM-3 (40 mg/kg)	8	2.02 ± 0.50[a,c]
NM-3 (10 mg/kg) + carplatin	10	4.96 ± 0.37[a]
NM-3 (20 mg/kg) + carplatin	10	4.80 ± 0.39[a]
NM-3 (40 mg/kg) + carplatin	8	1.78 ± 0.42[a,c]
Carplatin	10	6.98 ± 0.45

[a]$P < 0.05$ *vs* normal saline control group and carboplatin group, [c]$P < 0.05$ *vs* NM-3 (10 mg/kg) group.

critical role in solid tumor growth and its development[8]. Antiangiogenic agents inhibit tumor growth by preventing proliferation, migration and sprouting of tumor endothelial cells and formation of new blood vessels. Antiangiogenic therapy plays an important role in improving prognosis of patients with gastric carcinoma[12-15]. NM-3, a small molecule isocoumarin, is a recently discovered angiogenesis inhibitor. It has been shown that NM-3 enhances the antitumor effects of some chemotherapeutic drugs in breast and prostate tumor models[11]. The increased antitumor effects of chemotherapy in combination with NM-3 can be achieved without any apparent increase in toxicity. To date, the effect of antitumor and induction of cell apoptosis of NM-3 on human gastric cancer have not been reported. It is worthwhile, therefore, to further research its antitumor mechanisms underlying gastric cancer.

In the present study, NM-3 significantly inhibited the growth of human gastric cancer in mice. Compared with the controls, growth of the tumor implanted subcutaneously was remarkably reduced in size and weight in the mice treated with NM-3 at the doses of 10 mg/kg, 20 mg/kg, 40 mg/kg. These doses of NM-3 in combination with carboplatin delayed the growth of SGC-7901 human gastric cancer in mice, compared with NM-3 or carboplatin alone. NM-3 alone at the dose of 10 mg/kg or in combination with carboplatin had no obvious effects on body changes. However, two mice died of diarrhea in each of the two groups treated with 40 mg/kg NM-3 or with 40 mg/kg NM-3 in combination with carboplatin, suggesting that the toxicity and doses of NM-3 used in patients need further studies. Although tumor growth was inhibited by NM-3 in combination with carboplatin as compared with normal saline group, tumor weight increased. This may be due to the lower dose of carboplatin used. In our study, carboplatin at the dose of 5 mg/kg showed minimal effects on tumor growth. Previous studies have demonstrated that chemotherapy in combination with an angiogenesis inhibitor can enhance tumor growth inhibition. Reimer *et al*[11] demonstrated that NM-3 can significantly enhance tumor growth inhibition in breast and prostate carcinoma xenografts at nontoxic doses in combination with 5-fluorouoacil, paclitaxel or cyclophosphamide given at subtherapeutic doses. These effects were particularly marked when NM-3 was combined with cyclophosphamide. Vinblastine in combination with

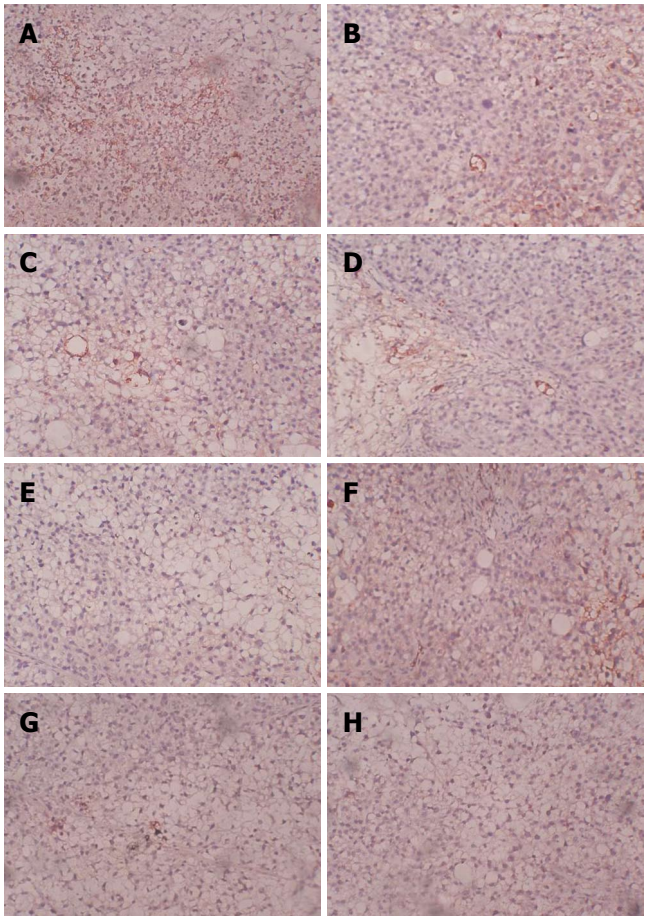

Figure 2 Immunohistochemical method for detection of microvessel density in mice injected with normal saline (**A**); carboplatin alone (**B**); 10 mg/kg NM-3 (**C**); 20 mg/kg NM-3 (**D**); 40 mg/kg NM-3 (**E**); 10 mg/kg NM-3 in combination with 5 mg/kg carboplatin (**F**); 20 mg/kg NM-3 in combination with 5 mg/kg carboplatin (**G**); 40 mg/kg NM-3 in combination with 5 mg/kg carboplatin (**H**); × 200.

VEGF receptor-2 antibody could cause sustained tumor regression. Salloum *et al*[10] studied the antitumor effects of NM-3 in combination with radiotherapy, and found that the tumor is significantly regressed after combined treatment compared with radiotherapy alone with no increase in systemic or local tissue toxicity, suggesting that NM-3 in combination with chemotherapy or radiotherapy can increase the efficacy of cancer treatment.

Cell apoptosis is an active death process of cells, its imbalance or changes are related to the occurrence of many diseases. Gastric cancer is not only a disease with abnormal cell proliferation and differentiation, but also a disease with abnormal apoptosis[16,17]. Increased apoptosis in human gastric cancer cells could be observed after treatment with 5-fluorouracil, cisplatin, *etc*. The results suggest that these drugs can be used in the treatment of patients with gastric cancer by inducing apoptosis of cancer cells. The results obtained by TUNEL method and cytometry analysis indicate that apoptosis is induced by NM-3. Matsuhashi *et al*[18] investigated the relationship between p53 expression and apoptosis induction of 5-fluorouracil and cisplatin on gastric cancer cells, and found that combined administration of 5-fluorouracil and cisplatin does not induce apoptosis of MKN-28 (mutant-type p53), while apoptotic cells can be observed in the case

of MKN-45 (wild-type p53). Browder *et al*[19] demonstrated that TNP-470 at a low dose in combination with cyclophosphamide can eradicate drug-resistant Lewis lung carcinoma. Agata *et al*[20] revealed that NM-3 potentiates dexamethasone-induced apoptosis of human multiple myeloma cells. Moreover, NM-3 is effective against dexamethasone-resistant RPMI8226 and U266 multiple myeloma cells. NM-3 enhances dexamethasone-induced release of mitochondrial apoptogenic factors (cytochrome c and smac/DIABLO) and dexamethasone-induced activation of intrinsic caspase-9→caspase-3 apoptotic pathway. These results suggest that NM-3 inhibits the growth of gastric cancer by enhancing apoptosis of cancer cells.

Angiogenesis has been implicated in the growth and metastasis of gastric cancer. MaCarty *et al*[21] reported that ZD6474, a vascular endothelial growth factor receptor (tyrosine kinase inhibitor) inhibits orthotopic growth and angiogenesis of gastric cancer and increases tumor cell apoptosis. Stoeltzing *et al*[22] demonstrated that inhibition of hypoxia-inducible factor 1 activity can inhibit gastric cancer growth and angiogenesis. Kamiya *et al*[23] showed that the antitumor effect of VEGF Ab on gastric cancer is exerted by inducing mild hypoxia and apoptosis. Reimer *et al*[11] reported that the antitumor effects of NM-3 in combination with chemotherapeutic agents are mediated through decreased proliferation of endothelial cells. The present study indicated that NM-3 significantly inhibited angiogenesis in gastric cancer, suggesting that apoptosis of gastric cancer is mediated by NM-3 through decreased angiogenesis and that the inhibitory effect of NM-3 on gastric cancer growth is related to the induction of apoptosis.

In conclusion, NM-3 in combination with carboplatin is effective against gastric cancer. The toxicity and mechanism of NM-3 underlying apoptosis of gastric cancer need further studies.

COMMENTS

Background

Gastric carcinoma is one of the most frequent malignancies in China. Angiogenesis plays a crucial role in tumor growth and metastasis. Recently, induction of apoptosis by antiangiogenic therapy has been suggested as a new anticancer strategy.

Research frontiers

2-(8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid (NM-3) is a synthetic derivative of cytogenin. In vitro studies have demonstrated that the inhibitory effects of NM-3 at lower concentrations are stronger on human umbilical vein endothelial cells than on normal fibroblasts or tumor cells. NM-3 alone inhibits endothelial sprouting and tube formation *in vitro*.

Innovations and breakthroughs

There is some experience of NM-3 as an apoptotic and antiangiogenic inducer in other types of tumors such as lung and prostatic cancers, but not in gastric cancer which is associated with high chemotherapy resistance. Inhibitory effects of NM-3 on gastric cancer growth are mediated through decreased angiogenesis and the increased induction of apoptosis. NM-3 at the dose of 10 mg/kg alone or in combination with carboplatin has no obvious effects on body changes.

Applications

NM-3 in combination with carboplatin may be effective against gastric cancer.

Peer review

This is a well designed experimental study of apoptosis and effects of NM-3 and carboplatin on gastric cancer model (SGC-7901). There is some experience in NM-3 as an apoptotic and antiangiogenic inducer in other types of tumors such as lung and prostatic cancers, but not in gastric cancer which is associated with high chemotherapy resistance. The paper is interesting.

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Usefulness of two independent histopathological classifications of tumor regression in patients with rectal cancer submitted to hyperfractionated pre-operative radiotherapy

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Abstract

AIM: To assess the usefulness of two independent histopathological classifications of rectal cancer regression following neo-adjuvant therapy.

METHODS: Forty patients at the initial stage cT3NxM0 submitted to preoperative radiotherapy (42 Gy during 18 d) and then to radical surgical treatment. The relationship between "T-downstaging" versus regressive changes expressed by tumor regression grade (TRG 1-5) and Nasierowska-Guttmejer classification (NG 1-3) was studied as well as the relationship between TRG and NG versus local tumor stage ypT and lymph nodes status, ypN.

RESULTS: Complete regression (ypT0, TRG 1) was found in one patient. "T-downstaging" was observed in 11 (27.5%) patients. There was a weak statistical significance of the relationship between "T-downstaging" and TRG staging and NG stage. Patients with ypT1 were diagnosed as TRG 2-3 while those with ypT3 as TRG5. No lymph node metastases were found in patients with TRG 1-2. None of the patients without lymph node metastases were diagnosed as TRG 5. Patients in the ypT1 stage were NG 1-2. No lymph node metastases were found in NG 1. There was a significant correlation between TRG and NG.

CONCLUSION: Histopathological classifications may be useful in the monitoring of the effects of hyperfractionated preoperative radiotherapy in patients with rectal cancer at the stage of cT3NxM0. There is no unequivocal relationship between "T-downstaging" and TRG and NG. There is some concordance in the assessment of lymph node status with ypT, TRG and NG. TRG and NG are of limited value for the risk assessment of the lymph node involvement.

Key words: Rectal cancer; Adenocarcinoma; Neoadjuvant therapy; Preoperative radiotherapy; Neoplasm staging

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INTRODUCTION

Colorectal cancer is the third most common malignancy diagnosed in the USA[1]. The estimated colorectal cancer mortality in the USA in 2006 is 55170[2]. The primary treatment method for rectal cancer is surgery, namely anterior rectal resection, abdomino-perineal resection or local excision[3-6]. Preoperative radiotherapy and radiochemotherapy play an increasing role in the treatment of rectal cancer[7-13]. The effectiveness of neo-adjuvant therapy may be assessed and monitored by means of long-term survival follow up, incidence of local recurrence, estimation of the percentage of patients with primary high stage tumor suitable for radical surgery, estimation of the percentage of patients suitable for sphincter-saving surgery or by monitoring the tumor stage using visualizing diagnostic methods[14,15]. Transrectal ultrasound (TRUS) is a useful method for the assessment of the local tumor stage and the regional lymph node status prior to neo-adjuvant therapy[3,4,6,16]. Basing on TRUS and histopathological examination one can define the tumor regression parameter "T-downstaging". Lower ypT parameter value (local tumor stage assessed by the pathologist in surgical specimen following neo-adjuvant therapy) than uT (local

tumor stage assessed by surgeon with use of TRUS prior to neo-adjuvant therapy) is considered an evidence of tumor regression. The value of ypT parameter equal or higher than uT indicates lack of tumor regression[11,14,17-21].

This parameter may also be applied to the regression of metastatic regional lymph nodes in rectal cancer, "N-downstaging"[17,20,22-26].

Preoperative radiotherapy and radiochemotherapy evokes a range of morphological changes in the microscopic picture of rectal cancer including increased tumor necrosis, cellular and nuclear atypia, endocrine differentiation of tumor cells, increased stromal fibrosis, quantitative and qualitative changes of the stromal inflammatory exudates, formation of mucin pools, surface ulceration, peritumoral eosinophilic infiltrate, dysplastic and adenomatous changes (high-grade dysplasia, and low-grade adenoma component in the intestinal mucosa)[5,27,28]. Several histopathological classifications of rectal carcinoma response to neo-adjuvant therapy have been proposed[5,20,29-33]. However, none of these classifications is used in routine histopathological diagnostics. This results from the fact that macro- and microscopic changes within the tumor structure and surrounding tissues are not a specific response to ionizing radiation but also may result from the non-specific inflammation, hormonal therapy and local immune reaction[5,7]. Tumor regression grade (TRG) is a semi-quantitative parameter describing a relative proportion of residual tumor and stromal fibrosis. It is regarded a useful parameter for the assessment of histopathological changes in tumor following neo-adjuvant therapy[14,18,19,21,22,29,34-38]. There are five grades of cancer response to treatment in TRG staging, ranging from TRG 1-no residual cancer cells in the intestinal wall, replaced by fibrous tissue, through TRG 2-presence of occasional residual cancer cells, scattered in fibrous stroma, TRG 3-fibrosis dominating over residual cancer, TRG 4-residual cancer outgrowing fibrosis, to TRG 5-no tumor response or regression, no fibrosis with extensive residual cancer[29]. Another classification, proposed by Nasierowska-Guttmejer (NG) distinguishes three degrees of cancer response to neo-adjuvant therapy depending on the intensity of the morphological changes. At present one should assess cancer cell degeneration (no cancer cells, high, moderate and low-grade degeneration), mucus pools (present or absent) and necrosis (absent, $\leqslant$ 50% cancer tissue, > 50% cancer tissue). Point scores are designated to each parameter of tumor response to neo-adjuvant therapy and then are summarized[5].

Some authors believe that "T-downstaging" does not precisely reflect cancer regression following neo-adjuvant therapy. They state that residual cancer has a form of rather small foci surrounded by fibrous tissue and they are localized in all layers of the rectal wall. Such a deep localization results in diagnosis of high tumor stage despite a good response to radiotherapy. This phenomenon justifies the search for the histopathological tumor regression grading systems[14,20,35,39]. Rodel *et al*[40] suggest that tumor regression following radiotherapy reflects its less aggressive potential resulting from the molecular profile. Particular biological properties of a tumor influencing its chemo-radiosensitivity may also prove to be of long-term prognostic significance, especially in cases submitted to neo-adjuvant therapy.

TRG classification is probably superior versus "T-downstaging" in terms of the evaluation of neo-adjuvant therapeutic effects[14]. Reports on the relationship between "T-downstaging" or ypT and TRG are not numerous, and are with regard to preoperative long-term radiotherapy and chemoradiotherapy[21,22,35,36]. The relationship between TRG and the probability of lymph nodes involvement has been described in detail only in patients with rectal cancer submitted to long-term radio-chemotherapy[22,41]. So far, no results have been published comparing the NG with other rectal cancer regression assessment systems following neo-adjuvant therapy.

The aim of the present study is to evaluate if two independent histopathological classifications based on semiquantitative assessment of regressive changes may prove useful for the monitoring of patients with rectal adenocarcinoma, initial stage cT3NxM0 submitted to preoperative hyperfractionated radiotherapy.

Our particular aim was to assess whether there is any relationship between: (1) "T-downstaging" and histopathological staging systems of cancer response to neo-adjuvant treatment (TRG and NG systems); (2) "T-downstaging" and local tumor stage and lymph node status; (3) TRG and NG classification and local tumor stage and lymph node status; (4) mutual relationships between TRG and NG systems.

MATERIALS AND METHODS

Patients

The study encompassed patients with rectal adenocarcinoma submitted to hyperfractionated preoperative radiotherapy, with perirectal tissue invasion assessed with ultrasound examination prior to neo-adjuvant treatment (TRUS: uT3). Patients' general performance status according to the Eastern Cooperative Oncology Group classification ranged from 0 to 2 points. Patients with distant metastases found on chest X-ray, and abdominal and pelvis CT examination were excluded from the study. Also, patients formerly submitted to radiotherapy due to present disease or another neoplasm were not included into the study. None of the patients had a history of inflammatory bowel disease.

Forty patients were included into the study. Median age was 64 (range 45-75) years. Ultrasound examination protocol has been described previously[6]. All patients were submitted to preoperative hyperfractionated radiotherapy. A total dose of 42 Gy in 28 fractions during 18 d (twice a day, 1.8 Gy, 5 d/wk, and with a minimum 6 h interval between doses) using a three-field isocentric technique-one posterior and two lateral portals. Photon rays of 20 (10-23) MV were used. The edge of the posterior field was situated 5 cm below the lower tumor margin. The lateral margins of the lateral fields extended beyond the pelvic inlet. The upper edge was at the top of the fifth lumbar vertebra. The target volume included the tumor and regional lymph nodes. The standard size of the posterior

Table 1 Clinicopathological characteristics of study patients

Median age (mean ± SD) (yr)	64 (61.75 ± 10.0)	
M:F	1:1	
Lymph node involvement prior to radiotherapy (cN)		
cN0	25	62.5%
cN+	15	37.5%
Tumor stage		
ypT0	1	2.5%
ypT1	4	10%
ypT2	6	15%
ypT3	29	72.5%
Lymph node status		
ypN0	26	65%
ypN1	8	20%
ypN2	6	15%
Number of lymph node assessed-median (mean ± SD)	16 (18 ± 11.5)	
Number of affected lymph nodes-median (mean ± SD)	0 (2.6 ± 6.9)	
Tumor histological grade (G)[1]		
G1	5	12.8%
G2	32	82.1%
G3	2	5.1%
Median tumor diameter[1] (mean ± SD) (mm)	33.5 (33.7 ± 16.1)	

[1]One case ypT0 (2.5%) had not been taken into account.

field was 12 cm × 15 cm and 10 cm × 15 cm for the lateral fields. Surgery was performed 1-7 d (mean, 5 d) following radiotherapy. Thirty one (77.5%) anterior resections, 8 (20.0%) abdomino-perineal resections and 1 (2.5%) Hartmann's operation, were performed.

Pathological examination

Surgical specimens were submitted to histopathological examination according to standard protocol[42]. Special attention was paid to definite, probable and potential prognostic factors[43]. The following pathological parameters were evaluated: local tumor stage (ypT), regional lymph node status (ypN), tumor grade (G1, G2, G3), number of metastatic lymph nodes, and parameters of the tumor response to radiotherapy. The latter included: cancer cell degeneration (severe, moderate, mild), mucin pools (absent, present), tumor necrosis (absent, $\leqslant$ 50%, > 50% of the tumor), tumor response to radiotherapy according to NG (1-3)[5], and TRG (1-5)[29] classification. In cases with non-homogeneous tumor response pattern to radiotherapy, the area of the weakest response was taken into account[38]. Routine surgical specimens submitted for histopathological examination were evaluated retrospectively. Concerning radiotherapy and surgery, the nature of the study was observatory and not experimental.

Statistical analysis

A study population was divided into 2 groups upon the "T-downstaging" tumor regression parameter. A group with features of cancer regression, ypT < uT (R group) and with no regression, ypT $\geqslant$ uT (NR group) were distinguished. The differences between groups in parameters studied were tested using Pearson's χ^2 test, Fisher's exact test, and Mann-Whitney's *U* test. Correlation was assessed with Spearman's rank correlation. $P < 0.05$ was considered statistically significant.

Table 2 Relationship between "T-downstaging" and prognostic parameters

Feature	Group R (n = 11)	Group NR (n = 29)	P
Median age (range) (mean ± SD) (yr)	70 (55-77) (67.7 ± 7.2)	61 (45-70) (59.4 ± 9.9)	< 0.05
Tumor stage			< 0.000
ypT0	1 (9.1%)	0	
ypT1	4 (36.4%)	0	
ypT2	6 (54.6%)	0	
ypT3	0	29 (100.0%)	
TRG			< 0.08
1	1 (9.1%)	0	
2	2 (18.2%)	1 (3.5%)	
3	6 (54.6%)	11 (37.9%)	
4	2 (18.2%)	14 (48.3%)	
5	0	3 (10.3%)	
NG			< 0.08
1	5 (45.5%)	4 (13.8%)	
2	2 (18.2%)	4 (13.8%)	
3	4 (36.4%)	21 (72.4%)	

RESULTS

Demographic data and staging parameters are presented in Table 1. Local tumor stage, ypT3 was found in 29 (72.5%) patients. "T-downstaging" was observed in 11 out of 40 (27.5%) patients. Six (15.0%) of them showed downstaging to ypT2, and 4 (10.0%) to ypT1. In one case, histopathological examination has shown no evidence of carcinoma in the intestinal wall (ypT0, TRG 1). Also, no lymph node involvement was found in this patient (ypT0N0). TRUS examination showed features of lymph node involvement in 15 (37.5%) patients. In 8 (20%) of 15 patients in whom TRUS examination showed lymph node involvement, microscopic examination revealed stage ypN0. In 7 (17.5%) out of 25 patients with no evidence of lymph node involvement in TRUS examination, histopathological examination showed presence of metastases. TRG grades 2, 3 and 4 were diagnosed in 3 (7.5%), 17 (42.5%) and 16 (40.0%) patients, respectively. No tumor regression (TRG 5) was found in 3 (7.5%) patients. Features of moderate or severe cancer cell degeneration were observed in 17 (42.5%) patients. Mucus lakes were seen in 22 (55.0%) cases. Necrosis was present in 27 (67.5%) of cases including 1 case with more than 50% of tumor involvement. Stage 1, 2, and 3 of NG classification was reported in 9 (22.5%), 6 (15.0%), and 25 (62.5%) patients, respectively.

Median age (range) in the group with tumor regression was higher than those of patients with no evidence of regression (Table 2). Groups R and NR included 5 (45.5%) and 15 (51.7%) men (NS), respectively. TRUS examination performed prior to neo-adjuvant therapy revealed lymph node involvement in groups R and NR in 4 (36.36%) and 11 (37.93%) patients (NS), respectively. Stage ypN0, ypN1 and ypN2 was found in 9 (81.8%), 2 (18.2%), and

Table 3 Relationship between tumor stage and TRG

	TRG 1	TRG 2	TRG 3	TRG 4	TRG 5
Local tumor stage[1,a]					
ypT0	1	-	-	-	-
ypT1	-	2	2	-	-
ypT2	-	-	4	2	-
ypT3	-	1	11	14	3
Lymph nodes involvement[2,c]					
ypN0	1	3	14	8	-
ypN1	-	-	1	5	2
ypN2	-	-	2	3	1

[1]Spearman R correlation $r = 0.47$; [a]$P < 0.005$, comparison between different local tumor stages. [2]Spearman R correlation $r = 0.47$; [c]$P < 0.005$, comparison between different lymph nodes involvements.

Table 4 Relationship between tumor stage and NG

	NG 1	NG 2	NG 3
Local tumor stage[1,b]			
ypT0	1	-	-
ypT1	3	1	-
ypT2	1	1	4
ypT3	4	4	21
Lymph node involvement[2,a]			
ypN0	9	5	12
ypN1	-	-	8
ypN2	-	1	5

[1]Spearman R correlation $r = 0.42$; [b]$P < 0.01$, comparison between different local tumor stages. [2]Spearman R correlation $r = 0.45$; [a]$P < 0.005$, comparison of lymph node involvement.

0 patients in group R and 17 (58.6%), 6 (20.7%) and 6 (20.7%) in group NR (NS). A trend ($P < 0.12$) indicating a relationship between the number of lymph nodes assessed and "T-downstaging" was found. Median (range) number of lymph nodes in group R was 11 (3-35), and 17 (3-41) in group NR. The number of involved lymph nodes in the group R (median, range) did not differ from the number of nodes in group NR, 0 (0-1) and 0 (0-35) (NS), respectively. Tumor grade G1 was found in 2 (20.0%) patients, G2 in 7 (70.0%), and G3 in 1 (10.0%) patient in the R group, and in 3 (10.3%), 25 (86.2%), and 1 (3.5%) patients in the NR group (NS); one (2.5%) case at the ypT0 stage had not been taken into account. Median (range) tumor diameter in groups R and NR was 26 (10-65) mm and 35 (10-70) mm, respectively (NS). The relationship between "T-downstaging" and TRG staging as well as the NG stage was at the borderline of statistical significance. The relationship between TRG and NG *vs.* local tumor stage and lymph node status is shown in Tables 3 and 4. Patients with ypT1 were diagnosed as TRG 2-3. Patients with TRG5 were classified as ypT3. No lymph node metastases were found in patients with TRG 1-2 (ypN0). None of the patients without lymph nodes metastases were diagnosed as TRG 5. Patients in the ypT1 stage were diagnosed as NG 1-2. No lymph node metastases were found in NG 1. There was a relationship between TRG and NG (correlation $R = 0.58$, $P < 0.01$). Patients with TRG 1-2 were classified as NG 1. Patients with TRG 5 were diagnosed as NG 3.

DISCUSSION

Ultrasound-histopathological tumor regression parameter, "T-downstaging" represents a simple marker of rectal cancer radiosensitivity both in patients submitted to short-term preoperative radiotherapy[11,18,19,44,45] as well as in patients with surgery delayed by 1 to 8 wk following irradiation[17,20,21,23,25-28,34-36,39,46-49]. Reports have been published showing the prognostic value of "T-downstaging" for overall survival[17,28,34], cancer-specific survival[48], recurrence-free survival[48] , disease-free survival[25,28], local recurrence risk[26,34,48], and the risk of distant metastases[48]. Read *et al*[50] showed that the local staging following neo-adjuvant therapy enables the risk assessment of lymph node metastases. This finding may prove to be of significance during planning of surgical treatment. The percentage of patients with "T-downstaging" in the group submitted to long-term radiotherapy and radiochemotherapy ranged from 23/88 (26.0%) to 15/20 (75.0%)[14,17,20-24,27,34-36,39,46-49,51,52]. Among patients submitted to short-term preoperative radiotherapy "T-downstaging" ranged between 10/28 (35.7%) and 44/104 (43%)[11,14,18,19]. An alternative way for the assessment of local tumor stage decrease is comparison of ypT in patients from study groups and control groups in randomized trials on the effects of neo-adjuvant therapy[53]. Results of randomized studies on effects of short-term preoperative radiotherapy with a dose of 25 Gy on local tumor stage were discrepant[8,44]. In the presented material, "T-downstaging" was achieved in 11/40 (27.5%) patients. No correlation between "T-downstaging" and lymph node involvement, tumor grade and its diameter were found. In patients submitted to neo-adjuvant therapy the number of assessed lymph nodes is usually lower than in patients treated with surgery only [54]. In the present study, a tendency towards statistical significance ($P < 0.12$) of the correlation between "T-downstaging" and the number of evaluated lymph nodes was observed. More lymph nodes were found in patients with local stage ypT3 (group NR) than in those with ypT0-2 stage. Joseph *et al*[55] showed that in patients with colon cancer at T1/T2 stage more lymph nodes must be studied than in patients with T3/T4 in order to reliably define stage pN0. However, frequently the surgical approach is completely different in patients with lower local stage a limited lymph node resection is performed[57].

In the presented study, "downstaging" parameter was evaluated exclusively in order to show cancer regression within the rectal wall ("T-downstaging"). This results from the fact that the sensitivity of ultrasound evaluation of affected lymph nodes prior to radiotherapy is probably not sufficient to make a reference point for other, strictly histopathological tumor regression classifications. The accuracy of ultrasound examination in the evaluation of lymph node involvement is 65%-81% and the accuracy of the local tumor stage assessment is 82% to 93%[3]. Another argument against uN parameter in the evaluation of rectal cancer regression is that uN is of

no prognostic significance[17,49]. Some authors studied "N-downstaging" parameter[17,20,22-26,36,47] and showed its prognostic value[17]. The percentage of patients submitted to radiochemotherapy or radiotherapy with long time intervals between neo-adjuvant treatment and surgery, in which "N-downstaging" was noted, ranged from 13/26 (50.0%) to 38/42 (90.4%)[17,20,22-26,36,47]. Tumor size decrease, 'sterilization' and lymph node atrophy are the classic effects of radiotherapy[20,44,45]. Graf *et al*[53] showed that short-term preoperative radiotherapy results in decreased risk of lymph node involvement.

TRG 1 indicates that no cancer cells have been identified in the rectal wall[18,19,21,29]. Some researchers refer TRG 1 to patients with no cancer cells in the entire post-surgical specimen[37]. The term-pathological complete response (pCR) of rectal cancer to preoperative radiotherapy regards the situation in which histopathological examination does not show the neoplasm in the rectal wall, lymph nodes and mesorectum[25,26,35-38,46,48-50,57-61]. This is in accordance with the definition developed by the WHO initiative[62]. A stage of pCR is sometimes identified with ypT0N0 - the situation in which there is no evidence of neoplastic tissue in the rectal wall and in the lymph nodes[17,23]. Cases with only a few residual cells or small clusters of cells detected in histopathological examination of surgical specimens are by some authors classified as pCR[63]. In the presented study, the authors have assumed that the term pCR represents the situation in which no cancer cells were found in the surgical specimen. There is no absolute concordance between pCR and clinical complete response (assessed by per rectum digital examination and in proctoscopy): pCR may regard barely 25.0% of patients submitted to long-term preoperative chemoradiotherapy with clinical complete response[61]. A complete response to radiotherapy in comparison with the presence of residual cancer tissue is associated with better overall survival rate[46], longer disease-free survival[25], and lower risk of local recurrence[46]. However, some authors claim that complete regression is of no prognostic significance[37]. Guillem *et al*[59] did not show any differences in long-term prognosis among patients with complete cancer regression in comparison with almost complete response ($\geqslant$ 95.0% regression) to neo-adjuvant therapy.

Demonstrating a complete remission is important not only because of its prognostic value but also because of the need of assessment of indications for the postoperative chemotherapy or radiotherapy, for the decision about the appropriate method of surgery[20,37,45,46,49,57,58,63,64] or to compare the effects of different treatment methods[45]. Zmora *et al*[58] showed that metastases to regional lymph nodes and cancer cells in the mesorectal tissue may be present in patients with complete tumor regression within the rectal wall (TRG 1, ypT0)[58]. However, neo-adjuvant therapy makes it possible to reduce the percentage of patients submitted to abdomino-perineal resection and, in some cases, to perform local tumor excision[15,25,41,49,64-67]. Randomized study conducted by Polish researchers on a group of 316 patients treated with long-term radiochemotherapy or short-term preoperative radiotherapy did not show differences in terms of sphincter preservation rate (58% *vs* 61%, P = 0.57)[67]. Appropriate selection of the study patients treated with local excision is a very important issue[41,64-66,68]. The local tumor stage seems to be a reliable predictor of lymph node regression in these patients[41,64]. The assessment of eventual residual cancer, local stage (ypT), surgical clearance in the resection margins in patients submitted to local resection may reveal the necessity of immediate radical resection (performed within 30 d after the primary surgery)[65,66,68]. Also, intraoperative frozen section may prove useful for the assessment of tumor stage and margins' status. In cases in which a more advanced stage (pT2 or pT3) is likely to be found at the time of surgery or where the surgical clearance could be doubtful, the patient should be prepared for the possibility of wide excision at the same operation[66].

Another interesting issue is the assessment of cancer regression following neo-adjuvant therapy with use of TRG classification on intraoperational microscopic examination. In particular, this regards patients with an evident but incomplete regression. One could expect that the lacking concordance between local tumor stage ypT and TRG in post-operative histopathological examination, as mentioned above, apply also to intra-operation evaluation[20,35,39]. Considering the fact, that local excision following neo-adjuvant treatment is a therapeutic option for carefully selected patients, it could be eventually considered in patients with an evident but incomplete tumor regression. These patients are characterized by a low risk of local recurrence[18,22]. In the present study we have observed 1 case of coincidence of ypT3 and TRG 2. In the absence of reliable alternative methods, microscopic examination plays an important role in the evaluation of cancer regression following neo-adjuvant treatment. Digital rectal examination, computerized tomography, transrectal ultrasound examination and magnetic resonance are of limited value in terms of assessment of residual cancer following long-term pre-operative radio- and radiochemotherapy, especially to demonstrate pCR[61,69]. However, Gavioli *et al*[70] believe that TRUS is a very useful tool, when the same experienced operator performs it before and after neo-adjuvant treatment since it leads to demonstrate tumor regression in a qualitative and quantitative way. Moreover, they proposed that TRUS performed 6-8 wk following irradiation makes it possible to visualize fibrous changes only, which does not, however, disqualify this diagnostic method. The extent of fibrosis indicates the possible depth of residual cancer infiltration-cancer cells are believed to be present within fibrous areas only[70]. The use of magnetic resonance volumetry may also be useful in quantitative assessment of cancer regression following neo-adjuvant treatment[71]. The difficulties in achieving high level of reliability of visualizing diagnostic methods result from similar signal intensity (echogenity) between residual cancer, fibrous tissue, mucus pools and peritumoral inflammatory infiltration[71]. The use of 18-fluorodeoxyglucose positron emission tomography may prove effective in assessment of tumor response to neo-adjuvant therapy[33]. Full thickness local excision still

remains an experimental treatment method[64].

The significance of pCR following radiotherapy has not been ultimately confirmed. It is possible that better long-term survival in patients with pCR results from different biological properties of the tumor. Also, interesting reports have been presented, showing that patients submitted to neo-adjuvant chemotherapy and receiving statins showed higher a pCR rate[72]. The percentage of patients with complete regression following long-term radiochemotherapy and radiotherapy ranged from 1/43 (2.3%) to 7/20 (35.0%)[5,17,20-21,23-27,35-37,47-52,57-61,67,73]. The percentage of patients presenting complete cancer regression following short-term radiotherapy ranges between 0% and 10/191 (5.2%)[10,11,18,19,50]. In the present study we have observed 1 case (2.5%) with complete tumor regression.

The period between the termination of neo-adjuvant therapy and surgery in long-term radiochemotherapy and radiotherapy schemes is a few days and a few weeks, respectively. One may assume that short-term radiotherapy will result in relatively lesser tumor regression[10,23,63]. The results obtained in large study groups indicate that the short-term radiotherapy results not only in a decrease of the tumor diameter[44,45,53], but also in decreased number of affected lymph nodes[53]. A decrease in tumor diameter is not, however, equivalent with the decrease in local extent of tumor. Some authors believe that the period shorter than 10 d is insufficient to achieve tumor regression following radiotherapy with a dose of 25 Gy[45]. The proposition of the role of the time period between neo-adjuvant treatment and the percentage of pCR has its supporters[26,63] and opponents[24,73]. It was, however, shown that the period of a few days between the termination of neo-adjuvant therapy and surgery is sufficient enough for the development of morphological changes within the tumor and in its gene expression profile[44,74].

It was demonstrated that there is a relationship between TRG and overall survival, disease-free survival, and the risk of local tumor recurrence[18,22]. Other authors suggest that TRG estimation does not enable long-term prognosis in patients with rectal cancer[37]. There are some doubts regarding the reliability of this classification due to its subjective nature[20]. Interobserver variability of the TRG system was found to be satisfactory (kappa 0.64) or mediocre (kappa 0.44). It is higher when a 5-point system is simplified to 3-point[21,38]. For the reliability assessment it is important that significant fibrosis may accompany neoplastic tissue even when no neo-adjuvant therapy had been administered[38]. In the presented study, we have not shown unequivocal correlation between "T-downstaging" and TRG. We have found a correlation of TRG and ypT parameters. The reliability of TRG as a lymph node predictor is not unequivocal. Veccio *et al*[22] showed that lymph node involvement is not observed in 41/45 (91.0%) patients submitted to long-term preoperative chemoradiotherapy at TRG 1-2 stage. Kim *et al*[41] showed that histopathological assessment of tumor response to preoperative long-term radiochemotherapy (performed with use of the method described by Dworak *et al*[32], similar to the TRG system) is, along with ypT, an independent predictor of lymph node involvement. In the present study, ypN0 stage was observed in all patients with TRG 1-2. We have not found any definite relationship between "T-downstaging" and the NG stage. The results indicate the correlation between TRG and NG. However, these classifications are based on the evaluation of different morphological parameters. Rectal Cancer Regression Grade (RCRG) classification proposed by Wheeler *et al*[20,39] is next to the TRG system and Dworak *et al*[32] classification, one of the most widely used in studies documenting rectal cancer regression following neo-adjuvant therapy. It defines 3 degrees of tumor regression: 1, no cancer nests or microscopic collections of cancer cells embedded in fibrous stroma; 2, residual neoplasm seen grossly but with evident fibrosis; 3, carcinoma seen grossly with discreet or absent fibrosis. According to some researchers, such distinguishing of neoplastic tissue and fibrosis is not reliable[38,58]. Due to these reservations and the retrospective nature of the study, this grading system had not been taken into consideration in the present study.

At present, there is no uniform, widely accepted histopathological classification used for the evaluation of rectal cancer regression following preoperative radiotherapy. As far as the need for evaluation of residual cancer raises no objections, its interpretation and clinical consequences of radiation-induced changes in the rectal wall and within the tumor are not clear[5,27,44,74]. It is also unclear to what extent the presence of necrosis one may assign to its radiotherapeutic effect and to what extent it is a result of ischemic changes due to local perfusion disturbance. Fibrosis that accompanies neoplastic tumor may reflect both natural protective body mechanisms as well as being a result of chronic inflammation[7]. Mucin pools in tissues previously occupied by neoplastic tissue are qualitatively different from changes described as colitis cystica profunda, which may develop within the normal intestinal wall following radiotherapy[20]. The presence of mucin pools (induced mucinous carcinoma, colloid response) should be taken into account in the differential diagnosis of mucus-secreting adenocarcinoma[28,74]. The prognostic value of other morphological changes observed within the residual neoplastic tissue (the intensity and the nature of inflammatory infiltrations accompanying fibrous tissue and cancer cell clusters, cancer cell nuclear pleomorphism and hyperchromasia, mucinous cancer component, low tumor histological grade) and in the intestinal wall (surface ulceration, dysplastic changes, low-grade adenoma component) has not been unequivocally established[27,44,74]. Figures 1-6 show examples of neo-adjuvant therapy induced changes.

The retrospective nature of the presented study and the relatively small group of study patients impose careful interpretation of the presented results. Few reports on "T-downgrading", TRG and NG in patients submitted to short-term radiotherapy according to the regimen presented make the presented results suitable for further prospective studies on a larger population.

In conclusion, histopathological classifications based on the assessment of regressive changes may be useful in the

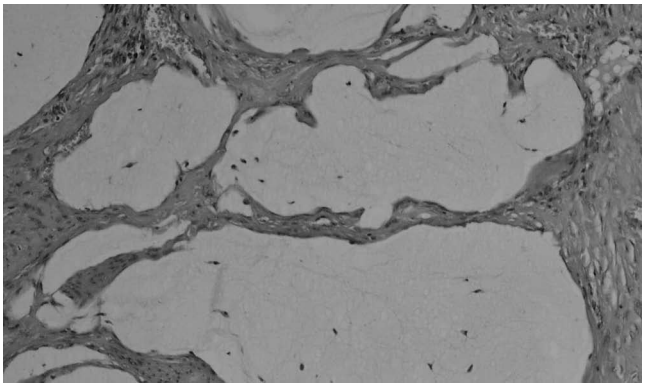

Figure 1 Acellular mucin pools in the intestinal wall (HE x 200).

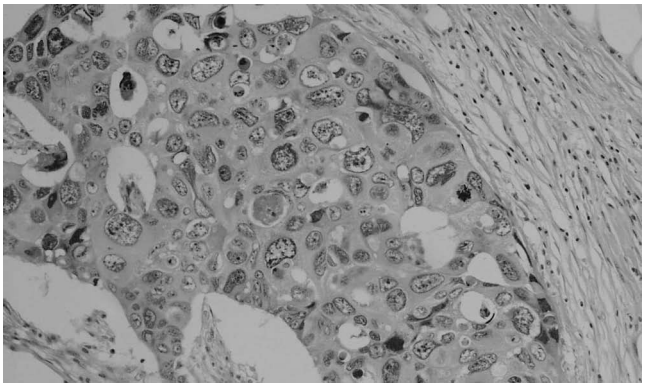

Figure 4 Degenerated adenocarcinoma cells following radiotherapy (HE x 125).

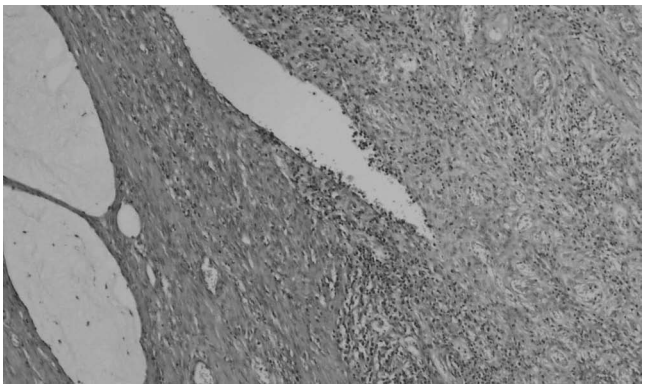

Figure 2 Complete tumor regression following radiotherapy. Inflammatory infiltrations, mucin pool and focal fibrosis in the stroma (HE x 64).

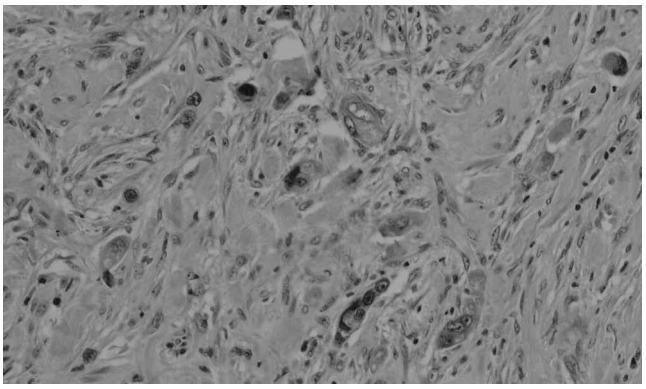

Figure 5 Dispersed degenerated adenocarcinoma cells following radiotherapy (HE x 125).

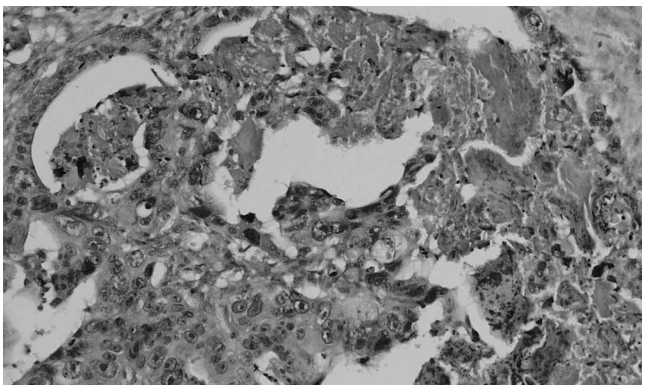

Figure 3 Degeneration and necrosis of tumor cells following radiotherapy (HE x 250).

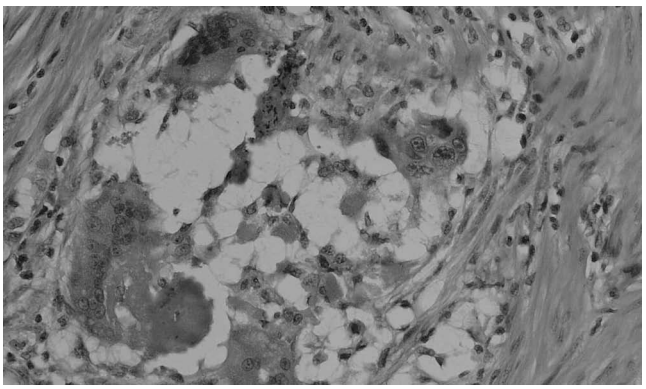

Figure 6 Macrophages and multinucleated (giant) cells close to necrotic tumor areas (HE x 200).

monitoring of effects of hyperfractionated preoperative radiotherapy in patients with rectal cancer at the initial stage of cT3NxM0. There is no unequivocal relationship between "T-dowstaging" and the tumor regression assessed with TRG and Nasierowska-Guttmejer classification. Poor tumor regression was seen more frequently in patients with no evident "T-downstaging". No relationships have been found between "T-downstaging" and lymph node involvement, tumor histological grade or tumor diameter. There is a clear but limited concordance in the assessment of regressive changes with ypT and TRG or NG. TRG and NG classifications are probably of limited predictive value in terms of lymph node involvement. There is a non-coincidental relationship between the assessment of radiation-induced regressive changes with use of TRG and NG classifications.

It is possible that immunohistochemical evaluation or molecular biology techniques applied to pre-operative biopsy samples may prove to be of predictive value in the future[21,34,52,75]. Undoubtedly, histopathological evaluation of the neoplastic tissue regression following preoperative radiotherapy is very important and necessary,

since ultrasound examination here is of limited reliability. Histopathological evaluation of rectal cancer regression may also prove to be useful for the evaluation of the effectiveness of future radio- and radio-chemotherapeutic treatment methods. It might also enable to isolate the population of rectal cancer patients in whom the adjuvant treatment would be especially justified.

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Sequential algorithms combining non-invasive markers and biopsy for the assessment of liver fibrosis in chronic hepatitis B

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Abstract

AIM: To assess the performance of several non-invasive markers and of our recently proposed stepwise combination algorithms to diagnose significant fibrosis (F $\geqslant$ 2 by METAVIR) and cirrhosis (F4 by METAVIR) in chronic hepatitis B (CHB).

METHODS: One hundred and ten consecutive patients (80 males, 30 females, mean age: 42.6 ± 11.3) with CHB undergoing diagnostic liver biopsy were included. AST-to-Platelet ratio (APRI), Forns' index, AST-to-ALT Ratio, Goteborg University Cirrhosis Index (GUCI), Hui's model and Fibrotest were measured on the day of liver biopsy. The performance of these methods and of sequential algorithms combining Fibrotest, APRI and biopsy was defined by positive (PPV) and negative (NPV) predictive values, accuracy and area under the curve (AUC).

RESULTS: PPV for significant fibrosis was excellent (100%) with Forns and high (> 92%) with APRI, GUCI, Fibrotest and Hui. However, significant fibrosis could not be excluded by any marker (NPV < 65%). Fibrotest had the best PPV and NPV for cirrhosis (87% and 90%, respectively). Fibrotest showed the best AUC for both significant fibrosis and cirrhosis (0.85 and 0.76, respectively). Stepwise combination algorithms of APRI, Fibrotest and biopsy showed excellent performance (0.96 AUC, 100% NPV) for significant fibrosis and 0.95 AUC, 98% NPV for cirrhosis, with 50%-80% reduced need for liver biopsy.

CONCLUSION: In CHB sequential combination of APRI, Fibrotest and liver biopsy greatly improves the diagnostic performance of the single non-invasive markers. Need for liver biopsy is reduced by 50%-80% but cannot be completely avoided. Non-invasive markers and biopsy should be considered as agonists and not antagonists towards the common goal of estimating liver fibrosis.

INTRODUCTION

Chronic hepatitis B (CHB) remains a serious global health concern. Approximately 350 million people are chronically infected, and 500 000 to 1.2 million deaths per year are attributed to HBV-associated complications[1]. Among patients with active viral replication, cirrhosis will develop in 15 to 20 percent within five years[2]. For patients with cirrhosis, acute exacerbation can occur and the disease may progress to end stage complications[2]. The histopathological pathway of progressive liver disease is characterised by the formation and accumulation of fibrosis, leading to increasing distortion of the hepatic architecture, that is the hallmark of evolution to cirrhosis. Liver fibrosis is the result of chronic injury and plays a direct role in the pathogenesis of hepatocellular dysfunction and portal hypertension. Current guidelines recommend that patients with HBV-DNA > 10^5 copies/mL and persistent or intermittent elevation in aminotrasferase levels should be evaluated further with liver biopsy, that is the gold standard for the assessment of fibrosis[3]. This procedure provides information on the severity of necroinflammatory activity and on the stage of fibrosis, features which are essential for estimating prognosis and the need for antiviral therapy[2,4,5]. However, biopsy is a costly procedure associated with side effects and some risks[6-8]. It also has limitations in underestimating liver fibrosis with small samples and is prone to intra- and inter-observer variation[9-12]. Moreover, several studies suggested that liver biopsy is far away from being a perfect gold standard since its performance is size-dependent[9,13-14]. Some studies would suggest that an adequate liver biopsy sample should contain more than 5 portal tracts and be at least 15 mm in length[11,15,16]. In a critical review of the literature concerning the use of liver biopsy in chronic viral hepatitis,

Guido and Rugge suggest that in an era of evidence-based medicine the use and interpretation of liver biopsy is very often flawed by unacceptable methodological limits and that a biopsy sample of 20 mm or more containing at least 11 complete portal tracts should be considered reliable for adequate grading and staging[14]. Other authors have recommended even bigger samples[17]. The pathologist need for obtaining a liver sample of adequate size is in contrast with the patient's need of a procedure causing limited pain and with the clinician's need of a safe procedure. A French survey which interviewed 1177 general practitioners concluded that liver biopsy may be refused by up to 59% of patients with chronic hepatitis C and that 22% of the physicians share the same concern regarding this invasive procedure[18]. In this regard, a recent survey assessing the consensus among Italian hepatologists on when and how to take a liver biopsy in chronic hepatitis C showed great divergence in the management of the same subgroup of patients[19]. Considering these limitations and patient reluctance to undergo liver biopsy, a great interest and many studies have been recently dedicated to the development of non-invasive markers as surrogates of liver biopsy. Most of the studies on non-invasive markers of liver fibrosis have been conducted in chronic hepatitis C and few data are available on the applicability of this approach to patients with CHB. Several markers have been described with variable diagnostic accuracy in hepatitis C, but the expected rate of misdiagnosis for each single test is still around 20%[11,20]. To overcome this limitation, recently we have developed and validated sequential algorithms that combine non-invasive markers with liver biopsy[21]. This approach allowed us to reach excellent diagnostic accuracy (> 95%) for both significant fibrosis and cirrhosis in patients with chronic hepatitis C with around 50%-70% reduced need of taking a liver biopsy. We have now assessed the performance of several non-invasive markers and of our stepwise algorithms in patients with CHB.

MATERIALS AND METHODS

Patients

This study included 110 consecutive patients with a diagnosis of chronic HBV infection, as defined by positive hepatitis B surface (HBsAg) for at least 6 mo, who underwent a diagnostic percutaneous liver biopsy at the Department of Clinical and Experimental Medicine at the University of Padova between March 2003 and June 2005. All patients were positive for serum HBV-DNA by polymerase chain reaction (PCR) and had compensated chronic HBV infection. The exclusion criteria were any other cause of chronic liver disease, and clinical signs of liver cirrhosis, co-infection with HCV or HIV and co-morbidities that could confound the results of the non-invasive markers adopted, clinical signs of liver cirrhosis. These included current alcohol intake (> 20 g/die), haemolysis, Gilbert's syndrome, and hematologic causes of thrombocytopenia. All biopsies were obtained with the 16G Menghini type needle. To limit the risk of fibrosis underestimation, patients with biopsy samples shorter than 1.5 cm or containing less than 7 portal tracts were excluded[11,15,16,22]. Informed consent was obtained from all patients participating in the study that was conducted according to the rules of the Declaration of Helsinki.

Virologic assays

HBsAg, hepatitis Be antigen (HBeAg), and antibodies to HBeAg and HDV were determined using commercial assays (Roche Diagnostics, Basel, Switzerland). HBV DNA level was measured by real-time PCR and expressed as $\log_{10}$ copies/mL.

Histological assessment

Liver biopsies were fixed in formalin and embedded in paraffin. The slides were stained with hematoxylin-eosin, van Gieson stain for collagen, PAS after diastase digestion and Perls' Prussian blue method. The slides were evaluated by a single Pathologist (MG) who was unaware of the clinical data. Fibrosis was scored according to the METAVIR system, which was previously applied in other reports on CHB[23-25]. Fibrosis was staged from F0 to F4: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Significant fibrosis was defined as a METAVIR score of F2 or more ($F \geqslant 2$), cirrhosis was defined as a METAVIR score of F4.

Non-invasive markers of liver fibrosis

All patients were evaluated for AST-to-Platelet Ratio Index (APRI), Forns' index, AST-to-ALT ratio (AAR), Hui's model, Goteborg University Cirrhosis Index (GUCI), Fibrotest. The rationale for the choice of the non-invasive markers was their simplicity together with a reported good performance for APRI, Forns' index, AAR and GUCI, and the high number of validation studies reported together with a good performance for Fibrotest[26-30]. Hui's model, based on a combination of body mass index, total bilirubin, platelets and albumin, was chosen since it is the only non-invasive marker developed in patients with hepatitis B[31]. The markers were all calculated using fasting serum samples obtained on the same day of liver biopsy. For this purpose platelet count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamiltranspeptidase (γGT), cholesterol levels, haptoglobin, apolipoprotein A1, alpha-2-macroglobulin, total bilirubin, prothrombin time (international normalised ratio, INR), albumin were routinely determined using validated methods. Fibrotest results were kindly provided by T. Poynard, Universite Paris VI, Paris, France. For all the non invasive methods the cut-off values indicated in the original reports were applied[26-31].

Stepwise combination algorithms for liver fibrosis

The algorithms recently developed by us for patient with chronic hepatitis C were applied to this cohort of hepatitis B patients. The diagnostic algorithms were developed by modelling the best algorithm for liver fibrosis in different clinical scenarios, as described in a previous study[21]. Algorithm A (for significant fibrosis, $F \geqslant 2$ by METAVIR) and algorithm B (for cirrhosis, F4 by METAVIR) are described in Figure 1A and B.

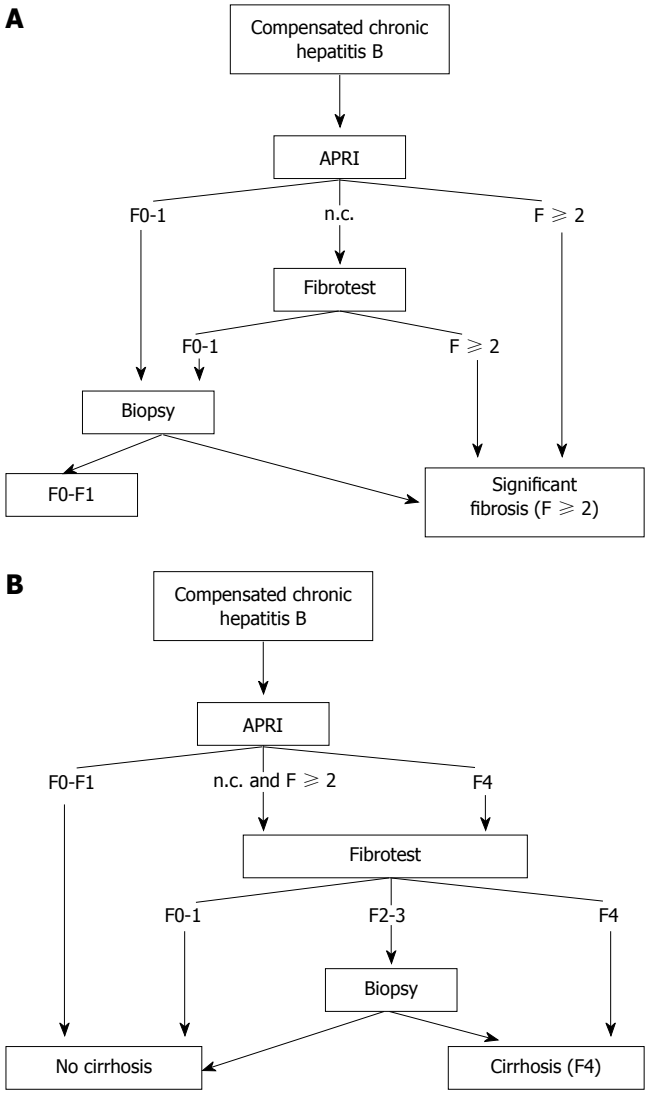

Figure 1 **A**: Algorithm A for detection of significant fibrosis (F ⩾ 2 by METAVIR) in HBV patients. F0-1 by APRI is intended as APRI < 0.5. F ⩾ 2 by APRI is intended as APRI > 1.5. n.c. by APRI is intended as APRI > 0.5 and < 1.5; **B**: Algorithm B for detection of cirrhosis (F4 by METAVIR) in HBV patients. F0-1 by APRI is intended as APRI < 0.5. F ⩾ 2 by APRI is intended as APRI > 1.5. F4 by APRI is intended as APRI ⩾ 2. n.c. by APRI is intended as APRI > 0.5 and < 1.5.

Statistical analysis

The primary endpoints were the detection of significant fibrosis (F ⩾ 2) and cirrhosis (F4). These thresholds were selected since the first is generally considered an indication for antiviral therapy and the second requires a specific management and follow-up. Descriptive results were expressed as mean ± standard deviation (SD) or number (percentage) of patients with a condition. Kappa statistics was used to measure intra-observer variation in the histopathological evaluation of the degree of fibrosis. The performance of the non-invasive methods for liver fibrosis was measured as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, and likelihood ratios (LR). Sensitivity, specificity, PPV, NPV and accuracy were expressed as percentage. The diagnostic value of the non-invasive methods was expressed using the Area Under the receiver operating characteristic Curve (AUC) and its corresponding 95% confidence intervals (CI).

Table 1 Demographic, laboratory and histological characteristics of the 110 patients with chronic hepatitis B

Males *n* (%)	80 (72.7)
Age (mean yr ± SD)	42.6 ± 11.3
BMI (kg/m^2)	24.2 ± 3.3
AST (mean IU/L ± SD)	73.4 ± 61.2
AST / ULN ratio (mean ± SD)	1.75 ± 1.47
ALT (mean IU/L ± SD)	144.5 ± 148.0
ALT / ULN ratio (mean ± SD)	3.14 ± 3.3
γGT (mean IU/L ± SD)	46.4 ± 49.1
γGT / ULN ratio (mean ± SD)	0.81 ± 0.84
Bilirubin (mean μmol/L ± SD)	13.9 ± 6.48
PLT (mean 10^9/L ± SD)	194.6 ± 56.9
Albumin (mean g/L ± SD)	42.7 ± 5
Cholesterol (mean mg/dL ± SD)	177.5 ± 32.9
INR (mean value ± SD)	1.12 ± 0.1
Haptoglobin (mean g/L ± SD)	1.05 ± 0.6
α_2M (mean g/L ± SD)	2.67 ± 0.84
ApoA1 (mean g/L ± SD)	1.48 ± 6.48
Viral load (mean log$_{10}$ cp/mL ± SD)	2.15 ± 1.18
HBeAg positive cases (%)	20 (18.2)
HDV co-infected cases (%)	8 (7.3)
Staging *n* (%)	
F0	15 (13.6)
F1	20 (18.2)
F2	40 (36.4)
F3	13 (11.8)
F4	22 (20.0)

SD: standard deviation; ULN: upper limits of normal;PLT: platelets; INR: international normalised ratio; α_2M: alpha-2-macroglobulin; ApoA1: apolipoprotein A1.

RESULTS

Demographic, laboratory and histological features of the 110 patients with CHB are described in Table 1. Mean age was 42.6 ± 11.3 years and 80 patients (72.7%) were males. Twenty cases (18.2%) were HBeAg positive and 8 cases (7.3%) were co-infected with HDV. Prevalence of significant fibrosis (F ⩾ 2) and cirrhosis was 68.2% and 20%, respectively. The mean length of liver specimens was 1.69 ± 0.29 cm and mean complete portal tracts number was 9.9 ± 3.6. Intra-observer agreement was assessed by re-evaluating a subset of 50 randomly chosen samples: k value was higher than 0.90.

Performance of non-invasive methods for the diagnosis of significant fibrosis (F ⩾ 2)

Seventy-five patients (68.2%) had significant fibrosis as defined by METAVIR fibrosis stage F ⩾ 2. The performance of the non-invasive markers in diagnosing significant fibrosis is shown in Table 2. AAR was not included here since it identifies cirrhosis but does not discriminate significant fibrosis. Fibrotest, GUCI and Hui' s model classified all cases while both APRI and Forns' index were unable to classify one third of the patients. All the methods showed high PPV (> 90%) for significant fibrosis. Forns' index had an excellent 100% PPV with a 6.9 cut-off but its diagnostic value was quite low, at 0.63 AUC (95% CI: 0.50-0.76). The NPV was quite low for all the non-invasive markers (always < 65%), so that significant fibrosis could not be reliably excluded by any of these markers. Fibrotest, APRI and GUCI showed good overall

Table 2 Performance of the non-invasive methods and of the algorithm A in detecting significant fibrosis (≥ F2 by METAVIR) in patients with CHB

	Fibrotest	Forns		APRI		GUCI	Hui's model	Algorithm A
Classified cases (%)	100	63.3		66.2		100	100	100
Cut-off	F2	4.2	6.9	0.5	1.5	0.2	0.15	na
Sensitivity (%)	80.8	58.3	14.6	70.8	27.1	66.7	50	100
Specificity (%)	90	78.3	100	87	95.7	95.7	90.9	91.3
PPV (%)	95.5	90.6	100	94.1	97.9	97.9	96.3	96
NPV (%)	64.3	53.5	35.9	62.2	39.7	58.9	45.8	100
LR +	8.1	2.69	0.146	5.45	6.3	15.5	5.49	11.49
LR -	0.21	0.53	0.85	0.36	0.76	0.35	0.55	0
Accuracy (%)	83.3	64.8	42.3	76.1	49.3	76.1	62.2	97.2
AUC (95% CI)	0.85 (0.75-0.95)	0.63 (0.50-0.76)		0.72 (0.58-0.86)		0.81 (0.70-0.92)	0.71 (0.56-0.86)	0.96 (0.92-1)

APRI: aspartate aminotransferase to platelets ratio; GUCI: Goteborg University Cirrhosis Index; na: not available; PPV: positive predictive value; NPV: negative predictive value; LR+: positive likelihood ratio; LR-: negative likelihood ratio; AUC: area under the curve; CI: confidence interval.

Table 3 Performance of the non-invasive methods and of the algorithm B in detecting cirrhosis (F4 by METAVIR) in patients with CHB

	Fibrotest	APRI	AAR	GUCI	Algorithm B
Classified cases (%)	100	66.2	100	100	100
Cut-off	F4	2	1	1	na
Sensitivity (%)	55.6	42.9	7.1	21.4	92.9
Specificity (%)	96.3	85.4	94.7	91.2	96.5
PPV (%)	90	53.8	82.4	73.7	87.5
NPV (%)	87.1	91.1	81.4	83.8	98.3
LR +	15	2.94	1.34	2.43	26.5
LR -	0.46	0.67	0.98	0.86	0.07
Accuracy (%)	86.1	79.2	77.5	77.5	95.8
AUC (95% CI)	0.76 (0.67-0.85)	0.64 (0.53-0.75)	0.51 (0.39-0.62)	0.56 (0.47-0.65)	0.95 (0.90-1)

accuracy (83.3%, 76.1% and 76.1% respectively). Among the non-invasive methods, Fibrotest showed the best diagnostic value as indicated by an AUC of 0.85 (95% CI: 0.75-0.95).

Performance of non-invasive methods for the diagnosis of cirrhosis (F4)

Twenty-two patients (20%) had cirrhosis as defined by METAVIR fibrosis stage F4. The performance of the non-invasive markers in diagnosing cirrhosis is shown in Table 3. Forns' index and Hui's model were not considered here since they do not discriminate between significant fibrosis and cirrhosis. Fibrotest had the best PPV (90%) and very good accuracy (86.1%). The overall diagnostic value of Fibrotest was quite good, with 0.76 AUC (95% CI: 0.67-0.85). APRI showed good NPV and accuracy but the diagnostic value, described by AUC, was rather low (0.64; 95% CI: 0.53-0.75). The other markers showed an even lower diagnostic value, with AUCs around 0.5.

Performance of stepwise algorithms for the diagnosis of significant fibrosis and cirrhosis

The performance of stepwise algorithms for the detection of significant fibrosis (algorithm A) and cirrhosis (algorithm B) is reported in Tables 2 and 3, respectively. Table 4 describes in details the number of tests and of liver biopsies needed when the two algorithms were applied to our cohort of patients with CHB. The stepwise algorithm for significant fibrosis (algorithm A) excluded the presence of F ≥ 2 by METAVIR with excellent 100% NPV. It also showed a very high accuracy, with excellent 97.2%, and it presented with an excellent diagnostic value, with 0.96 AUC (95% CI: 0.92-1). This algorithm permitted avoidance of liver biopsy in about half of the cases (Table 4). Algorithm B showed excellent 95.8% accuracy and 0.95 AUC (95% CI: 0.90-1) in the identification of cirrhosis. Furthermore, this algorithm reduced by more than 80% the need for liver biopsy (Table 4).

DISCUSSION

Several non-invasive markers of liver fibrosis have been recently described, mainly in patients with hepatitis C, but their implementation in clinical practice as a substitute for invasive liver biopsy has been delayed by lack of adequate accuracy in assessing individual patients. Indeed, according to the most recent International Guidelines and Recommendations, inter-laboratory variability, lack of reproducibility and, most important, an expected rate of misdiagnosis of at least 20% do not yet allow the use of these methods in clinical practice[11,32]. Since the diagnostic performance of described non-invasive markers is variable depending on the stage of fibrosis and other patient characteristics, they can be used to reduce rather than completely substitute the need for liver biopsy. Recently we have described stepwise combination algorithms based on

the use of two non-invasive markers (APRI and Fibrotest) and liver biopsy[16]. When applied to patients with chronic hepatitis C these algorithms were proven to correctly identify significant fibrosis and cirrhosis with high (> 95%) accuracy and 50%-70% reduction in liver biopsy. Very few studies have investigated the role of non-invasive markers of liver fibrosis in hepatitis B. Indeed, significant differences exist between CHB and chronic HCV infection in natural history, laboratory parameters, liver histology and associated comorbidities. For example, elevated ALT reflects accurately the necroinflammatory activity of CHB and is used as one of the criteria for antiviral therapy while the same could not be applied to hepatitis C[3]. Steatosis is an important feature of chronic HCV infection while its role in CHB is unclear[33]. The association of diabetes mellitus with chronic hepatitis C has not been found in CHB[34]. Since CHB has specific pathogenetic mechanisms and is associated strongly with liver disease, the results of the studies on hepatitis C cannot be directly transferred to hepatitis B and a dedicated validation of the markers should be provided. The latest AASLD guidelines on management of chronic hepatitis B recommend that patients with HBV-DNA > 10^5 copies/ml and persistent or intermittent elevation in transaminase levels should be evaluated further with liver biopsy[3]. Moreover, prior to consider of antiviral treatment, liver biopsy is still recommended. Assessment of the stage of liver disease is indeed fundamental for treatment decision in any patient presenting with compensated chronic HBV infection. The available evidences suggest that non-invasive markers of liver fibrosis in hepatitis B present with a similar accuracy to hepatitis C. Lebensztejn *et al*[35] assessed the value of some non-invasive markers of liver fibrosis in few children with chronic hepatitis B and found that a combination of hyaluronan and laminin had 0.84 AUC. Hui and colleagues developed a predictive model based on body mass index and three routine laboratory tests, which showed 0.79 AUC[31]. Two recent reports applied Fibrotest in CHB showing 0.77 and 0.78 AUC for detection of significant fibrosis and cirrhosis, respectively[24,25]. Our results, based on an independent application of Fibrotest to CHB patients, showed an accuracy that is similar to that reported by Poynard's group. A very recent study by Zeng *et al*[36] proposed a non-invasive combination model based on alpha-2-macroglobulin, hyaluronan, age and γGT and it showed an AUC between 0.77 and 0.84. For all these markers, the expected rate of misdiagnosis was around 20%, thus similar to that reported for hepatitis C which is considered not satisfactory by many clinicians. Very recently the use of "proteome" technology has been introduced in studying liver fibrosis. In 46 patients with chronic hepatitis B, 30 features predictive of significant fibrosis and cirrhosis were identified. The AUC for this analysis was very promising, being 0.906 and 0.921 for advanced fibrosis and cirrhosis, respectively[37]. However, this is a quite complicated method that might not be available for large scale testing. Moreover, the excellent performance reported in that preliminary study should be confirmed by others. In our study we found that Fibrotest had the best performance when compared to other non-invasive methods. However, none of the investigated non-invasive markers of liver fibrosis had adequate accuracy for universal use in substitution of liver biopsy, the expected rate of misdiagnosis being 15%-35% for significant fibrosis and 25%-45% for cirrhosis. On the other hand, when APRI and Fibrotest were combined with liver biopsy in sequential algorithms, we could reach > 95% accuracy for detecting significant fibrosis or cirrhosis, with a 50%-80% reduced need for liver biopsy, as already described previously in patients with compensated chronic hepatitis C. With this approach, the number of liver biopsies needed decreased especially for the patients at higher risk of cirrhosis and this appears particularly important since the risk of liver biopsy complications is increased in cirrhotic cases. The overall cost of these algorithms appears favourable compared to universal use of liver biopsy. Indeed, for a cohort of one hundred patients algorithm A requires 100 APRIs, 34 Fibrotests and 52 biopsies while algorithm B requires 100 APRIs, 52 Fibrotests and 19 biopsies (Table 4). A cost-benefit analysis indicates that in the US a liver biopsy costs 1032 USD, which increases to 2745 USD when a complication occurs[8]. Fibrotest-Fibrosure is a commercialised method with a cost of around 90 euros (Biopredictive, Houilles, France). According to these values, algorithm A and algorithm B would result in a 50% and 75% reduction in cost compared to liver biopsy, respectively.

Table 4 Features of clinical interest of stepwise algorithms in chronic hepatitis B

	Algorithm A	Algorithm B
Saved biopsies (%)	48	81
APRI performed (%)	100	100
Fibrotest performed (%)	34	52
Under-diagnosed and unclassified (%)	0	0
Over-diagnosed (%)	3	3

Algorithm A: algorithm for significant fibrosis (F ⩾ 2 by METAVIR); Algorithm B: algorithm for cirrhosis (F4 by METAVIR); APRI: aspartate aminotransferase to platelets ratio.

There are some limitations in our study. This was in fact a retrospective study, with a quite limited number of cases. Another limitation could be in the choice of the dimension of biopsy sample. We have here included specimens of at least 1.5 cm length and containing 7 portal tracts on the basis of the recommendations of some authors[11,15,16,22]. However, several observations from the pathologists would suggest even bigger samples for a correct staging of liver fibrosis[13,14,17]. Finally, recent criticisms suggested that liver biopsy is not a perfect gold standard for fibrosis evaluation due to its large variability (sampling error plus observer error). Indeed, Bedossa *et al*[13] indicated that biopsy is an estimate of liver fibrosis which, when compared with the whole liver, showed a coefficient of variation greater than 40% with length greater than 15 mm with 80% accuracy.

In conclusion, this study suggests that in hepatitis B currently available non-invasive tests do not show a diagnostic performance that would be considered adequate by many clinicians. However, their stepwise combined use can be most useful to reduce the need for liver biopsy

without loosing diagnostic accuracy. In this respect liver biopsy and non-invasive markers should be considered as agonists and not as antagonists towards the common goal of correctly classifying the stage of liver fibrosis. Priority should be given to large scale validation studies of these algorithms in different patient populations inclusive of all major etiologies of chronic liver disease and most frequent cofactors, which may affect the diagnostic performance of fibrosis markers.

COMMENTS

Background

Non-invasive markers of liver fibrosis have been recently proposed as substitutes for liver biopsy but their reported accuracy was around 80%. They have been mostly validated in hepatitis C while few studies have been conducted in hepatitis B. We have recently shown that stepwise combination of non-invasive markers and liver biopsy permitted to obtain excellent accuracy (> 95%) by saving 50%-70% liver biopsies in hepatitis C. We applied our method to a cohort of patients with chronic hepatitis B.

Research frontiers

Nowadays many clinicians show concerns about the role of liver biopsy in chronic viral hepatitis due to side effects, intra- and inter-observer variation and costs. Some non-invasive methods for liver fibrosis have been proposed but International Guidelines still do not recommend a routine use of the markers due to lack of reproducibility and an expected misdiagnosis rate of 20%. Thus, a trusted method that avoides a number of liver biopsies by maintaining excellent accuracy is urgently needed.

Innovations and breakthroughs

In this article we validated in hepatitis B a recently proposed method for the detection of liver fibrosis and cirrhosis in hepatitis C. This is the first sequential approach based on a first line assessment by non-invasive markers of liver fibrosis followed by liver biopsy in unclassified cases or cases in which non-invasive methods do not reach a satisfactory accuracy. The overall accuracy of this method is > 95% and it saved 50%-80% liver biopsies. This is a rational and practical way to apply non-invasive markers in hepatitis B and it introduces a new concept: non-invasive markers and liver biopsy are agonists and not antagonists towards the common goal of classifying liver fibrosis.

Applications

The most accurate non-invasive markers should be used as a first line assessment, limiting liver biopsy to the cases in whom they are unclassified or show low predictive value. For the future, priority should be given to large scale validation studies of these algorithms and the most promising non-invasive markers in different patient populations inclusive of all major etiologies of chronic liver disease and most frequent cofactors which may affect the diagnostic performance of fibrosis markers.

Terminology

(1) Fibrotest: a commercial panel of serum markers combining γGT, alpha-2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin for the non invasive assessment of liver fibrosis. It has been extensively validated in hepatitis C. The overall accuracy of the panel is good but it combines also uncommon parameters. Only two, not independent, validation studies on hepatitis B have been so far conducted. (2) APRI: a simple test combining AST and platelet count group for the non-invasive prediction of significant fibrosis and cirrhosis in hepatitis C. It is a very simple and economic tool but it is somehow less accurate than fibrotest and it presents with a significant percentage of unclassified cases. To our knowledge, this is the first validation of APRI in an independent series of HBV patients. (3) Forns' index: an index combining, age, platelet, γGT, cholesterol for the non-invasive prediction of significant fibrosis in hepatitis C. It is a quite simple index, combining common parameters (except for cholesterol) but it showed a significant number of unclassified cases. To our knowledge, this is the first application of Forns' index to a cohort of patients with CHB. (4) GUCI: a simple index combining AST, platelets and INR. It showed good accuracy in hepatitis C for both significant fibrosis and cirrhosis. It has never been applied to HBV cases. (5) Hui's model: a panel combining albumin, BMI, total bilirubin and platelet count for the prediction of significant fibrosis. It has been developed for hepatitis B patients and no validation study has to date been conducted.

Peer review

Evaluate the applicability and prognostic value of a previously developed algorithm that includes a combination of two different serum marker tests for the detection of liver fibrosis to avoid liver biopsies in patients with chronic HBV infection. This is an excellent paper that investigates the performance of non-invasive tests for estimating liver fibrosis in patients with chronic hepatitis B. The study is timely and provides useful information.

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Serum-free culture of *H pylori* intensifies cytotoxicity

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Abstract

AIM: To perform a long culture passage of *H pylori* without serum, taking into account its cytotoxicity and the presence of the probable new cytotoxic factor.

METHODS: One sample of *H pylori* 60190 (ATCC 49503) was grown on Brain Heart Infusion (BHI) agar containing 0.5% 2,6-di-O-methyl-β-cyclodextrin without any serum, being passaged 70-100 times every 3-4 d for approximately 2 h, while another sample of *H pylori* contained 70 mL/L fetal calf serum without 2,6-di-O-methyl-β-cyclodextrin. Their supernatant and extract after 16 h in culture were evaluated for changes in cell morphology and for cell viability using HeLa cells. Furthermore, the characteristics of the probable cytotoxic factor in the extract were examined on partial purification studies and its cytotoxicity was evaluated in various human cells.

RESULTS: The supernatant and the extract of the bacterium grown on serum-free medium had strong cytotoxicity compared with those grown on serum-containing medium. They irreversibly damaged HeLa cells without vacuolation that was altogether different from that of the bacterium when grown with serum. Their cytotoxicity was easily measured by cell viability assay. The probable cytotoxic factor partially purified and detected by chromatography had characteristics difference from that of vacuolating toxin and a broad cytotoxicity toward various cell lines.

CONCLUSION: Serum-free long culture method of *H pylori* makes its supernatant and its extract cytotoxic enough to be easily measured by cell viability assay. The probable cytotoxic factor has a unique characteristic and might be a new cytotoxin.

Key words: *H pylori*; Cytotoxicity; Serum-free long culture

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INTRODUCTION

Irreversible degeneration and cell death were induced in gastric mucous epithelium infected with *H pylori* both in an *in vitro* and an *in vivo* study, including electron microscopic examination. These cells deteriorated without vacuolations, and biopsy specimens containing the organism obtained from gastric mucosa demonstrated a loss of microvilli and irreversible cell damage[1-4]. Even after eradication therapy of the bacterium, there were no significant differences in the histological scores and atrophic scores in the inflammation of the gastric body and the atrophy of the antrum[5-9]. However, the only known cytotoxic factor of *H pylori* is vacuolating toxin (Vac A), which causes vacuolations in assay cells 18-24 h after addition, but is a reversible and weak cytotoxin[10,11]. The clinical findings that the bacterium caused irreversible and severe destruction to the gastric lineage appear incompatible with the *in vitro* observations that *H pylori* produces only a reversible and comparatively weak cytotoxin that induces vacuolations. Whether there is any direct irreversible cytotoxic factor of *H pylori* remains to be determined. Although, so far, none of the studied factors met these requirements, there is some evidences that indicated cytoskeletal cell death or round-formed cell death differed from vacuolation-formed cell death[3,12,13].

To our best of knowledge, this is the first report about the cytotoxicity of *H pylori* after a long duration of culture without serum. We cultured *H pylori* 60190, plating many passages without serum for approximately two years, considering that the cell growth environments should be as near as possible to the actual intragastric ones. Here, we showed serum-free long period culture made the supernatant and the extract of *H pylori* dramatically cytotoxic such that it induced irreversible decayed cell death easily measurable with a cell viability assay. Cell death induced by these samples showed a rapid development of the cell death process and was rarely accompanied by vacuolation. These observations might explain clinical findings and develop into a new field of the bacterium's cytotoxicity.

MATERIALS AND METHODS

Materials

The *H pylori* strain 60190 was obtained from American Type Culture Collection. Brain Heart Infusion (BHI) was purchased from DIFCO Laboratories and 2,6-di-O-methyl-β-cyclodextrin was purchased from Wako Pure Chemical Industries Ltd.

Culture conditions and preparation of bacterial extract

H pylori 60190 (ATCC49503) was the source for purification. Bacterial cells were grown on Brain Heart Infusion (BHI) agar containing 5% fetal calf serum for 7-10 d, then transferred to BHI broth containing 2,6-di-O-methyl-β-cyclodextrin with gradual stepwise decreases, i.e., 5%, 2%, 1%, 0.5%, of the concentration for 2-4 d without serum and maintaining stable growth conditions, being passaged 100-120 times every 3-4 d. Then bacterial cells were scraped and cultured for 16 h at 37℃ in the liquid medium of BHI containing 0.5% 2,6-di-O-methyl-β-cyclodextrin without serum in an ambient atmosphere containing 50 mL/L CO_2, while agitating with a rotary shaker. Bacterial cells were collected by centrifugation at 12000 *g* for 20 min at 4℃ and suspended in 10 mmol/L Tris-HCl buffer (pH 7.7). The cells were washed once and sonicated at 54% effect for 30 s × 6 sets in Ultrasonic Disruptor (Tomy Seiko Co., Ltd.) and stored at -80℃ overnight. Sonically disrupted cells were thawed and sonicated again under the conditions previously described and centrifuged at 100000 *g* for 60 min at 4℃. The aliquots of the most upper layer were stored at -80℃ until use.

Cytotoxicity assay with cell viability

HeLa cells (Cell Resource Center for Biomedical Research, Tohoku University) cultured in Eagle's modified minimal essential medium containing 100 mL/L fetal bovine serum (MEM-FBS) were seeded into 96-well plates at a density of 10^4 cells per well. After 24 h, the cells were washed twice with PBS and challenged with 10 μL of aliquots of serial fraction or serial dilution of protein solution with 90 μL of MEM-FBS for a total volume of 100 μL per well. Cells were incubated at 37℃ for 24 h and cell viability was evaluated by WST-1 assay using Cell Counting kits (DOJINDO Laboratories). Each determination was performed in triple wells.

Purification of cytotoxic factor

Proteins in the bacterial extract were precipitated with a 700 g/L saturated solution of ammonium sulfate. After centrifugation at 12000 *g* for 20 min, the pellets were resuspended in 10 mmol/L Tris-HCl buffer (pH 7.7) and dialyzed against the same buffer.

The anion exchange chromatography was performed on DEAE Sephacel (Amersham Pharmacia Biotech UK Ltd.) with 10 mmol/L Tris-HCl buffer (pH 7.7). The cation exchange chromatography was CM Sepharose Fast Flow (Amersham Pharmacia Biotech UK Ltd.) with 10 mmol/L Tris-HCl buffer (pH 5.8). The proteins were eluted with the same buffer containing a linear gradient of 0-0.3 mol/L NaCl and 0-1.0 mol/L NaCl, respectively. Size exclusion chromatography was performed on a Superose 12 HR 10/30 column (Pharmacia) with buffer containing 10 mmol/L Tris-HCl (pH 7.7) and 0.15 mol/L NaCl at a flow rate of 0.25 mL/min.

RESULTS

Morphological change of HeLa cells

We cultured *H pylori* 60190 with or without serum for approximately two years. The serum-free culture method using 0.5% 2,6-di-O-methyl-β-cyclodextrin was less favorable for the bacterium and made it inclined to be easily autolysed in the culturing process. In this study, we defined the serum-free method to culture the bacterium using Brain Heart Infusion (DIFCO) and 0.5% 2,6-di-O-methyl-β-cyclodextrin without serum for approximately two years in semisolid medium, and the conventional method using Brain Heart Infusion and 70 mL/L serum without cyclodextrin in semisolid medium. After more than approximately 100 passages, both the supernatant and the extract of the bacterium cultured with the serum-free method induced characteristic morphological changes in HeLa cells, compared to the supernatant and the extract cultured using serum; the cells exposed to its supernatant showed a round-formed shape or small debris with a destroyed contour without vacuolation and the cells exposed to its extract showed an elongated or spindle-like shape with a decrease in cell number in 24 h, while the control cells exposed to the supernatant and the extract had scarcely changed, except for some vacuolations that were similar to the previously reported morphological change (Figure 1).

We observed that both HeLa cells exposed to the supernatant and the extract of the bacterium cultured without serum showed the round-formed change or round debris through the elongated and spindle-like shape without vacuolation. We examined whether HeLa cells exposed to the extract had undergone the same changes using a denser extract. We found that both of the HeLa cells exposed to the supernatant and the extract finally showed the round-formed or shrunken-formed change through the elongated or spindle-like form after 24 h. We compared the morphologic change of HeLa cells exposed to the extract cultured with serum and cultured for approximately one year without serum (Figure 2), and considered that both contained probably a similar cytotoxic factor.

The conventional search of cytotoxin in *H pylori* used the uptake of neutral red into the vacuole of sample-treated cells because the supernatant of the bacterium caused some vacuolations in cells[14], but scarcely any degeneration or remarkable decrease of the number of assay cells. No direct cell viability assay, therefore, was used in search of the cytotoxic factor of *H pylori*. However, we were able to successfully adopt this cell viability assay in place of the neutral red uptake method because of the radical cytotoxicity of the supernatant and the extract cultured without serum.

Cytotoxicity measured by cell viability assay

The cytotoxicity was determined with an assay using HeLa cell viability with WST-1 tetrazolium and a lactate

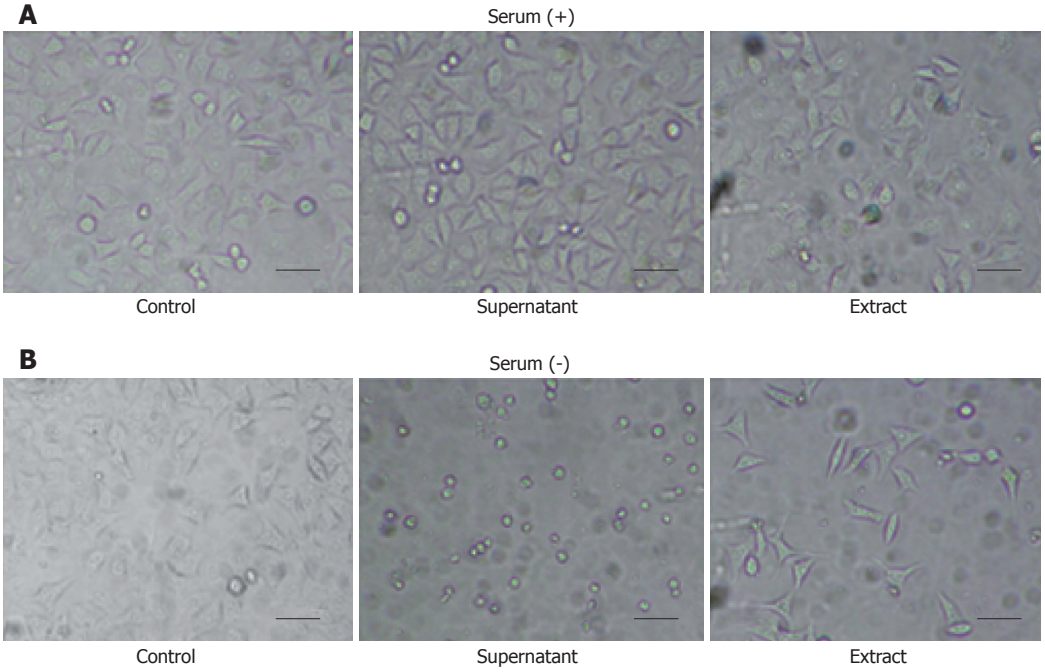

Figure 1 Morphologic change of HeLa cells caused by the supernatant and the extract of *H pylori* with or without serum after 24 h. HeLa cells were seeded at 5000 cells per well in every plate. The concentration of the supernatant cultured with or without serum was 2.1 mg/mL and the concentration of the respective extract was 0.5 mg/mL. **A**: HeLa cells exposed to the supernatant of the method using serum showed little change and the ones exposed to the extract also showed no remarkable changes but included a few minor vacuolations compared to the control; **B**: HeLa cells exposed to the supernatant of the method without serum showed a round-formed shape and a decayed contour with greatly decreased cell number, and those exposed to its extract showed an elongated or spindle-like shape with a decrease in cell number. Scale bar, 200 μm.

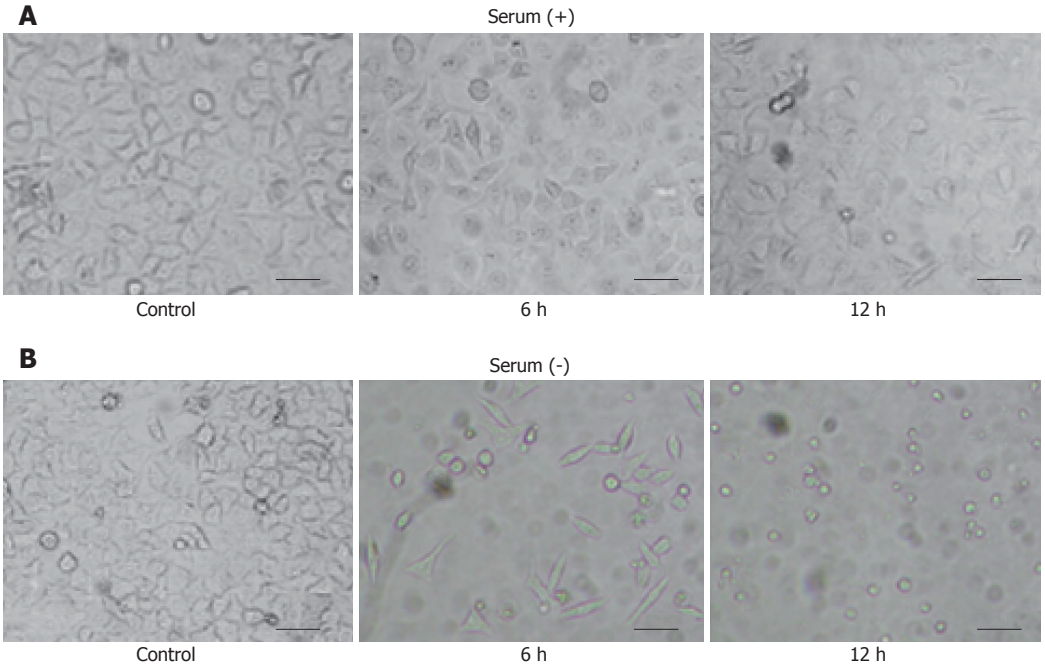

Figure 2 Morphologic change of HeLa cells caused by *H pylori* 1.0 mg/mL cell extract with or without serum. HeLa cells exposed to the extract cultured without serum showed the characteristic morphological changes in 12 h. **A**: HeLa cells exposed to the extract cultured with serum 6 h and 12 h after addition of the extract showed no remarkable changes but a small decrease in cell number; **B**: HeLa cells exposed to the extract cultured without serum 6 h and 12 h after addition of the extract showed the round-formed shape or round debris with a decayed contour through the elongated or spindle-like shape without vacuolations. Scale bar, 200 μm.

dehydrogenase release assay. This cell viability assay revealed the intense cytotoxicity toward HeLa cells, as we predicted. Both the supernatant and the extract cultured without serum had a greater cytotoxicity toward HeLa cells than the ones cultured with serum. The extract, especially of that cultured without serum, had a far greater cytotoxicity than that cultured with serum (Figure 3). This cytotoxicity was thought to be irreversible because the viability assay used measured the intracellular enzyme in the dead or destroyed cells. No cell reversibility could be ascertained for certain when we removed the supernatant and the extract from the cells at 6 h and 12 h after the addition and replaced it with the control lysate for a further 24 h observation (data not shown).

Purification in accordance to the passage time

We pursued the growing cytotoxicity of the extract using a cell viability assay and anion exchange chromatography in accordance to the passage time without serum. The extract at the onset of culturing without serum showed two minor dips of cell viability at approximately 0.1 mol/L NaCl and 0.2 mol/L NaCl with gradient elution, respectively. The extract after approximately 6 mo of culture (approximately 50 passages) from the onset indicated clearer minor dips at the same concentration of the NaCl linear gradient elution; while approximately one year (approximately 100 passages) from its onset without serum, the extract had a major dip at 0.2 mol/L NaCl and a minor dip at 0.1 mol/L NaCl with far less elution protein. We found that the putative cytotoxic

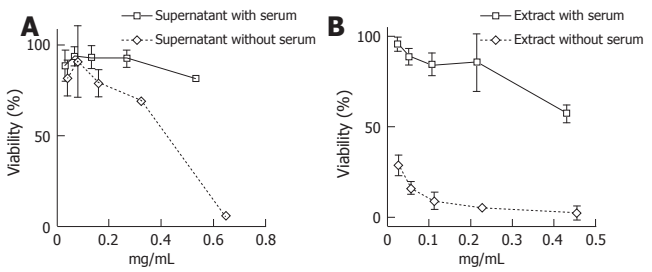

Figure 3 Cell viability assay of the supernatant and the extract of serum-free cultured *H pylori* at the onset of culturing and long cultured *H pylori*, approximately 100 passages after the onset of culturing. **A**: Long serum-free cultured supernatant showing dose-dependent decrease curves of cell viability; **B**: Long serum-free cultured extract showing further dose-dependent decrease curves of cell viability. Each point represents the mean ± SD from three separate experiments.

factor had an isoelectric point less than pH 7.7 due to the use of the buffer with pH 7.7 (Figure 4).

Purification on cation exchange chromatography and size exclusion chromatography

In addition, we examined the extract cultured approximately one year (approximately 100 passages) from its onset without serum using cation exchange chromatography and size exclusion chromatography. Cation exchange chromatography showed one clear dip at around 0.3 mol/L NaCl in the gradient elution that indicated that the putative cytotoxic factor had an isoelectric point of more than pH 5.8 due to the use of the buffer with pH 5.8. Size exclusion chromatography showed a sharp dip at around 37-45 ku, which was different from the molecular mass of the vacuolating toxin (87 ku) (Figure 5).

Cytotoxicity toward human cells

These extracts partially purified by anion exchange chromatography showed robust cytotoxicity toward HeLa cells and other human cells, including AZ521 (gastric carcinoma cells), HLF (hepatocellular carcinoma cells), Caco2 (colorectal carcinoma cells), and HL60 (promyeloblastic leukemia cells) (Figure 6).

DISCUSSION

This is probably the first report about serum-free long culture method of *H pylori*. We showed that the established strain, *H pylori* 60190, could be converted to a far more cytotoxic strain with a serum-free long culture method that was altogether different from that obtained using conventional culture methods including serum. This cytotoxicity was acquired by the long process of culturing it without serum. Both the supernatant and the extract cultured without serum caused cytotoxicity toward HeLa cells that was distinctly different from the cytotoxicity of H pylori cultured using serum of the conventional method to help the feeble growth of *H pylori*. The conventional method using serum certainly may ultimately help the growth state[15] but was considered to be very much unlike the actual growing environment. 2,6-Di-O-methyl-β-cyclodextrin (CD) is considered to reduce the toxic effect

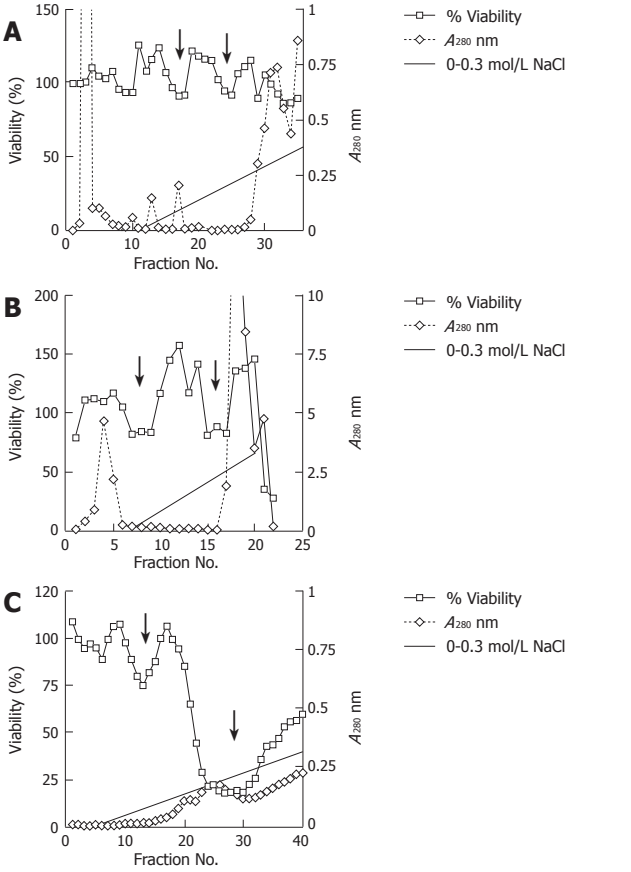

Figure 4 Analysis of the column anion exchange chromatography of the extract cultured without serum. Cell viability that represents the putative cytotoxic factor was measured for cell viability at A_{415} using the WST1 method. Column eluates that represent the protein densities were monitored at A_{280} for each of the filtrated fractions. Proteins were eluted with a linear gradient of 0-0.3 mol/L NaCl. **A**: Anion exchange chromatography of the cell extract protein at the onset of culturing without serum. Two minor dips of cell viability are indicated (arrows); **B**: Anion exchange chromatography of the cell extract protein approximately 50 passages (approximately 6 mo) after the onset of culturing without serum. Two clearer minor dips of cell viabilities are indicated (arrows); **C**: Anion exchange chromatography of the cell extract protein approximately 100 passages (approximately one year) after the onset of culturing without serum. One minor dip and one major dip are at 0.1 mol/L NaCl and 0.2 mol/L NaCl, respectively (arrows).

of fatty acids as well as bovine serum albumin (BSA)[16,17]. Although blood and serum may contain growth-stimulatory factors required by the organism[14], the strain 60190, we used, grew in media with CD without any serum, but did not grow in media without sera or CD as the other strains were reported[18].

The supernatant of the serum-free culture was reported to cause epithelial cytoskeletal disruption[13] after as little as 48 h culture, while 72 h culture decreased the apoptotic signaling of cells, as compared to serum-containing cultures[19]. Serum-free culture method may be less favorable to the organism but more useful with the research of its cytotoxic factors.

Most *in vitro* studies hitherto have used *H pylori* strains cultured using serum, and if any serum-free culture of the bacterium was used for a short time[10,14], it may have been that the supernatant caused the morphological cytotoxic change with vacuolations and without any remarkable cell viability loss or any decrease of assay cell number. However, we discovered the supernatant and the extract of the bacterium cultured for long periods of

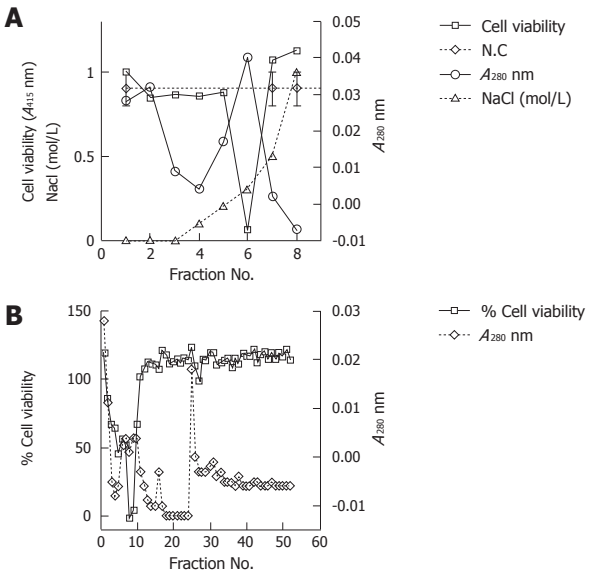

Figure 5 Analysis of the extract cultured without serum. Cell viability that represents the putative cytotoxic factor was measured for cell viability at A_{415} using the WST1 method. Column eluates that represent protein densities were monitored at A_{280} for each filtrated fraction. **A**: Cation exchange chromatography of the cell extract protein approximately 100 passages (approximately one year) after the onset of culturing without serum. The cell extract proteins were eluted with a stepwise gradient of 0-0.5 mol/L NaCl; **B**: Size exclusion chromatography of the cell extract protein approximately 100 passages (approximately one year) after the onset of culturing without serum.

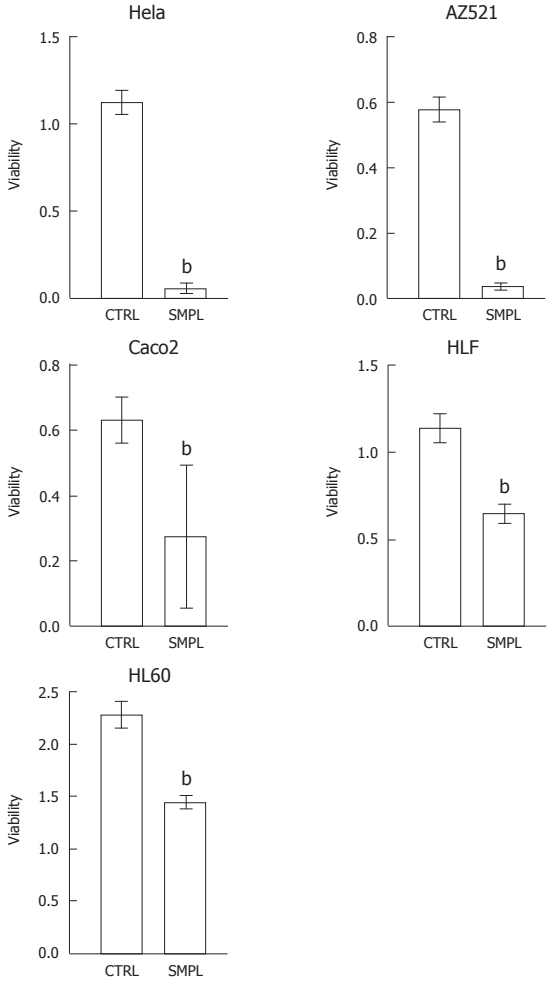

Figure 6 Cytotoxicity of the extract partially purified by anion exchange chromatography. Each bar represents mean + SE of three separate experiments. CTRL: control lysate; SMPL: sample lysate. $^{b}P < 0.01$ *vs* CTRL.

time without serum had a different cytotoxicity in both the cell morphology and the activity that scaled up with the passage duration for a year or more, Furthermore, the shape of the organism cultured in serum-free medium had seemed to be more cocoid form than that would be in serum-containing medium.

In addition, our chromatography study showed that putative cytotoxic factor had an isoelectric point between pH 5.8 to 7.7, with a molecular mass of approximately 37-45 ku, which was different from that of the vacuolating toxin (87 ku). The anion exchange chromatography study appeared to indicate two cell-viability dips, a minor dip and a major dip. Further investigation will be required to determine the number of possible factors involved.

This experimental method might represent a state closer to that of the actual intragastric state, rather than that obtained by using conventional serum-using studies. The experimental method reported herein may also produce results more closely related to clinical observations, which would redress the conventional concept that *H pylori* has only a weak and reversible cytotoxin. *H pylori* might, in fact, be more insidious and more fierce than the researchers had ever conceived.

Our result may provide a new approach to the cytotoxin research and elucidate a pathological mechanism in *H pylori* using this new method, which is different from that of the vacuolating toxin, the only presently known cytotoxin.

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BASIC RESEARCH

Pretreatment with adenosine and adenosine A1 receptor agonist protects against intestinal ischemia-reperfusion injury in rat

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Abstract

AIM: To examine the effects of adenosine and A1 receptor activation on reperfusion-induced small intestinal injury.

METHODS: Rats were randomized into groups with sham operation, ischemia and reperfusion, and systemic treatments with either adenosine or 2-chloro-N^6-cyclopentyladenosine, A1 receptor agonist or 8-cyclopentyl-1,3-dipropylxanthine, A1 receptor antagonist, plus adenosine before ischemia. Following reperfusion, contractions of ileum segments in response to KCl, carbachol and substance P were recorded. Tissue myeloperoxidase, malondialdehyde, and reduced glutathione levels were measured.

RESULTS: Ischemia significantly decreased both contraction and reduced glutathione level which were ameliorated by adenosine and agonist administration. Treatment also decreased neutrophil infiltration and membrane lipid peroxidation. Beneficial effects of adenosine were abolished by pretreatment with A1 receptor antagonist.

CONCLUSION: The data suggest that adenosine and A1 receptor stimulation attenuate ischemic intestinal injury via decreasing oxidative stress, lowering neutrophil infiltration, and increasing reduced glutathione content.

Key words: Adenosine; Adenosine A1 receptor; Intestinal ischemia; Pharmacological preconditioning

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INTRODUCTION

Ischemia-reperfusion (I/R) injury of the intestine is a significant problem in a numerous situations such as abdominal aortic aneurysm surgery, small bowel transplantation, cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis[1]. Decreased contractile activity, increased microvascular permeability, and dysfunction of mucosal barrier are all associated with intestinal I/R[2,3]. I/R injury of the intestine is an intricate and multifactorial pathophysiological process that involves the formation and action of oxygen free radicals (OFRs)[3-9], inflammatory cytokines, the complement system[1,3] and neutrophil infiltration[1-3,6,7,10] at the site of damage.

The purine nucleoside adenosine is one of the major local regulators of normal tissue function, acting in both an autocrine and paracrine fashion. Its regulatory function becomes pronounced especially when energy supply ceases abruptly as in the case of ischemia, and fails to meet cellular energy demand. Adenosine exerts its effects by interacting with its receptors, four of which have been cloned and characterized as adenosine A_1, A_{2a}, A_{2b}, and A_3[11-13]. The physiological role of adenosine in the gastrointestinal tract is still poorly understood, particularly with regard to colonic and ileal motor functions. It has been reported that A_1 adenosine receptor (A_1AR) antagonists increase defecation in rats[13] and that A_1AR agonists can inhibit intestinal fluid secretion and peristalsis *via* adenosine A_{2B} and A_1 receptors, respectively[14].

One of the cellular events observed during ischemia is the increased consumption of ATP, leading to accumulation of adenosine with thereby elevating extracellular adenosine. The accumulation of adenosine is believed to contribute to cytoprotection in the ischemic tissue[11,12]. Furthermore, adenosine which is released during short periods of ischemia followed by reperfusion, provides cytoprotection against a subsequent sustained ischemia in heart, resulting in reduced infarct size[15-20]. This is known as the preconditioning effect of adenosine, which is mediated mostly through the activation of cardiac A_1ARs before ischemia[11,12,18]. It is well documented that the early[19,20] and late[11,15,18] phases of ischemic tolerance are mediated by adenosine in myocardium. That adenosine exerts anti-ischemic actions is indicated by

a number of studies using adenosine receptor agonists and antagonists[15-18] as well as animals overexpressing or lacking A_1AR[21,22]. Administration of adenosine either prior to ischemia or during reperfusion has been shown to attenuate myocardial injury[23,24]. Treatment with adenosine A_1AR agonist initiates preconditioning not only in heart[15-18,25] but also in tissues such as kidney[26,27] and brain[28,29], resulting in attenuation of ischemic injury. One of the underlying mechanisms suggested for adenosine receptor-mediated preconditioning in the heard is through involvement of protein kinase C (PKC) in heart[11,12,30]. Activation of PKC induces opening of ATP-sensitive K^+ channels[11,12,31]. Among other effectors that most likely contribute to the cytoprotection by adenosine are mitogen activated protein (MAP) kinases[25,31-33], heat shock proteins (HSPs)[11-15,34], antioxidant enzymes[15,34] and inducible nitric oxide synthase (iNOS)[11,12]. Moreover, induction and activation of manganese superoxide dismutase (Mn-SOD) is also believed to be a significant factor in mediating myocardial adaptation in response to activation of A_1AR[15].

In the phenomenon of ischemic preconditioning (IPC), a short period of ischemia protects the organs (e.g. heart) against a subsequent more substantial ischemic injury[35]. In fact, IPC has been one of the most promising strategies against reperfusion injury during the last few years. It appears to elevate the tolerance of the intestine to I/R injury. A number of experimental studies have shown that reperfusion injury in small intestine is prevented by IPC[36-40]. IPC conducted in small intestine reduces postischemic leukocyte adhesion by maintaining the bioavailability of nitric oxide[41]. Moreover, it lowers the expression of P-selectin[38], which is a downstream effector target of the adenosine-initiated, PKC dependent, signalling pathway in intestine. Although activation of PKC triggered by adenosine has been a crucial factor for initiating the beneficial actions of IPC in most tissues, the effector of the preconditioning phenomenon appears to vary among tissues. Activated-K^+ channels[42], nitric oxide[39,41] and endogenous opioid peptides[43] have reported to be the other downstream effectors of IPC in intestine. Based primarily on animal experiments, the identification of the molecular mechanisms that are responsible for protection by IPC, has provided opportunities to consider several rational targets for pharmacological intervention. Consequently, a variety of drugs have been demonstrated to be able to mimic IPC when applied instead of ischemia. This is known as pharmacological preconditioning (PPC). Recently, various studies carried out in rat small intestine have demonstrated that establishing PPC by administration of either adenosine[37] or A_1AR agonist[38] mimics the protective effects of IPC. Intensive investigation has been focused on explaining how adenosine accomplishes the beneficial effect of preconditioning. For instance, currently published studies suggest important anti-ischemic roles of the A_1[18,25,30], A_3[17,21] or A_{2a}[44,45] adenosine receptors in heart. On the other hand, relatively little data are available on the role of the different adenosine receptors in mediating cytoprotection in intestinal tissue which is exposed to I/R. The majority of studies strongly suggest that adenosine can promote protection against I/R injury via activation of different adenosine receptors in various tissues. A substantial number of studies report that IPC has been beneficial in human heart and the liver. However, both prospective controlled studies in human and experimental studies in animals are lacking[1]. Furthermore, research based on administration of drugs that can mimic the effects of IPC is required further to explore the cellular events during I/R injury of the intestine. To date, there is no direct evidence showing possible effects of adenosine and A_1AR activation on reduced contractility of intestinal smooth muscle due to I/R injury. Therefore, the present study was constructed to explore the possible effects of adenosine and A_1AR activation on reperfusion injury of small intestinal tissue by evaluating contractile response and levels of thiobarbituric acid-reactive substances (TBARS, a marker of lipid peroxidation), reduced glutathione (GSH, an endogenous antioxidant), and myeloperoxidase (MPO an index of neutrophil infiltration), in terminal ileum subjected to I/R.

MATERIALS AND METHODS

Animals

Following Ethical Committee approval, forty adult male Wistar rats, weighing 200-230 g, were obtained from the Experimental Research Section of Zonguldak Karaelmas University, where animals have been reared and maintained under standard conditions, such as stable room temperature (23 ± 2℃), a 12 h light: 12 h dark cycle, and feeding with commercial rat chow and tap water *ad libitum*. Experimental manipulations and surgical operations were approved by the Animal Ethical Committee of the University. Maximum care and a humane approach to use of animals was of primary consideration.

Experimental groups and operative procedures

The surgical goal was to induce mesenteric ischemia in rats for 30 min followed by a 180 min reperfusion period. On the day before surgery, each animal was fasted overnight with unlimited access to water. Briefly, each animal was anesthetized by an intraperitoneal injection of 50 mg/kg sodium thiopenthal followed by a midline incision made into the peritoneal cavity. The small bowel was exteriorized gently to the left onto moist gauze, and then the superior mesenteric artery (SMA) was carefully exposed, isolated, and clamped using a microvascular clamp. Intestinal ischemia was confirmed by obvious lack of pulse in the SMA and paleness of the jejunum and ileum. The intestines were then meticulously placed back into the abdomen which was closed with two small clamps. Following 30 min of occlusion time, the clamping was gently released and the intestine inspected for proper reperfusion characterized by regular pulsation. Throughout the surgical procedure, each animal was placed under a heating lamp to maintain constant body temperature (e.g. 37℃). For the purpose of assessing the roles of adenosine and A_1AR agonist, animals were randomly divided into five groups: (1) Sham-operated group, subjected to laparatomy without performing the occlusion of the SMA; (2) I/R group, subjected to the occlusion of SMA followed by reperfusion; (3) CPA-treated group (0.1 mg/kg, 5 min prior to ischemia) + I/R; (4) Adenosine-treated group (10 mg/kg, 5 min prior

to ischemia) + I/R; (5) DPCPX pretreatment (1 mg/kg, 15 min prior to adenosine administration) + adenosine treatment (10 mg/kg, 5 min prior to ischemia) + I/R. In the last group, confirming the possible effect of selective A_1AR agonist CPA on reperfusion injury, the selective A_1AR antagonist DPCPX was administered 15 min prior to adenosine treatment. The route and volume for drug administration were the tail vein and 200 μL, respectively. To animals in both sham-control and I/R-control groups were given sterile serum physiological solution in the same volume instead. Choice of dose regimen for the drugs was based on published studies in the literature[22,37,38].

Preparation of terminal ileum

Upon completion of the I/R period, and whilst still unconcious, the animals were sacrificed by exsanguination of the abdominal aorta. Strips of terminal ileum of 10 mm length were immediately removed 10 cm oral to the ileocecal junction and transferred into a Petri dish containing Krebs solution (in mmol/L: NaCl 118, $NaHCO_3$ 24.88, KH_2PO_4 1.18, KCl 4.7, $MgSO_4$ 1.16, $CaCl_2$ 2.52 and glucose 11.1). Then, tissue was longitudinally suspended in a standard organ chamber, and continuously perfused with 20 mL of preoxygenated Krebs solution (pH 7.4), which was bubbled constantly with a mixture of 950 mL/L O_2 and 50 mL/L CO_2 gas and maintained at a temperature of 37℃. One end of the tissue strip was tied to a fixed post and the other attached to an isometric force transducer under a resting tension of 2 g. Isometric responses were monitored by external force displacement transducer (FDA-10A, Commat Iletisim Co., Ankara, Turkey) and recorded on the computer using MP 30 software (Biopac Systems Inc., Santa Barbara, CA, USA). In the organ bath, each strip was allowed to equilibrate for 1 h with intervening washes every 15 min before adding any compound. Tissue samples also obtained from small intestine approximately 10 cm proximal to the ileocecal area were frozen immediately and stored at -40℃ for biochemical measurements.

Concentration-response curves

At the beginning of each experiment to observe dose-contractile response relationship, KCl was added to the organ chamber to a final concentration of 30 mmol/L. For the preparation of high K^+ solutions, NaCl was exchanged for an equimolar amount of KCl to maintain the physiological osmolarity of the Krebs solution. The contraction recorded in response to KCl was considered as a reference response. Afterwards, the contractions in response to carbachol and substance P at various final concentrations ranging from 10^{-9} mol/L to 10^{-2} mol/L were recorded by pipetting these compounds into the organ bath in a cumulative fashion at equal intervals. At the end of the experiment, the response to 30 mmol/L KCl was measured again to confirm and evaluate the degree of tissue viability. The amplitude of all contractions was then normalized for each g of tissue and expressed as percentage of the initial KCl-reference response. The number of experiments, represented as "*n*", indicates that each experiment was performed with a tissue sample taken from one animal. All experiments were conducted in a paired way. For the purpose of evaluating the effects of ligand, agonist, and antagonist, the maximum response (E_{max}) and pD_2 values (e.g. the negative logarithm of the concentration for the half-maximal response, ED_{50}) were computed by using GraphPad Prism Software 3.02 (GraphPad Prism Inc., San Diego, CA, USA)[46]. The pD_2 values (apparent agonist affinity constants) were calculated from each agonist concentration–response curve by linear regression of the linear median part of the sigmoid curve and taken as a measure of the sensitivity of the tissues to each agonist.

Drugs

Adenosine, CPA, DPCPX, carbachol and substance P were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). They were dissolved in double distilled water, except for CPA and DPCPX which were initially prepared in dimethyl sulphoxide and then diluted in physiological saline. Adenosine, CPA, and DPCPX were prepared fresh just before usage. Carbachol and substance P were made up at different concentrations and kept frozen in aliquots. Compounds, which were used for preparing Krebs solution, were purchased from Merck (Merck KGaA, Darmstadt, Germany). All other reagents, including trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxy toluene (BHT), and dithiobisnitrobenzoate (DTNB) were obtained from Sigma.

Determination of tissue TBARS and GSH

Tissue TBARS content was measured in order to estimate the extent of lipid peroxidation in the injured terminal ileum. Samples obtained from each group were stored at -40℃ until assayed. Tissue samples were washed in ice-cold Krebs solution, blotted on absorbent paper and weighed. Afterwards, each sample was minced followed by homogenization with 10 mL of 100 g/L TCA per g of tissue, using a motor-driven homogenizer (Heidolph Diax 900, Heidolph Elektro GmbH&Co.KG, Kelheim, Germany). Then, the tissue TBARS levels were measured spectrophotometrically based on a method described by Casini *et al*[47] and expressed as nmol/g of tissue weight. Briefly, following two consecutive centrifugations at 3000 g for 15 min, 750 μL supernatant was added to equal volume of 6.7 g/L TBA and heated to 100℃ for 15 min. The absorbance of the samples was then measured spectrophotometrically at 535 nm (Smart Spectro, LaMotte Co., Chestertown, MD, USA).

The GSH content of the samples were measured by applying a modified Ellman method[48]. In brief, 2 mL of 0.3 mol/L Na_2HPO_4 solution was mixed with 0.5 mL of supernatant obtained by employing the homogenization procedure described above. Into the mixture, 0.2 mL of DTNB solution was added followed by reading absorbance at 412 nm. The tissue GSH levels were expressed as μmol/g of tissue weight.

Measurement of tissue MPO activity

The degree of neutrophil accumulation in the intestinal tissue samples was measured by assaying MPO activity as described by Bradley *et al*[49]. Briefly, upon thawing, each

Table 1 E_{max} and pD_2 values of carbachol and substance P (n = 8, means ± SE)

	Sham Control	I/R Control	CPA-I/R	ADO-I/R	DPCPX + ADO-I/R
Carbachol					
E_{max}	488.67 ± 47.01	157.05 ± 41.35[a]	395.05 ± 32.62[c]	372.21 ± 54.68[c]	212.35 ± 40.09[a]
pD_2	6.30 ± 0.19	7.02 ± 0.31	6.24 ± 0.40	6.94 ± 0.13	5.81 ± 0.18
Substance P					
E_{max}	255.94 ± 31.17	115.00 ± 13.36[a]	148.19 ± 13.80[a]	242.93 ± 46.55[c]	259.61 ± 24.62[c]
pD_2	6.51 ± 0.05	8.29 ± 1.08	7.00 ± 0.22	6.69 ± 0.28	6.51 ± 0.07

[a]P < 0.05 *vs* sham-operated control group, [c]P < 0.05 *vs* I/R control group.

sample was very finely minced with surgical blade in a petri dish containing 50 mmol/L potassium phosphate buffer (PB, pH 6.0) at a volume 20 times the tissue weight (e.g. 1 mL) followed by homogenization for 5 min in ice-cold PB by means of motor driven homogenizer. The homogenate was centrifuged at 40 000 g for 15 min at 4℃. The homogenized tissue pellet was suspended in 50 mmol/L PB containing 5 g/L hexadecyltrimethylammonium bromide (HETAB) and then homogenized again. Following three freeze and thaw cycles with sonication (Bandelin Sonopuls HD2070, Bandelin Electronic GmbH&CO.KG, Berlin, Germany) between cycles, the samples were centrifuged at 40 000 g for 10 min. Aliquots of supernatant (0.1 mL) were added to 2.9 mL of reaction mixture containing 0.167 mg/mL of *o*-dianisidine, and 20 mmol/L H_2O_2 solution, which were prepared in 50 mmol/L of PB. Immediately after adding the aliquot to the mixture, the change in absorbance at 460 nm was measured for 5 min. One unit of MPO activity was defined as that degrading 1 μmol of peroxide per min at 25℃. The activity was then normalized as unit per mg of tissue (U/mg).

Statistical analysis

Values for the experiments dealing with contractility were normalized for per g of tissue followed by expressing them as percentage of KCl response. Each data point represents mean ± SE. For statistical evaluation, SPSS 11.0 statistical software package programme was used (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was applied for statistical comparison of groups, followed by analysis with Tukey-Kramer test so as to determine differences between the groups. Probability value (P) of 0.05 or less was considered statistically meaningful.

RESULTS

Ileal longitudinal muscle contractility

For longitudinal ileum muscle collected from sham control, CPA-treated, adenosine-treated, and DPCPX-adenosine-treated animals, mean contraction responses to 30 mmol/L KCl were measured as 0.59 ± 0.01 g; 0.40 ± 0.09 g; 0.50 ± 0.22 g; and 0.44 ± 0.21 g, respectively, which were statistically indistinguishable (Figure 1). In I/R control group however, the contractile response (0.26 ± 0.08 g) was significantly reduced when compared to that in sham-operated control group (P = 0.012).

The addition of carbachol at concentrations from 10^{-9} mol/L to 10^{-2} mol/L into the organ bath resulted

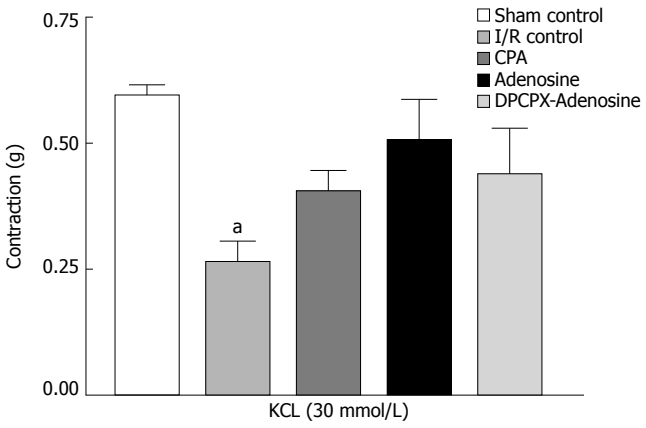

Figure 1 Mean contraction of longitudinal ileum muscle isolated from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats in response to 30 mmol/L KCl. Data are expressed as means ± SE (n = 8). [a]P < 0.05 *vs* sham-operated control group.

in a dose-dependent contractile effect on the terminal ileum segments from all groups (Figure 2), providing sigmoid curves with E_{max} and pD_2 values (Figure 3). E_{max} value for carbachol was significantly lower in the I/R control group than in the sham-operated control group (157.04% ± 41.35% *vs* 488.66% ± 47.01%, respectively). In other words, contraction in response to carbachol was significantly reduced by induction of I/R. Statistical difference between the groups appeared to be meaningful at 10^{-6} mol/L (P = 0.02), reaching a maximal level at 10^{-3} mol/L of carbachol (P = 0.0001). The I/R-induced reduction in contractility was significantly restored by treatments with both CPA and adenosine but not by pretreatment with DPCPX. Amelioration of reduced contractions with CPA and adenosine therapies became statistically significant at millimolar doses of carbachol (P = 0.03 at 10^{-3} mol/L and 10^{-2} mol/L).

Comparison of the E_{max} values showed that average contraction of ileum samples in I/R group was just 32% of that in sham-operated control group, while those in CPA- and adenosine-treated groups were approximately 81% and 76%, respectively (Table 1). In the group pretreated with DPCPX, E_{max} was found to be approximately 60% of that in sham control group, which was statistically significant (P < 0.05). On the other hand, no statistically significant change was detected in the corresponding pD_2 values in any group (Table 1).

In response to various concentrations of substance

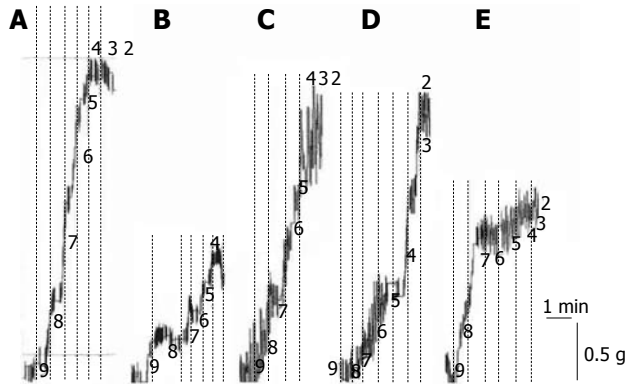

Figure 2 Representative traces showing responses generated by various concentrations of carbachol in longitudinal ileum muscle isolated from sham-operated control (**A**), I/R control (**B**), CPA–I/R (**C**), adenosine-I/R (**D**), and DPCPX-adenosine-I/R (**E**) rats.

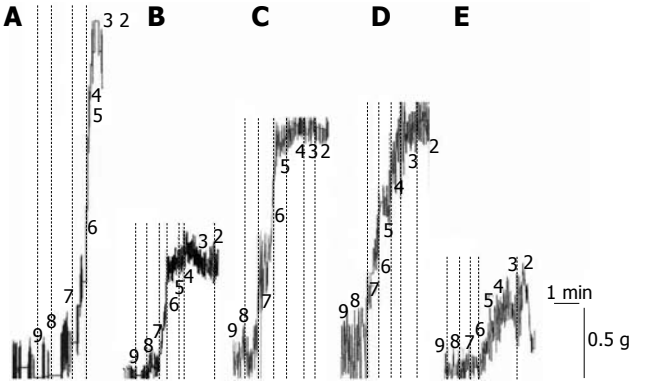

Figure 4 Representative traces showing responses generated by various concentrations of substance P in longitudinal ileum muscle isolated from sham-operated control (**A**), I/R control (**B**), CPA-I/R (**C**), adenosine-I/R (**D**), and DPCPX-adenosine-I/R (**E**) rats.

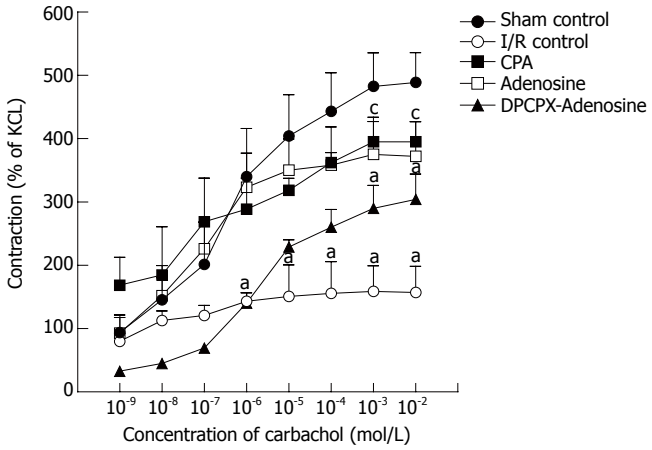

Figure 3 Dose-response curves of carbachol in longitudinal ileum muscle isolated from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats. Data are expressed as means ± SE (n = 8). $^{a}P < 0.05$ *vs* sham-operated control, $^{c}P < 0.05$ *vs* I/R control groups.

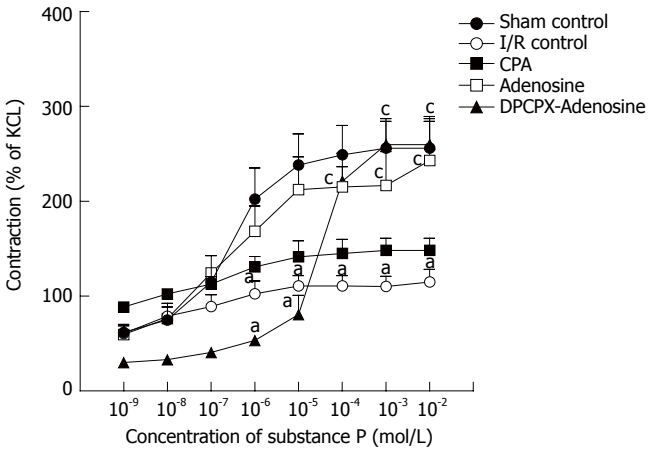

Figure 5 Dose-response curves of substance P in longitudinal ileum muscle isolated from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats. Data are expressed as means ± SE (n = 8). $^{a}P < 0.05$ *vs* sham-operated control, $^{c}P < 0.05$ *vs* I/R control groups.

P ranging from 10^{-9} mol/L to 10^{-2} mol/L, terminal ileum samples contracted in a dose-dependent fashion in all groups (Figure 4), rendering sigmoid curves with E_{max} and pD_2 values (Figure 5). The contractile response induced by substance P was significantly and dose-dependently inhibited by induction of I/R. Statistical difference between sham-operated control rats and I/R control animals was significant at 10^{-6} mol/L and over doses of substance P ($P < 0.05$). Reduced contractility due to I/R was alleviated significantly by adenosine treatment ($P < 0.05$). This effect of adenosine was completely lost once DPCPX was given prior to adenosine administration. However, the exacerbating effect of DPCPX was significantly evident in response to substance P at doses lower than 10^{-5} mol/L (Figure 5). Above this concentration, as shown in the ascending part of the curve, responses in both adenosine- and DPCPX-adenosine-treated groups were statistically indistinguishable. Accordingly, there was a statistically significant difference between I/R control group and DPCPX-adenosine-treated group in response to 10^{-4} mol/L ($P = 0.022$), 10^{-3} mol/L ($P = 0.004$), and 10^{-2} mol/L ($P = 0.011$) of substance P. Regarding the corresponding pD_2 values, no statistically significant change was detected in any group (Table 1).

TBARS level

Average TBARS content of intestinal samples from sham-operated animals was 54.18 ± 3.26 nmol/g tissue, while that from I/R control rats was 78.27 ± 7.60 nmol/g tissue (Figure 6). I/R caused approximately 1.45 fold increase in TBARS content of the tissue, which was significantly different from that measured in samples from sham-operated animals ($P = 0.002$). Administration of either CPA or adenosine prior to the induction of ischemia significantly reduced the elevated TBARS content to the levels observed in sham control rats. Mean values of the both groups (39.87 ± 11.02 nmol/g tissue and 56.49 ± 7.03 nmol/g tissue, respectively) were significantly different from that of the I/R control group ($P = 0.001$). On the other hand, in the case of DPCPX pretreatment before

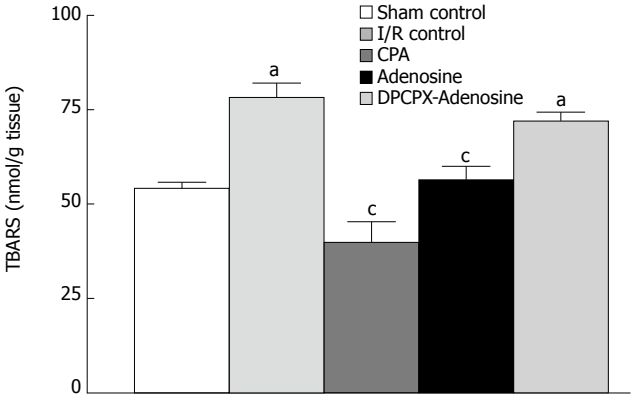

Figure 6 TBARS content of ileum samples from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats. Data are expressed as means ± SE (n = 8). $^{a}P < 0.05$ *vs* sham-operated control, $^{c}P < 0.05$ *vs* I/R control groups.

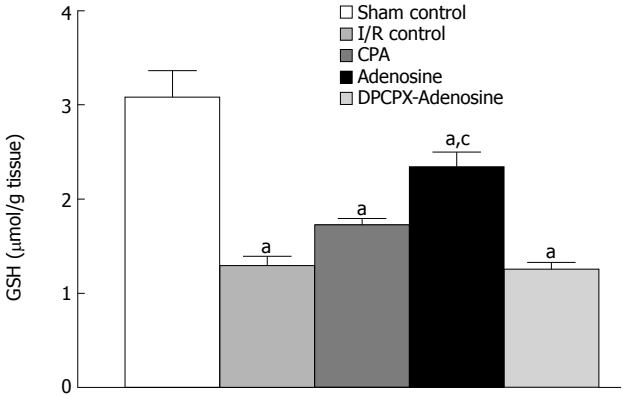

Figure 7 GSH content of ileum samples from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R rats. Data are expressed as means ± SE (n = 8). $^{a}P < 0.05$ *vs* sham-operated control, $^{c}P < 0.05$ *vs* I/R control groups.

adenosine administration followed by I/R, the average TBARS content was 72.02 ± 4.34 nmol/g tissue, which was not significantly different from that in the I/R control group.

GSH level

As shown in Figure 7, the amount of GSH measured in tissues subjected to I/R (1.29 ± 0.19 μmol/g tissue) decreased approximately 58% compared to that measured in the tissues from the sham-operated group (3.08 ± 0.27 μmol/g tissue) ($P < 0.001$). Levels of tissue GSH were statistically indistinguishable when comparing the samples from I/R control group with those from CPA-treated group. In contrast, treatment with adenosine significantly ameliorated the decreased amount of GSH. Mean GSH content was 2.34 ± 0.31 μmol/g tissue, which was significantly different from that measured in I/R control animals ($P = 0.002$). However, pretreatment with DPCPX prevented this effect of adenosine, reducing GSH content to the levels observed in I/R control animals.

MPO activity

MPO enzyme activities in the terminal ileum samples from animals subjected to sham operation, I/R, CPA treatment, adenosine treatment, and DPCPX-adenosine treatment averaged 6.09 ± 1.04 U/mg tissue, 18.19 ± 6.57 U/mg tissue, 10.80 ± 3.66 U/mg tissue, 5.58 ± 2.89 U/mg tissue, and 21.45 ± 9.61 U/mg tissue, respectively (Figure 8).

I/R caused approximately a 3 fold increase in MPO activity of terminal ileum tissue compared to the basal level of the activity ($P < 0.0001$), which was measured in tissues of sham control animals (Figure 8). As MPO activity of samples from animals treated with CPA or adenosine were significantly different from that in I/R group ($P = 0.056$ and $P = 0.0001$, respectively), it appeared that pretreatment with DPCPX completely abolished the reducing effect of adenosine on MPO activity. Clearly, no statistical difference was observed in MPO activity between I/R control animals and DPCPX-pretreated rats, while there was a significant difference between CPA-treated animals and DPCPX-pretreated animals ($P = 0.023$) as well as between adenosine-treated group and DPCPX-pretreated group ($P < 0.0001$).

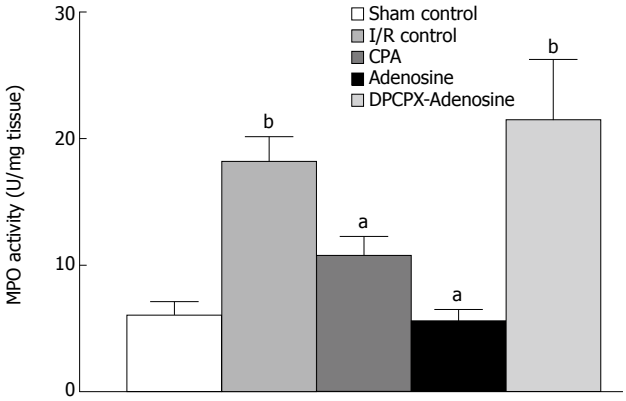

Figure 8 MPO level of ileum samples from sham-operated control, I/R control, CPA-I/R, adenosine-I/R, and DPCPX-adenosine-I/R animals. Results are the means ± SE of 6 to 8 animals in each group. $^{a}P < 0.05$ *vs* I/R control, $^{b}P < 0.001$ *vs* sham-operated control.

DISCUSSION

The major findings of the present study can be summarized as follows: (1) I/R resulted in reduced ileal contractility in response to KCl, carbachol, and substance P as well as elevating oxidative stress and neutrophil infiltration; (2) These disturbances were significantly ameliorated by either adenosine administration or A_1AR activation in the preischemic period; (3) Adenosine and A_1AR-mediated protection against I/R injury seemed to be associated with decreased oxidative stress and MPO activity; (4) A_1AR antagonist DPCPX diminished the injury-sparing effect of PPC with adenosine as observed in other tissues. Pharmacological blockade of A_1ARs exacerbated the contractile response of small intestinal smooth muscle.

I/R results in disrupted exogenous electrical activity and contractile response of ileum[8-10]. A substantial amount of evidence indicates that the pathogenesis of I/R and I/R-induced motor alterations have been related to OFRs[1-3,6,8] and activated neutrophils[1-3,10]. Intestinal I/R sets the groundwork for an inflammatory response in

the vicinity of muscularis cells, provoking the recruitment and extravasation of leukocytes into smooth muscle syncytium[2,10]. A number of experimental studies have been conducted in order to test various pharmacological agents that might reduce reperfusion injury of the intestinal mucosa[4,5,7,9,50,51] with the intention of improving life-span after acute mesenteric ischemia,.

Our results showed that intestinal I/R resulted in decreased ileal contractility in response to carbachol, subtance P, and KCl; therefore influencing both receptor-mediated induction and non-receptor-mediated induction. That the pD_2 values in all groups were statistically indifferent from each other however, suggest that I/R does not alter agonist-receptor interaction. Hence, the reduced E_{max} value in I/R group may be dependent partly on change in the regulation of postreceptor processes (e.g. excitation-contraction coupling)[9,46]. Furthermore, the decreased contraction response also observed in non-receptor-mediated induction strongly supports this possibility.

IPC makes reference to a phenomenon in which the harmful effects of prolonged ischemia is prevented by exposure of a tissue to brief periods of ischemia[35]. In spite of the fact that it is highly complicated in nature, IPC has been successfully applied to various animal models of intestinal I/R, resulting in attenuation of the reperfusion injury[37-40,52]. However, although IPC has been shown to be beneficial in the human heart and liver, prospectively controlled studies in both humans and animals involving IPC and PPC of the intestine are inadequate. More research focused on the application of drugs that can mimic the effects of IPC is needed to analyze the cellular and molecular events during I/R injury of the intestine so as to attenuate I/R injury[1]. Both animal and human studies have revealed that adenosine is one of the major triggers for IPC. A study done by Unal *et al*[37] has demonstrated that administration of adenosine prior to ischemia is as effective as IPC for inducing ischemic tolerance in rats. Data gathered in the present study confirms this finding and show that the treatment with adenosine significantly restored I/R-reduced contractile response. Furthermore, the treatment also provided such beneficial effects such as elevating GSH content, lowering lipid peroxidation, and reducing neutrophil infiltration. In addition, that the A_1AR antagonist DPCPX significantly blocked these protective effects of adenosine is consistent with the hypothesis that PPC with adenosine is primarily mediated via A_1ARs.

The findings of the present study revealed that non-receptor mediated (e.g. KCl-induced) and receptor mediated (e.g. carbacol- and substance P-induced) ileal contractions that were reduced significantly due to I/R, were improved remarkably and returned to sham-control levels by systemic administration of adenosine or CPA. During the process of preconditioning, adenosine is generated in the ischemic tissue. The endogenous adenosine or selective pharmacological agonists activate A_1ARs. Preischemic activation of A_1ARs has been demonstrated to prevent from I/R damage in various organs including heart[15-18,25], kidney[26,27] and brain[28,29]. In these studies, the activation of A_1ARs has been strongly implicated in the mediation of IPC. Adenosine therapy before induction of ischemia has been reported to attenuate ischemic injury in heart[23,24] and intestine[37]. Furthermore, pharmacological blockade of A_1AR during preconditioning eliminates the achivement of protection[15-18]. The protective effect of A_1AR activation is accomplished through the activation of PKC, leading to translocation of PKC to sarcolemmal and to mitochondrial membranes. Activated PKC then induces an increase in opening of ATP-sensitive K^+ channels in heart[11,12]. Stimulation of A_1ARs also precedes the early activation of some other kinases such as tyrosine kinases, p38, MAPK[11,19,25,31,33], ERK[32], and Akt[20]. Additionally, in protection obtained by agonist-induced stimulation of A_1AR, elevated content or activity of many proteins have been demonstrated such as HSP 27[11] and Mn-SOD[15]. Despite the existence and involvement of relatively large number of effector molecules, it appears that they vary among tissues. In small intestine, for instance, Davis *et al*[38] report that pharmacological modulation of A_1ARs is involved in reduced expression of P-selectin, which is a downstream effector target of the adenosine-initiated, PKC dependent, anti-inflammatory signaling pathway in preconditioning. In our study, I/R of small intestine elevated the tissue TBARS content, indicating enhanced generation of OFRs; therefore, inducing lipid peroxidation. Systemic administration of adenosine or CPA appeared to be protective against I/R-induced reduction of contractility via, at least, inhibiting lipid peroxidation and neutrophil infiltration as confirmed by reduction of TBARS and MPO levels, respectively. Another significant observation reported recently is that activation of A_1ARs *in vitro* prevents cellular functions from H_2O_2-induced injury through signaling pathways related to PKC in renal proximal tubular cells[53]. The same observation has been demonstrated in other studies on heart[54,55] and kidney[53]. In these studies, activation of A_1ARs *in vivo* and *in vitro* is reported to be associated with protection against H_2O_2-induced oxidative injury by modulation of the detrimental increases in intracellular calcium concentration and by means of activation of cardiomyocyte K^+ channels after H_2O_2 exposure.

GSH is an endogenous antioxidant and present in all animal cells. Reacting with free radicals, it can provide protection from singlet oxygen, hydroxyl radical and superoxide anion[31]. Many published studies indicate that tissue injury, induced by various stimuli (e.g. I/R), is coupled with glutathione depletion[9]. In the present study, we showed that depleted GSH content in ischemic ileal tissue was recovered by adenosine or CPA therapies. In other words, inducing PPC with these drugs maintained GSH content during reperfusion. This effect may be related to activation of PKC since adenosine has been reported to induce the activation of antioxidant enzymes *in vitro* and since it is suggested that the stimulatory action of adenosine is likely involved in PKC-mediated phosphorylation. Such a mechanism could serve to decrease the levels of OFRs, which would otherwise be harmful to the cell. This very effect of adenosine is also evident *in vivo*, and may account for adenosine-induced reduction of lipid peroxidation in cochlea[34]. Although the

present study has not examined antioxidant enzymes or PKC, the elevated level of GSH implicates the potential involvement of a cytoprotective mechanism related to adenosine and A1 receptor activation.

Modulation of the inflammatory response following I/R injury is an important component of tissue defense, mostly because inflammation is the major component of cell death and motor alteration in intestine subjected to intestinal injury. In the initial period of I/R, generation of OFRs occurs, which is the most likely the initial factor responsible for the induction of neutrophil chemotactic activity. Afterwards an influx of leukocytes during reperfusion triggers an intricate cascade of proinflammatory events associated with cytokine/chemokine release and free radical-mediated intestinal injury[1,3]. Upon attachment to endothelium, neutrophils cause the secretion of additional OFRs, contributing to the damage. At this point, the enzyme MPO, found largely in leukocytes particularly in neutrophils, provides an opportunity to check the tissue level of the cells since it is a marker of neutrophil infiltration and accumulation into tissues[27]. In the present study, we have demonstrated that the therapy with A_1AR agonist CPA prevented neutrophil infiltration into the reperfused-intestine as shown by the decrese in MPO content. This finding is in agreement with those of previous studies which reports that A_1AR stimulation is associated with decreased inflammation and MPO levels[22,27,38].

In the present study, we have demonstrated that administration of adenosine and the A_1AR agonist CPA ameliorated intestinal contractile dysfunction induced by I/R. The outcome of the study suggests that preischemic administration of adenosine or CPA may protect intestine, as indicated by recovery of contractile response, possibly through decreasing oxidative strees and reducing neutrophil infiltration. In conclusion, our findings suggest the cellular mechanism by which adenosine and pharmacological stimulation of A_1ARs attenuate intestinal injury, which may indicate the possible therapeutic usage of adenosine as an adjunct for ischemia and ischemia related small bowel diseases.

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COMMENTS

Background

A number of experimental studies have shown that reperfusion injury in the small intestine is prevented by ischemic preconditioning (IPC). Moreover, various studies using rat small intestine have demonstrated that establishing pharmacological preconditioning (PPC) by administration of either adenosine or adenosine A1 receptor agonist mimic the protective effects of IPC. On the other hand, relatively little data is available on the role of the different adenosine receptors in mediating cytoprotection during intestinal I/R injury. There is no direct evidence which confirms the possible effects of adenosine and adenosine A1 receptor activation on I/R injury- related decreased in contractility of intestinal smooth muscle.

Research frontiers

That adenosine exerts anti-ischaemic actions is indicated by a number of studies using adenosine receptor agonists and antagonists as well as animals overexpressing or lacking the adenosine A1 receptor. Administration of adenosine either prior to ischemia or during reperfusion has been shown to attenuate myocardial injury. Treatment with adenosine A1 receptor agonist initiates preconditioning not only in heart but also in such tissues as kidney and brain, resulting in attenuation of ischemic injury.

Innovations and breakthroughs

IPC of the small intestine reduces postischemic leukocyte adhesion by maintaining the bioavailability of nitric oxide. Moreover, it lowers the expression of P-selectin, which is a downstream effector target of the adenosine-initiated, PKC dependent, signalling pathway in intestine. Although activation of PKC triggered by adenosine is a crucial factor for initiating the beneficial actions of IPC in most tissues, the effector of the preconditioning phenomenon appears to vary among tissues. Activated-K^+ channels, nitric oxide, and endogenous opioid peptides have reported to be the other downstream effectors of IPC in intestine. Furthermore, currently published studies suggest important anti-ischemic roles of the A_1, A_3 or A_{2a} adenosine receptors in heart.

Applications

The therapeutic efficacy of adenosine and the adenosine A1 agonist, 2-chloro-N^6-cyclopentyladenosine (CPA) should be examined for potential clinical application in the treatment of conditions related to intestinal ischemia-reperfusion injury, such as small bowel transplantation, strangulated hernias, and abdominal aortic aneurysm. In addition, it would be worthwhile to focus onthe possible effector molecules (e.g. involvement of PKC, opening of mitochondrial ATP-sensitive K^+ channels, or activation of Akt) which underlie the mechanism (s) responsible for the beneficial effects of adenosine and CPA observed in the present study.

Terminology

Ischemia: deficient supply of blood to a body part (e.g. any organ) that is due to obstruction of the inflow of arterial blood (for example, by narrowing of arteries as a result of spasm or disease); Ischemia-reperfusion: interruption of the blood flow to a tissue for a period of time followed by restoration of blood flow. During the ischaemic period , a sequence of events is initiated that may ultimately lead to cellular dysfunction or even cell death; Reperfusion injury: When ischemia is ended by restoration of blood flow, a second series of injurious events ensue producing additional damage. The injury produced by reperfusion is more severe that that induced by ischemia and is called reperfusion injury. The primary harmful events are the formation of cytotoxic oxidants (also commonly called oxygen free radicals) derived from molecular oxygen, oxygen free radical-mediated damage to cellular membranes via lipid peroxidation, loss of cellular calcium balance, and generation of inflammatory reaction at the site of damage; Oxidative stress: stress on the body or organism that results from the cumulative damage done by oxygen free radicals which are inadequately neutralized by antioxidants; Agonist: a chemical substance capable of combining with a receptor on a cell and initiating the same reaction or activity typically produced by the binding of an endogenous substance; Antagonist: a chemical substance that acts through receptor to reduce the physiological activity of another chemical or endogenous substance.

Peer review

The present study is interesting, well designed, and contained novel findings. The study is set up throughly and the paper is well written. The conclusions are well based and are of clinical value.

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BASIC RESEARCH

Influence of dexamethasone on inflammatory mediators and NF-κB expression in multiple organs of rats with severe acute pancreatitis

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Abstract

AIM: To observe the therapeutic effects of dexamethasone on rats with severe acute pancreatitis (SAP) and investigate the influences of dexamethasone on the inflammatory mediators and NF-κB expression in multiple organs of SAP rats as well as the mechanisms involved.

METHODS: Ninety Sprague-Dawley (SD) rats with SAP were randomly divided into the model group (n = 45) and dexamethasone treatment group (n = 45), and another 45 rats were selected for the sham operation group. All groups were randomly subdivided into the 3 h, 6 h and 12 h groups, each group containing 15 rats. The survival of all groups and pathological changes of multiple organs (liver, kidney and lung) were observed at different time points after the operation. The pathological score of multiple organs was carried out, followed by the determination of amylase, endotoxin and TNF-α contents in blood. The tissue microarray was used to detect the expression levels of NF-κB p65 protein in multiple organs.

RESULTS: There was no marked difference between the model group and treatment group in the survival rate. The amylase content of the treatment group was significantly lower compared to the model group at 12 h (P < 0.01, 7791.00 *vs* 9195.00). Moreover, the endotoxin and TNF-α levels of the treatment group were significantly lower than that of the model group at 6 h and 12 h (P < 0.01, 0.040 *vs* 0.055, 0.042 *vs* 0.059 and P < 0.05, 58.30 *vs* 77.54, 38.70 *vs* 67.30, respectively). Regarding the changes in liver NF-κB expression, the model group significantly exceeded the sham operation group at 3 h (P < 0.01, 1.00 *vs* 0.00), and the treatment group significantly exceeded the sham operation group at 12 h (P < 0.01, 1.00 *vs* 0.00), whereas no marked difference was observed between the model group and treatment group at all time points. The kidney NF-κB expression level in the treatment group significantly exceeded the model group (P < 0.05, 2.00 *vs* 0.00) and the sham operation group (P < 0.01, 2.00 *vs* 0.00) at 12 h. No NF-κB expression in the lung was found in any group.

CONCLUSION: Dexamethasone can lower the amylase, endotoxin and TNF-α levels as well as mortality of SAP rats. NF-κB plays an important role in multiple organ injury. Further studies should be conducted to determine whether dexamethasone can ameliorate the pathological changes of multiple organs by reducing the NF-κB expression in the liver and kidney. The advantages of tissue microarrays in pancreatitis pathological examination include time- and energy- saving, and are highly efficient and representative. The restriction of tissue microarrays on the representation of tissues to various extents due to small diameter may lead to the deviation of analysis.

Key words: Severe acute pancreatitis; Dexamethasone; NF-κB; Tissue microarrays; Mutiple organs

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INTRODUCTION

Severe acute pancreatitis (SAP), as one of the common presentations of clinically acute abdomen is a systemic disease in which the local inflammatory pathological changes of pancreas involve multiple organs[1]. It is recently believed the systemic inflammatory response syndrome (SIRS) due to the excessive inflammatory reactions plays an extremely important role in SAP pathogenesis, which is relatively complicated[2,3]. Although the exact pathogenesis remains unclear[4]. Studies in recent years have found that NF-κB (nuclear factor kappa-B) which is a main factor in the genetic transcription of inflammation, presents high expression state in acute pancreatitis and plays an important role in the onset and turnover of acute pancreatitis together with other inflammatory cytokines[5-7]. Dexamethasone is the antagonist of a non-specific inflammatory mediator[8]. Studies have shown that dexamethasone can lower the expression level of NF-κB by inducing the release of NF-κB profilin (IκB)[9]. In this study, we prepared rat SAP models by using the improved Aho's method[10], and investigated the therapeutic effects of dexamethasone on the SAP rats and examined the influences of dexamethasone on the inflammatory mediators and NF-κB expression in multiple organs of the rats.

MATERIALS AND METHODS

Materials

Clean grade healthy male Sprague-Dawley (SD) rats weighing 250-300 g were purchased from the Experimental Animal Center of Medical School, Zhejiang University, China. Sodium taurocholate and sodium pentobarbital were purchased from Sigma Company, USA. Dexamethasone injection was purchased from Zhejiang Xinchang Pharmaceutical Company. NF-κB p65 antibody was purchased from Santa Cruz Company, USA. The full automatic biochemical analyzer was used to determine the plasma amylase level (U/L). Plasma endotoxin Tachypleus Amebocyte Lysate Kit was purchased from Shanghai Yihua Medical Science and Technology Corporation (Institute of Medical Analysis in Shanghai, China), the calculation unit for content is EU/mL. TNF-α ELISA kit was purchased from Jingmei Bioengineering Corporation, China, the calculation unit for content is pg/mL (ng/L).

Animal grouping

We adopted the improved Aho's method[10] to prepare 90 SAP rat models and randomly divided into the model group (45 rats) and treatment group (45 rats). Another 45 rats were selected as the sham operation group. All groups were randomly subdivided into the 3, 6 and 12 h groups, each group containing 15 rats. The dexamethasone treatment group was injected once with dexamethasone (1 mL = 5 mg) *via* the vena caudalis, 0.5 mg/100 g body weight 15 min after successful preparation of SAP model. During the laparotomy in the sham operation group, we performed pancreas and duodenum manipulation, observed pathological changes of multiple organs and finally closed the abdomen. The sham operation group and model group were injected with same amount of normal saline (0.1 mL/100 g body weight) *via* the vena caudalis 15 min after the operation.

Animal model preparation

Fasting and water restriction was imposed on all rat groups 12 h prior to the operation. The rats were anesthetized by intraperitoneal injection of 20 g/L sodium pentobarbital (0.25 mL/100 g body weight) and the operation was performed under aseptic conditions. Model group: After entering the abdomen *via* median epigastric incision, the bile-pancreatic duct, hepatic hilus and common hepatic duct were identified; the duodenal papilla inside the duodenum duct wall was identified, and then a No. 5 needle was used to drill a hole in the mesenteric avascular area. A segmental epidural catheter was inserted into the duodenum cavity *via* the hole, and then inserted into the bile-pancreatic duct toward the direction of papilla in a retrograde way, a microvascular clamp was used to nip the catheter head temporarily. Meanwhile, another microvascular clamp was used to temporarily occlude the common hepatic duct at the confluence of the hepatic duct. After connecting the epidural catheter end with the transfusion converter, 3.5% sodium taurocholate (0.1 mL/100 g) was transfused *via* the microinjection pump at a speed of 0.2 mL/min, stayed for 4 min after injection, and then the microvascular clamp and epidural catheter were removed. After checking for bile leakage, the hole in the lateral duodenal wall was sutured. A sterile cotton ball was used to absorb up the anaesthetic in the abdominal cavity and then the abdomen was closed.

Preparation of tissue microarrays of mutiple organs

We fixed the tissue sample with neutral formalin, prepared the routine paraffin block (named donor block), cut the donor block into 5-μm thick tissue section and carried out routine hematoxylin-eosin (HE) staining as well as microscopic morphological observation under microscope, and then selected the required representative area, marked the locations on the HE sections and also on the corresponding part of the donor block. We prepared the blank block as the recipient block in the size of 45 mm × 20 mm × 15 mm and drilled the recipient block with the tissue microarrays section (Beecher Instruments, USA), the diameter of the drilling needle is 2.0 mm. In obtaining of the donor block tissue microarrays, we used another drilling needle (its inner diameter is equal to the outer diameter of the former drilling needle) to drill the marked location of paraffin block and collected the tissue microarray. Its length was about 0.1 mm shorter than the depth of hole. The tissue microarrays collecting method was just the same as that of drilling the recipient block. After pushing out the tissue microarrays, we directly

inserted it or inserted it with forceps into the hole of recipient block. After pressing the tissue microarrays downwards with common glass slide, we used the distance adjuster to correctly move the drilling needle to a proper distance forward and back or right and left. This process was repeated and could insert tens of tissue microarrays into the recipient block in an orderly fashion. Finally, we piled up three glass slides to press all the tissue microarrays and thus the prepared tissue microarrays section block had flat and smooth surface. We put the prepared tissue microarrays section block into the paraffin block again to make the mold, and put it into a 60℃ oven for 1 h so that the paraffin of the tissue microarrays and recipient block could be melted together. We took the mold out of the oven gently, cooled the half melted paraffin at the room temperature (about 30 min) and then cooled it in a -20℃ refrigerator for 6 min. Later, we took the tissue microarrays section block out of the mold and stored it in a 4℃ refrigerator for later use. We took out the standby paraffin block and rapidly nipped it on the sectioning machine for correction till all the tissue microarrays were on the same plane, then stuck the ice block on the paraffin block for about 5 min and cut into 10-30 successive sections of 5-μm thickness and used the ice block to freeze the paraffin block. We repeated the above process until finishing sectioning of the tissue, floated the successive sections on the cool water and let it spread naturally. Then we used the ophthalmic elbowed forceps and glass slide to separate the sections during which the first section on the head part of the successive sections could be stuck to the glass slide, fixed and separated it with forceps to avoid loss of tissue microarrays sample due to the leakage of tissue section during separation. The sections were transferred into 45℃ warm water to spread for 1 min to ensure their full spreading without scattering. The sections were backed by the glass slide processed by 10% APES acetone solution for staining. We incubated the prepared tissue microarrays sections into a 60℃ oven for 1 h, took them out, cooled it at room temperature and put it into a -20℃ refrigerator for later use.

NF-κB p65 immunohistochemical staining (supersensitive S-P method)

We baked the section at 60℃ for 16 h and dewaxed in a routine fashion. We carried out antigen retrieval at high temperature and high pressure for 2 min, dropped reagent A to block the endogenous peroxidase, incubated at room temperature for 10 min, followed by washing with distilled water thrice, with biotin blocking reagent A at room temperature for 10 min, twice with PBS for 5 min each, with biotin blocking reagent B, at room temperature for 10 min, and twice with PBS for 5 min each. We added the normal goat serum-blocking liquid, incubated at room temperature for 20 min and removed the extra liquid, then added primary antibody (1:100 dilution), incubated over night at 4℃, washed thrice with PBS for 5 min each, and again incubated with secondary antibody at room temperature for 10 min, washed thrice with PBS for 5 min each. We added streptomycete antibiotin-peroxidase solution, put at room temperature for 10 min, washed four times with PBS for 5 min each, and then added freshly prepared DAB solution for coloration. The sections were observed under microscope and washed with distilled water.

Table 1 Comparison of plasma amylase [M (Q_R)]

Group (time/h)	3 h	6 h	12 h
Sham operation	2038.00 (346.00)	2117.00 (324.00)	1725.00 (434.00)
Model	7423.00 (2275.00)	8149.00 (1540.00)	9195.00 (1298.00)
Treatment	6739.00 (2310.00)	7839.00 (2258.00)	7791.00 (1863.00)

Observational index

Survival rate: The rat mortality observed at 3 h, 6 h, and 12 h after operation and the survival rate was calculated.

Pathological changes of mutiple organs: After mercy killing the rats anesthetized by sodium pentobarbital in batches, the gross samples of mutiple organs (liver, kidney, lung) were collected and observed for the pathological changes.

NF-κB p65 protein expression in the mutiple organs: We applied tissue microarrays to prepare microarray sections of the mutiple organs, and, using immunohistochemical S-P method, observed the NF-κB p65 protein expression and carried out the comprehensive assessment according to the positive cell percentage: < 10% (-); 10%-20% (+); 20%-50% (++); > 50% (+++).

Statistical analysis

The statistical analysis was conducted using the SPSS11.5 software. The Kruskal-Wallis test was applied for comparison of the three groups. The Bonferroni test was applied to the two-group comparison. The likelihood ratio Chi-square test was applied to compare the survival rate. $P < 0.05$ was considered statistically significant.

RESULTS

Survival rate

The 3 h, 6 h and 12 h mortality of the model group were 0% (0/15), 0% (0/15), 13.33% (2/15), respectively. The entire survival rate was 86.67%, while the survival rate of sham operation group and treatment group at all time points were 100%. But the survival rate at different time points was not significantly different between the model group and treatment group.

Comparison of plasma amylase content of all groups

Plasma amylase content was significantly increased in the model group and dexamethasone treatment group compared to the sham operation group at all time points ($P < 0.001$). No marked difference was observed in plasma amylase content between the dexamethasone treatment group and model group at 3 h and 6 h. However, plasma amylase content was found to be significantly less in the dexamethasone treatment group than the model group at 12 h ($P < 0.01$) (Table 1).

Comparison of plasma endotoxin content of all groups

Plasma endotoxin content was significantly increased in

Table 2 Comparison of plasma endotoxin [M (Q_R)]

Group (time/h)	3 h	6 h	12 h
Sham operation	0.015 (0.007)	0.015 (0.007)	0.016 (0.005)
Model	0.035 (0.0170)	0.055 (0.025)	0.059 (0.020)
Treatment	0.030 (0.0140)	0.040 (0.012)	0.042 (0.018)

Table 3 Comparison of serum TNF-α [M (Q_R)]

Group (time/h)	3 h	6 h	12 h
Sham operation	3.30 (3.60)	4.90 (2.60)	3.70 (2.30)
Model	46.13 (37.95)	77.54 (42.16)	67.30 (32.13)
Treatment	38.40 (26.60)	58.30 (26.40)	38.70 (28.50)

the model group and dexamethasone treatment group than the sham operation group at all time points ($P < 0.001$). No marked difference was observed in plasma endotoxin content between the dexamethasone treatment group and model group at 3 h. However, plasma endotoxin content was found to be significantly less in the dexamethasone treatment group compared to the model group at 6 h and 12 h ($P < 0.01$) (Table 2).

Comparison of serum TNF-α content of all groups

Serum TNF-α content was obviously increased in the model group and dexamethasone treatment group compared to the sham operation group at all time points ($P < 0.001$). No obvious difference was observed in serum TNF-α content between the dexamethasone treatment group and model group at 3 h. Serum TNF-α content was found to be significantly less in the dexamethasone treatment group compared to the model group at 6 h and 12 h ($P < 0.05$) (Table 3).

Macroscopic and microscopic changes of the liver

Sham operation group: Macroscopically, we observed normal color without obvious swelling of the liver in all groups. Microscopically, roughly normal hepatic tissue, slight inflammatory cell infiltration in the portal area, normal morphous of most liver cells, some with acidophilia apomorphosis or slight expansion and congestion of sinus hepaticus were observed.

Model group: Macroscopically, in the 3 h group, slight swelling of the liver was observed, and some rats had local grey plaques with unclear boundary, while in the 6 h and 12 h groups, pale, turbid color or congestion on the liver, and some with scattered grey plague in irregular shape or necrosis were observed. Microscopically, we observed swelling or acidophilia apomorphosis of the liver cells, inflammatory cell infiltration in the portal area, expansion and congestion of sinus hepaticus, and scattered spotty necrosis in the hepatic lobule in the 3 h group, obvious swelling of the liver cells, increased range and area of the liver cell necrosis, visible focal or massive hemorrhagic necrosis, inflammatory cell infiltration in necrosis focus, obvious congestion of partial sinus hepaticus, bile duct proliferation and scattered necrosis of single cell in the portal area (concentration and fragmentation of nucelus) in the 6 h group, obviously damaged structure of the hepatic lobule, further increased necrosis range and area of the liver cells, more inflammatory cell infiltration in the lobule and/or portal area, and obvious congestion of sinus hepaticus in the 12 h group.

Dexamethasone treatment group: Macroscopically, the gross liver pathological changes of the dexamethasone treatment group at 6 h and 12 h were milder than those of the model group, most significantly at 12 h. Microscopically, we observed slight swelling of the liver cells, slight expansion and congestion of the sinus hepaticus, scattered inflammatory cell infiltration but with significantly less scale in the portal area at all time points in the dexamethasone treatment group, while more limited necrosis range of the liver cells, no obvious lamellar necrosis at 6 h and 12 h groups. The gross pathological changes of the dexamethasone treatment group were milder than those of the model group at 6 h and 12 h, most significantly at 12 h.

Macroscopic and microscopic changes of the kidney

Sham operation group: Macroscopically, no swelling of the kidney with normal morphous, and no bleeding point on the renal cortex surface were observed. Microscopically, normal structures of renal glomerulus, renal tubule and renal interstitium without any obvious pathological changes were observed in most of the rats, while unclear boundary of the renal tubular epithelial cells (especially proximal tubule), stenosis and atresia of lumens, congestion of renal glomerulus and interstitial edema were observed in a small number of rats.

Model group: Macroscopically, in the 3 h group, no obvious gross changes in the kidney, while in the 6 h and 12 h groups, renal swelling, tension of the kidney envelope, scattered bleeding points on surface of the kidney envelope in some rats and slight hemorrhagic urine within the pelvis in severe cases were observed. Microscopically, in the 3 h group, congestion of glomerular capillary, swelling of the renal tubular epithelial cells, scattered necrosis, unclear cell boundary, stenosis or atresia of lumens, visible protein cast, interstitial edema and inflammatory cell infiltration, while in the 6 h and 12 h groups, obvious congestion of glomerular capillary, swelling of the renal tubular epithelial cells, scattered necrosis, interstitial edema, inflammatory cell infiltration were observed. Moreover, eosinophilic staining floss, red cells and eosinophilic staining homogen cast or red cell cast in the glomerular capsule were observed. There were expansion of renal tubule lumens in medulla, atrophia of endothelial cells and the pathological changes grew worse with time; lamellar necrosis of the renal tubular epithelial cells in a small number of rats.

Dexamethasone treatment group: Macroscopically, in the 6 h and 12 h groups, the gross pathological changes of the dexamethasone treatment group were milder than those of the model group. Microscopically, we observed milder congestion of glomerular capillary, swelling of the renal tubular epithelial cells as well as less eosinophilic staining floss and red cells in the renal capsule and less inflammatory cell infiltration than those of the model group; edema of renal interstitium and scattered necrosis

Table 4 The changes of expression level of NF-κB in the liver

Group	(Time/h)	Cases	Pathologic grade			
			-	+	++	+++
Sham operation	3	15	15	0	0	0
	6	15	15	0	0	0
	12	15	15	0	0	0
Model	3	15	7	4	4	0
	6	15	10	3	2	0
	12	13	11	1	1	0
Treatment	3	15	12	1	2	0
	6	15	11	1	3	0
	12	15	9	3	3	0

Table 5 Comparison of the expression level of NF-κB in the liver [M (Q_R)]

Group	3 h	6 h	12 h
Sham operation	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Model	1.00 (2.00)	0.00 (1.00)	0.00 (0.00)
Treatment	0.00 (0.00)	0.00 (1.00)	0.00 (1.00)

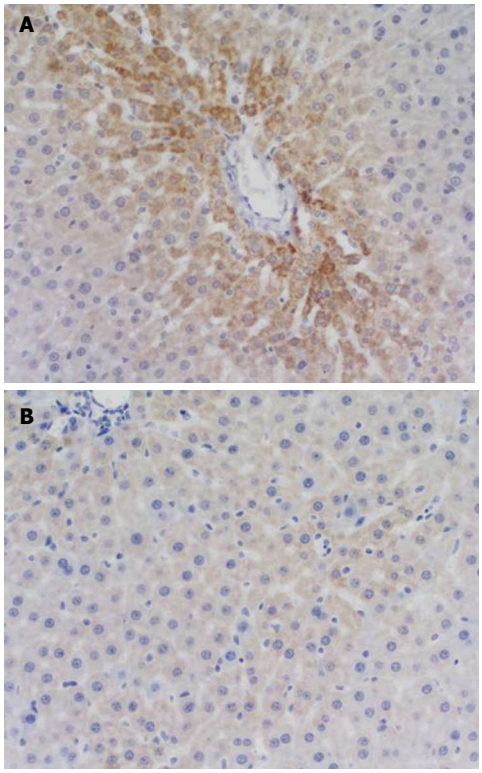

Figure 1 NF-κB expressions in the liver in the model group-3 h, (++) × 200 (**A**) and the treatment group-3 h, (-) × 200 (**B**).

in a small part of the renal tubular epithelial cells.

Macroscopic and microscopic changes of the lung

Sham operation group: Macroscopically, normal color and structure of the lung on both sides, no bleeding point on the surface and no effusion in the thoracic cavity were observed. Microscopically, normal function of the most lung tissues, but quite few with slight edema and inflammatory cell infiltration of interstitium were observed.

Model group: Macroscopically, in the 3 h group, obvious hyperemia and edema of the pulmonary lobes on both sides, dark red bleeding points on the local pulmonary lobe surface, small amount of amber and dilute effusion in the thoracic cavity, while in the 6 h and 12 h groups, aggravated pathological changes of the lung on both sides with prolonged time after modeling, lump-like pruinosus plaque on the lung surface, increased effusion in the thoracic cavity, and some hemorrhagic changes were observed. Microscopically, in the 3 h group, edema of the lung interstitium and alveolar space, widened interstitium of alveolar wall, visible inflammatory cell infiltration, telangiectasis and congestion of alveolar wall and widened alveolar septum, while in the 6 h and 12 h groups, further increased range of pathological changes of pulmonary lobes, obviously increased effusion in alveolar space, edema and bleeding of interstitium and alveolar space, obviously widened alveolar septum, more inflammatory cell infiltration and lucent kytoplasm of local tunica mucosa bronchiorum epithelium were observed.

Dexamethasone treatment group: Macroscopically, no obvious bleeding point on the pulmonary lobe surface, sound elasticity of pulmonary lobes, no obvious effusion in the thoracic cavity were observed; the gross lung pathological changes were milder than those of the model group at all time points, indicating obvious therapeutic effects. Microscopically, most lung tissue restored normal structures, and few with slight edema of interstitium and alveolar space were observed, indicating obvious therapeutic effects.

Changes of NF-κB expression levels in the liver of all groups

The positive NF-κB staining was located in the cytoplasm of the liver cells. The NF-κB expressions were all negative in the sham operation group at different time points, partly negative and partly + or ++ at 3 h in the model group. Most expressions of the model group were negative at 6 h and 12 h and a small part + or ++, while the negative rate of the model group at 12 h surpassed that at 6 h. There was no marked difference among all groups at 6 h. There was no marked difference between the model group and treatment group at all time points. However, the model group significantly exceeded the sham operation group at 3 h ($P < 0.01$) and the treatment group significantly exceeded the sham operation group at 12 h ($P < 0.01$) (Tables 4 and 5, Figure 1A and B).

Changes of NF-κB expression levels in the kidney of all groups

The positive NF-κB staining was located in the cytoplasm of renal tubular epithelial cells (Table 3). The expressions of the sham operation group were all negative at different time points. Most expressions of the model group were negative at 3 h and 6 h and a small part + or ++. The expressions of the model group were all negative at 12 h. Most expressions of the treatment group were negative at 3 h and a quite small part + or ++. Most expressions of the treatment group were negative at 6 h and a quite small part ++. The expressions of the treatment group were partly negative and partly + or ++ at 12 h. There were no marked differences between the model group and

Table 6 The changes of expression level of NF-κB in the kidney

Group	(Time/h)	Cases	Pathologic grade			
			-	+	++	+++
Sham operation	3	15	15	0	0	0
	6	15	15	0	0	0
	12	15	15	0	0	0
Model	3	15	13	1	1	0
	6	15	12	1	2	0
	12	13	13	0	0	0
Treatment	3	15	12	2	1	0
	6	15	13	0	2	0
	12	15	9	2	3	1

Table 7 Comparison of the expression level of NF-κB in the kidney [M (Q_R)]

Group	3 h	6 h	12 h
Sham operation	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Model	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Treatment	0.00 (0.00)	0.00 (0.00)	0.00 (2.00)

treatment group at all time points, moreover, no obvious differences among all groups at 3 h and 6 h. However, the treatment group significantly exceeded the model group ($P < 0.05$) and sham operation group ($P < 0.01$) at 12 h (Tables 6 and 7, Figure 2A, B and C).

Changes of NF-κB expression levels in the lung of all groups

NF-κB expressions in the lung of all groups were negative (Figure 3).

DISCUSSION

As one of the common clinical acute abdomen, severe acute pancreatitis (SAP) usually accompanied by the obvious inflammatory reactions besides local pathological injuries can lead to systemic inflammatory response syndrome (SIRS) or even the complication of multiple organ injury, further multiple organ dysfunction syndrome (MODS)[11], resulting in quite high mortality. Although people have conducted enormous studies on AP pathogenesis and brought forward many valuable theories such as the theory of oxygen-free radicals[12], the exact mechanism remains unclear. Studies in recent years have proven that the activation of NF-κB plays an extremely important role in the onset process of SAP[7,13,14].

NF-κB, a protein with multi-attribute transcription-regulating effect, consists of NF-κB/Rel protein family members. NF-κB is usually combined with NF-κB profilin (IκB) to form an inactive trimer that cannot enter the cell nucleus but exists in the cytoplasm[15,16]. A series of enzymes can be activated through signal transduction pathway after the stimulation of TNF-α, IL-1, LPS[17] to activate NF-κB and next IκB kinase to realize the phosphorylation of IκB. When IκB falls off the NF-κB complex, the activated NF-κB will move into the cell nucleus, bind to the κB structural domain of the promoter or enhancer of target gene and cause the transcription of many factors[18-20], including cytokines, chemotactic factor, macrophage chemotactic peptide, cellular adhesion molecule, growth factor, immune receptor and acute phase reactive protein.

The NF-κB p65 involved in this experiment is one of the important composing members of NF-κB/Rel family with 65 000 of relative molecular weight. Its most common

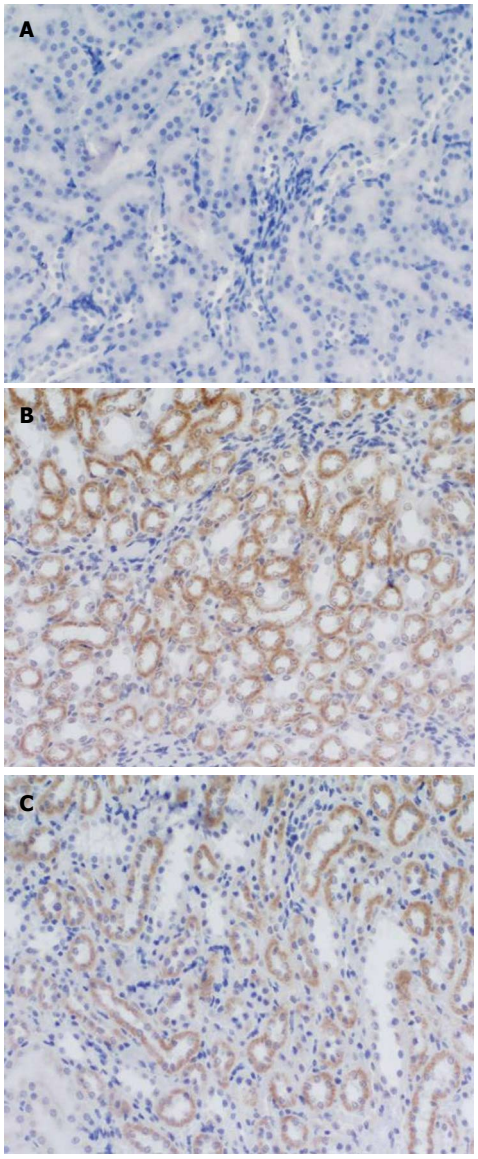

Figure 2 NF-κB expressions in the kidney in the sham operation group-12 h, (-) × 200 (**A**), model group-6 h, (++) × 200 (**B**) and model group-12 h, (++) × 200 (**C**).

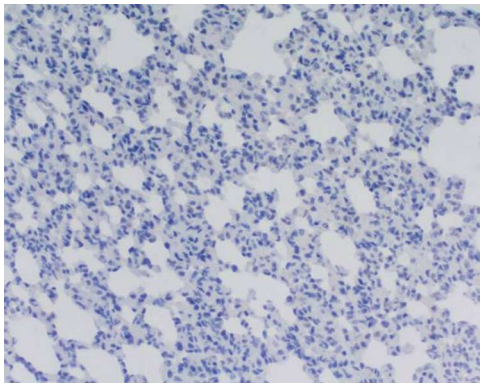

Figure 3 NF-κB expressions in the lung in the sham operation group-3 h, (-) × 200.

form in cell is the heterotic dimer of NF-κB consisting of p65 and p50. When resting, it is combined with its inhibitor IκB to exist in the cytoplasm. When stimulated, the NF-κB will be transferred into nucleus through IκB degradation to combine the κB sequence of the promoter and enhancer area of the regulated gene, promote the transcription of these genes[5] and participate in the tissue injury caused by manifold factors[21]. Tietz *et al*[22] respectively determined the serum concentration of TNF-α and IL-6 as well as their mRNA expression levels in mice with acute pancreatitis through ELISA and RT-PCR methods, and found that the genetic expression of inflammation promoting cytokines like TNF-α and IL-6 played an extremely important role in the progression of acute pancreatitis.

Dexamethasone, a kind of glucocorticoid, can inhibit the gene synthesis of manifold inflammatory mediators and inhibit inflammatory mediators by increasing the synthesis of anti-inflammatory protein[8]. The possible mechanism for dexamethasone inhibition of NF-κB activation could be: (1) As a hormone where the ligand binds to its corresponding receptor and activates it. The activated glucocorticoid receptor directly couples the RelA subunit of NF-κB in the cell nucleus to inhibit the functions of NF-κB. (2) The activated glucocorticoid receptor blocks the nucleus shifting of NF-κB and its combination with DNA by enhancing the genetic transcription of IκB and raising its level.

Acute liver injury is also a common complication of SAP. Studies found that NF-κB plays an important role in the liver injury process of SAP rats[23,24]. NF-κB after activation can promote liver injury by promoting the genetic transcription of TNF-α and IL-6[25]. NF-κB inhibitor can protect the liver by inhibiting the activation of NF-κB. Some studies believe liver cells are not only target cells of SAP liver injury but also could be the effector cells of inflammatory reactions secreting cytokines. They can act on the liver cells through autocrine or paracrine fashion to aggravate the liver injury[26]. In this experiment, we observed the rise of NF-κB p65- positive cell percentage and expression of manifold inflammation-inducing factors which participated in the liver injury complicated with SAP. The positive cell percentage of the dexamethasone treatment group dropped at 3 h and 6 h possibly because dexamethasone had inhibited the activation of NF-κB, reduced the expression of relevant cytokines and thereby alleviated the liver injury. It should be mentioned that the positive cell rate of the treatment group had risen compared with the model group at 12 h, while the statistical results showed no marked difference between the model group and treatment group at all time points. Theoretically, the positive cell rate should decline possibly due to the following factors: (1) With features like time- and energy- saving and high efficiency, the tissue microarray sections of 2.0 mm in diameter may not reflect the whole tissue picture and, to a certain extent, its representation is limited. (2) With different case numbers, the two groups cannot be compared directly. Statistical results showed no marked difference between the model group and treatment group at all time points; (3) There were also possible faults in reagent selection and staining.

Acute kidney injury is also one of the common complications of SAP with few relevant reports currently. In this experiment, the NF-κB expression level of the treatment group significantly exceeded that of the model group at 12 h after dexamethasone treatment, which is contradictory to the theory to certain extent and we can hardly understand. The reason could resemble that of the aforementioned liver. The main reason is still that the representation of tissue microarrays sections of 2.0 mm in diameter is doubtful and could lead to the deviation of the experimental results. We have planned to select three 2.0-mm points for each tissue in subsequent studies to ensure the representation of tissue. In common studies, the determination and analysis of a single index usually could pose many limitations. Therefore, we add the determination of multiple indexes into the experiment including the plasma amylase content, plasma endotoxin content and serum TNF-α content in order to completely evaluate the therapeutic effects and mechanism of dexamethasone.

Acute lung injury is one of the most common severe complications of SAP[27]. The mechanism responsible for acute lung injury caused by SAP is quite complicated and remains unclear till now. It is now believed that pancreatin, adhension molecule, neutrophil, various inflammatory mediators, etc play extremely important roles in the onset process[28-30]. It has been shown an increase in expression of manifold inflammation-inducing cytokines in the lung tissue at early injury stage, while the activation of transcription factor NF-κB can stimulate the expression of manifold cytokines. Studies show the selective use of NF-κB inhibitor can markedly lower the injury degree of the pancreas and lung, indicating the important role of NF-κB in SAP complicated with the lung injury[31-33]. This experiment has observed the NF-κB p65 expression in the lung tissue but the NF-κB expression levels in the lungs of all groups were negative, indicating that the non-expression of NF-κB p65 in the lung could be related to the reagents. The reagents used in this experiment did not stain in the lung.

In conclusion, NF-κB plays an important role in multiple organ injury. Further studies should be conducted to determine whether dexamethasone could alleviate the pathological changes of multiple organs by reducing the NF-κB expression of the liver and kidney. The advantages of tissue microarrays in pancreatitis pathological examination include saving time and energy, they are highly efficient and representative. The restriction on the representation of tissues to various extents due to small diameter may lead to the deviation of analysis.

Produced mainly by the pancreas, amylase (AMS) has an important effect on digestion of polysaccharide in food and acute pancreatitis is the most common cause of its rise. The rising degree of AMS activity is not certainly related to the injury degree of pancreatic tissue. But the more obviously AMS rises, the more severe the injury becomes. Our study showed that the amylase content of the model group increased with time and the amylase content in plasma dropped after treatment.

Endotoxin is a kind of compound of lipopolysaccharide (LPS) and small protein (Protein) on the cell wall of Gram-

negative bacteria. It is specific not because it is a bacteria or metabolite of bacteria but a substance with endotoxin bioactivity only released after death or disintegration of bacteria. Its chemical composition mainly consists of O-specific chain, core polysaccharide and lipoid A. Endotoxemia results when the endotoxin can be detected in the circulatory blood, which could cause a series of pathophysiological changes including sepsis, shock, and diffuse intravascular coagulation and multiple organ dysfunction syndrome (MODS). The role of endotoxin in onset of acute pancreatitis covers the following aspects: (1) Interfering the normal function of cell membrane by non-specific combination with it; (2) Directly destroying the lysosomal membrane within cells of mononuclear phagocytic system to cause cell damage; (3) Damaging mitochondria structure, affecting the coupling process of ATP enzyme and oxidative phosphorylation and causing disturbance in energy metabolism; (4) Changing the immune function of body; (5) Causing a series of pathological or pathophysiological changes of body, affecting the vasomotor function, activating vasoactive substance, reducing platelet and leukocyte, lowering blood pressure or even causing DIC, MOSF, etc.

Mainly generated from the activated mononuclear macrophage, TNF-α is a kind of polypeptide cytokine with extensive biologic activities. The secretion of appropriate amounts of TNF-α had protecting effects and can promote the chemotaxis and antimicrobial effects of PMN, macrophage and eosinophile granulocyte and is one of the defense mechanisms of the body. The excessive secretion of TNF-α could cause inflammatory reactions. High concentration of TNF-α entering the blood flow could also cause fever, drop of blood pressure and reduction of tissue perfusion through lowering myocardial contraction force and tension of vascular smooth muscle as well as metabolic disorder, organ injury and even multi-system damage. It has potentially lethal effects.

Our experiment found no marked difference in the plasma amylase content at 3 h and 6 h between the treatment group and model group, while the plasma amylase content of the treatment group was significantly lower than that of the model group at 12 h ($P < 0.01$). The plasma endotoxin content of the treatment group was significantly lower than that of the model group at 6 h and 12 h ($P < 0.01$). The serum TNF-α level of the treatment group was significantly lower than that of the model group at 6 h and 12 h ($P < 0.05$). Thus, it is concluded that dexamethasone can lower the plasma amylase, plasma endotoxin and serum TNF-α content of rats with SAP as well as their mortality.

The tissue microarrays (TMA) technology adopted by us in this study is exactly the new biochip technology currently extensively applied to basic and clinical applications as well as in other fields. The tissue microarray technology has exceeded the traditional histopathological section technology which is single in sample and low in efficiency[34]. The advantages of TMA are high-throughput, economic, time-saving, reliable result, convenient for experimental control, etc. Since the most distinguished feature of tissue microarrays is to combine the study of gene and its expression products with histomorphology, it possesses great potential in oncopathology studies[35,36], and current studies also mainly focus on this area[37-44]. The chip preparation, staining, examination, etc have restrained the application of this technology in non-tumor diseases. To the best of our knowledge, there was no study report in literature on applying tissue microarrays to pancreatitis pathological examination before our experiment was conducted. Thus this article has taken the lead.

We used the tissue microarray section maker (Beecher Instruments, USA) to drill a hole of 2.0 mm in diameter on recipient block and combined the immunohistochemical method to examine the NF-κB expression levels of the multiple organs. The experimental results are unsatisfactory mainly because the tissue representation has been restricted to different extents due to the small diameter. However, there are advantages of tissue microarrays shown in its application in the pathological examination of pancreatitis, including time- and energy-saving and high efficiency.

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BASIC RESEARCH

Inhibitory effects of saikosaponin-d on CCl_4-induced hepatic fibrogenesis in rats

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Abstract

AIM: To investigate the suppressive effect of saikosaponin-d (SSd) on hepatic fibrosis in rats induced by CCl_4 injections in combination with alcohol and high fat, low protein feeding and its relationship with the expression of nuclear factor-κB (NF-κB), tumor necrosis factor-alpha (TNF-α) and interleukins-6 (IL-6).

METHODS: Hepatic fibrosis models were induced by subcutaneous injection of CCl_4 at a dosage of 3 mL/kg in rats. At the same time, rats in treatment groups were injected intraperitoneally with SSd at different doses (1.0, 1.5 and 2.0 mg/kg) once daily for 6 wk in combination with CCl_4, while the control group received olive oil instead of CCl_4. At the end of the experiment, rats were anesthetized and killed (except for 8 rats which died during the experiment; 2 from the model group, 3 in high-dose group, 1 in medium-dose group and 2 in low-dose group). Hematoxylin and eosin (HE) staining and Van Gieson staining were used to examine the changes in liver pathology. The levels of alanine aminotransferase (ALT), triglyeride (TG), albumin (ALB), globulin (GLB), hyaluronic acid (HA) and laminin (LN) in serum and the content of hydroxyproline (HYP) in liver were measured by biochemical examinations and radioimmuneoassay, respectively. In addition, the expression of TNF-α and IL-6 in liver homogenate was evaluated by enzyme-linked immunosorbent assay (ELISA) and the levels of NF-κBp65 and I-κBα in liver tissue were analyzed by Western blotting.

RESULTS: Both histological examination and Van Gieson staining demonstrated that SSd could attenuate the area and extent of necrosis and reduce the scores of liver fibrosis. Similarly, the levels of ALT, TG, GLB, HA, and LN in serum, and the contents of HYP, TNF-α and IL-6 in liver were all significantly increased in model group in comparison with those in control group. Whereas, the treatment with SSd markedly reduced all the above parameters compared with the model group, especially in the medium group (ALT: 412 ± 94.5 IU/L *vs* 113.76 ± 14.91 IU/L, TG: 0.95 ± 0.16 mmol/L *vs* 0.51 ± 0.06 mmol/L, GLB: 35.62 ± 3.28 g/L *vs* 24.82 ± 2.73 g/L, HA: 42.15 ± 8.25 ng/mL *vs* 19.83 ± 3.12 ng/mL, LN: 27.56 ± 4.21 ng/mL *vs* 13.78 ± 2.57 ng/mL, HYP: 27.32 ± 4.32 μg/mg *vs* 16.20 ± 3.12 μg/mg, TNF-α: 4.38 ± 0.76 ng/L *vs* 1.94 ± 0.27 ng/L, IL-6: 28.24 ± 6.37 pg/g *vs* 12.72 ± 5.26 pg/g, respectively, $P < 0.01$). SSd also decreased ALB in serum (28.49 ± 4.93 g/L *vs* 37.51 ± 3.17 g/L, $P < 0.05$). Moreover, the expression of NF-κB p65 in the liver of treated groups was lower than that in model groups while the expression of I-κBα was higher in treated group than in model group ($P < 0.01$). The expression of NF-κBp65 and TNF-α had a positive correlation with the level of HA in serum of rats after treatment with CCl_4 ($r = 0.862$, $P < 0.01$; $r = 0.928$, $P < 0.01$, respectively).

CONCLUSION: SSd attenuates CCl_4-induced hepatic fibrosis in rats, which may be related to its effects of hepato-protective and anti-inflammation properties, the down-regulation of liver TNF-α, IL-6 and NF-κBp65 expression and the increased I-κBα activity in liver.

Key words: Saikosaponin-d; Hepatic fibrosis; Tumor necrosis factor; Interleukins-6; Nuclear factor-κB; Inhibitory κB alpha

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INTRODUCTION

Hepatic fibrosis represents the wound healing response of the liver to repeated liver injuries, and is associated with increased inflammatory cell infiltration and may involve the interplay of different inflammatory mediators, which is a common stage in most chronic liver diseases[1-5]. If treated properly in this stage, hepatic fibrosis can be

reversed and its progression to irreversible cirrhosis often leading to lethal complications and high mortality may be prevented[6-9]. Nuclear factor-κB (NF-κB) as a critical component in inflammatory conditions can produce proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which are involved in the process of fibrogenesis[10-13]. Therefore, suppressing the inflammatory response and reducing the release of proinflammatory cytokines such as NF-κB, TNF-α and IL-6, may prevent and reverse hepatic fibrosis. Saikosaponin-d (SSd) is a major active component extracted from the root of Bupleurum falcatum. It has been demonstrated that SSd has a wide variety of pharmacological activities, such as liver-protective activity, and anti-hepatic fibrosis or anti-microbial or anti-tumor and anti-inflammatory activities[14-17]. However, its molecular mechanism involved in therapeutic effects of SSd on hepatic fibrosis has not been completely elucidated. Our present study was designed to further evaluate the effect of SSd on hepatic fibrosis in rats induced by CCl_4 and its relationship with the expression of NF-κB, TNF-α and IL-6.

MATERIALS AND METHODS

Reagents

SSd was purchased from Jiangxi Herbfine Hi-tech Co. Ltd. CCl_4 (Xi'an Chemical Factory) was diluted into 400 g/L in olive oil before it was used. Enzyme-linked immunosorbent assay (ELISA) kit for mouse TNF-α and IL-6 was purchased from R&D Systems Co. Ltd (USA). Hydroxyproline (HYP) assay kit was a product of Nanjing Jiancheng Bioengineering Institute. Kits for HA and LN were bought from Senxiong Company, Shanghai, China. Polyclonal rabbit anti-rat P65 and I-κBα were purchased from Santa Cruz Biotechnology (USA). HRP-labeled goat-anti-rabbit IgG was obtained from HuaMei Company, Shanghai, China.

Animals

Seventy-five adult male SD rats weighing 160-200 g were provided by the Laboratory Animal Center of Medical College, Xi'an Jiaotong University. The rats were randomly divided into 5 groups (n = 15): control group, model group, and three treatment groups. Except for the control rats, all rats were subcutaneously injected with 400 g/L CCl_4 (CCl_4: olive oil = 2:3), 3 mL/kg, b.w, at every 3 d for 6 wk, and fed with high fat, low protein diet (75% pure maize plus 20% lard and 0.5% cholesterol) and 300 mL/L alcohol in the drinking water. In the 3 treatment groups, SSd was administered daily, *via* intraperitoneal injection at a dosage of 2.0, 1.5 and 1.0 mg/kg for 6 wk, respectively. After 6 wk, all rats were anesthetized with 200 g/L urethane (5 mL/kg, abdominal injection). Blood was taken from the abdominal aorta. Serum was separated by centrifugation at 4℃ and kept at -20℃ for assay. Liver tissue was homogenized in cold saline for pathological diagnosis.

Light microscopic examination

Liver tissue was fixed in a 40 g/L solution of formaldehyde in 0.1 mol/L phosphate-buffed saline (pH 7.4), and embedded in paraffin. Five-micrometer thick sections were prepared. All the sections stained with HE and standard Van Gieson (VG) were coded and scored by blind reading. Van Gieson's method was used to detect collagen fibers[18]. Liver condition was classified according to the standard formulated by China Medical Association in 1995[19], and fibrosis was graded from 0 to 4 (0: no fibrosis; 1: portal area fibrosis; 2: fibrotic septa between portal tracts; 3: fibrosis septa and structure disturbance of hepatic lobule and 4: cirrhosis).

Biochemical determination

Serum levels of alanine transaminase (ALT), albumin (ALB), triglyeride (TG) and globulin (GLB) were measured by routine laboratory methods using a 7170-automatic biochemistry analyzer (Tokyo, Japan). Serum hyaluronic acid (HA) and laminin (LN) were detected by radioimmunoassay, and the content of hydroxyproline (HYP) in liver was determined according to the method described by Jamall *et al*[20]. The contents of TNF-α and IL-6 protein in liver homogenate were determined by ELISA according to the corresponding protocols of the kits.

Western blotting detection

Nuclear and cytosolic protein extracts were prepared according to manufacturer's instructions provided with the kits (Active Motif Corp, USA). Nuclear or cytosolic proteins (100 μg each) were run on a 10% SDS-PAGE gel and transferred electrophoretically onto a nitro-cellulose membrane respectively (Shanghai Huashun Corp, China). The membrane was blocked overnight with 10% nonfat milk prior to incubation with polyclonal rabbit anti-rat I-κBα antibody (1:800) or anti-NF-κBp65 antibody (1:1000) at room temperature for 2 h. After washed with PBS, the blots were incubated with HRP-labeled goat-anti-rabbit serum for 1 h and colored on X-ray film by ECL.

Statistical analysis

Quantitative data were analyzed using ANOVA by SPSS 13.0 statistic package and RIDIT test was used for statistical analysis of the qualitative data. All data were expressed as mean ± SD. The correlation was analyzed by Spearman's correlation analysis. All P values were two-tailed. $P < 0.05$ was considered statistically significant.

RESULTS

Pathological assay

At the end of the experiment, liver tissue samples from control rats showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). Liver tissue samples from model group showed that more fibrous tissues were formed extending into the hepatic lobules to separate them completely. A large number of inflammatory cells infiltrated in the intralobular and interlobular regions. The liver structure was disordered and there were more necrotic and fatty degenerated liver cells compared with the controls (Figures 1B and 1D). In the 3 treatment groups, however, hepatocyte degeneration,

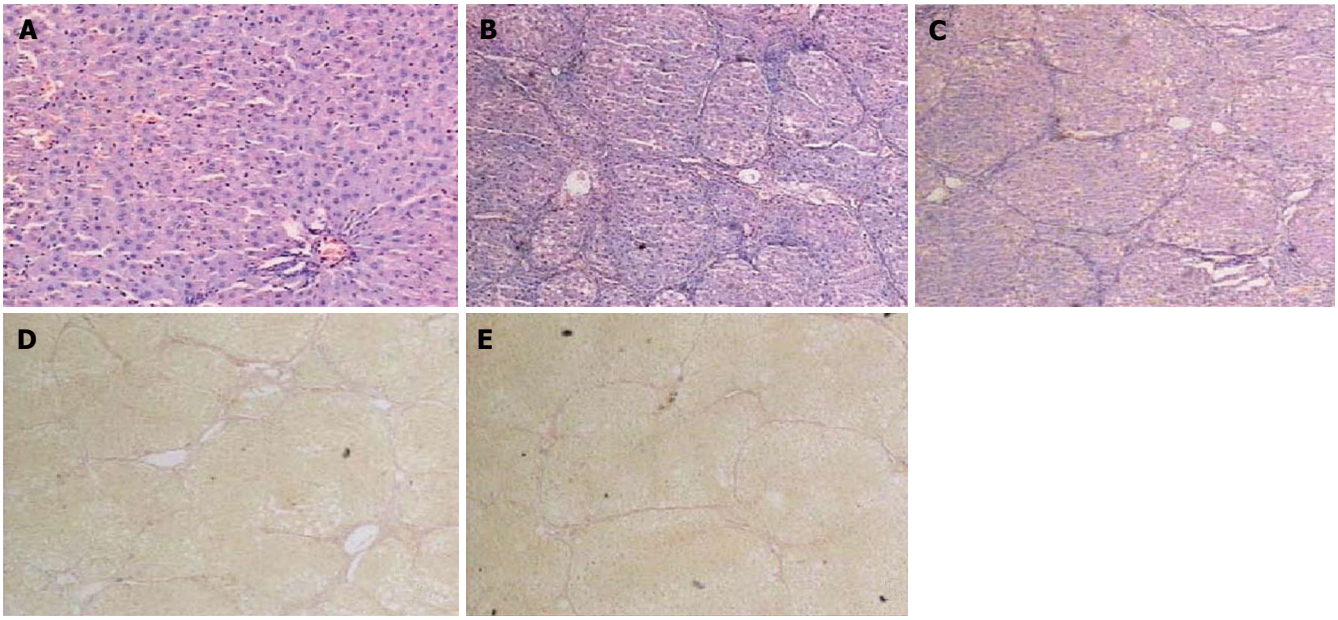

Figure 1 Light microscopy showing normal liver tissue in control group (**A**) (HE × 100), degenerated and necrotic liver cells associated with inflammatory cells in model group (**B**) (HE × 40), attenuated necrosis and infiltration of inflammatory cells after SSd treatment (**C**) (HE × 40), collagen fibers deposited in spaces of Disse and formation of pseudoloculi in model group (**D**) (Van Gieson × 40), and liver fibrosis tissue in SSd group (**E**). The pathological change of liver was much milder in SSd group than in model group (Van Gieson × 40).

necrosis and infiltration of inflammatory cells were all apparently ameliorated and collagen deposition was also markedly reduced (Figures 1C and 1E). Compared with model group, the liver condition of rats in SSd treatment groups was significantly improved (Table 1).

Detection of serum HA, LN and liver function

As is shown in Figure 2A, HA and LN levels in serum were significantly higher in model group than in the controls, but they were markedly decreased in 3 treatment groups compared with the model group. Compared with the controls, the serum ALT, TG and GLB levels in model group were all significantly increased while the level of ALB was decreased ($P < 0.001$, $P < 0.05$), respectively. However, the levels of ALT, TG and GLB were all in the 3 treatment groups, especially in the group receiving the middle dose of SSd, and the level of ALB was increased compared with the model group ($P < 0.05$) (Table 2).

TNF-α, IL-6 and HYP contents in liver tissue

The contents of TNF-α, IL-6 and HYP were all significantly lower in SSd treatment groups than in the model group (Figures 2B and 2C). Furthermore, among the 3 treatment groups the high-dose group showed the best effect. The liver HYP level in three SSd treatment groups and TNF-α, IL-6 content in high-dose group were higher than those in the controls, with no significant difference between them ($P > 0.05$). However, there was a significant difference in the contents of TNF-α, IL-6 between low- and medium-SSd treatment groups and control group ($P < 0.05$).

Western blot analysis

NF-κBp65 expression was increased significantly in model group compared with the control group, whereas it was markedly decreased in all SSd treatment groups ($P < 0.01$), especial in the high-dose group. There was no significant difference in the expression of NF-κBp65 between SSd treatment groups and control group ($P > 0.05$) (Figure 2D). On the contrary, its inhibitory IκBα was significantly decreased in model group while increased in SSd treatment group compared with the model group ($P < 0.01$) (Figures 3A and 3B). Therefore, SSd could significantly inhibit the activation of NF-κB, which might be associated with increased I-κBα degradation.

Table 1 Pathological observation of liver condition

Group	*n*	Liver condition					*U*
		0	Ⅰ	Ⅱ	Ⅲ	Ⅳ	
Model	13	0	0	0	4	9	
High-dose	12	0	5	3	2	2	3.26[a]
Medium-dose	14	0	6	4	3	1	4.17[b]
Low-dose	13	0	3	2	6	2	2.96[a]

U represents the RIDIT value of the two groups, $P < 0.05$ indicates $U > 1.96$, $P < 0.01$ indicates $U > 2.58$. [a]$P < 0.05$, [b]$P < 0.01$ *vs* model group.

Correlation analysis

Correlation analysis revealed that NF-κBp65 had a highly positive correlation with the expression of TNF-α protein ($r = 0.823$, $P < 0.01$). Both NF-κBp65 and TNF-α had a strong positive correlation with the levels of HA in serum of rats induced by CCl_4 ($r = 0.862$, $P < 0.01$; $r = 0.928$, $P < 0.01$, respectively).

DISCUSSION

Hepatic fibrosis is a chronic inflammation-associated

Table 2 Serum level of ALT, ALB, GLB, TG and liver HYP (mean ± SD)

Group	*n*	ALT (IU/L)	ALB (g/L)	GLB (g/L)	TG (mmol/L)	Liver HYP (μg/mg protein)
Control	15	67.58 ± 11.21	41.12 ± 2.54	21.48 ± 3.24	0.39 ± 0.08	9.80 ± 1.07
Model	13	412 ± 94.50	28.49 ± 4.93	35.62 ± 3.28	0.95 ± 0.16	27.54 ± 4.32
High-dose	12	173.09 ± 24.62[b,c]	35.73 ± 2.73[a]	25.59 ± 3.61[a]	0.61 ± 0.10[b]	12.83 ± 2.54[a,d]
Medium-dose	14	113.76 ± 14.91[a,d]	37.51 ± 3.17[a]	24.82 ± 2.73[a]	0.51 ± 0.06[a]	16.20 ± 3.12[b,d]
Low-dose	13	152.86 ± 19.19[b,c]	34.31 ± 4.52[b]	27.51 ± 2.41[b]	0.58 ± 0.07[b]	14.38 ± 2.18[b,d]

ALT: Alanine aminotransferase; ALB: Albumin; GLB: Globulin; TG: Triglyeride; HYP: Hydroxyproline. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* model group.

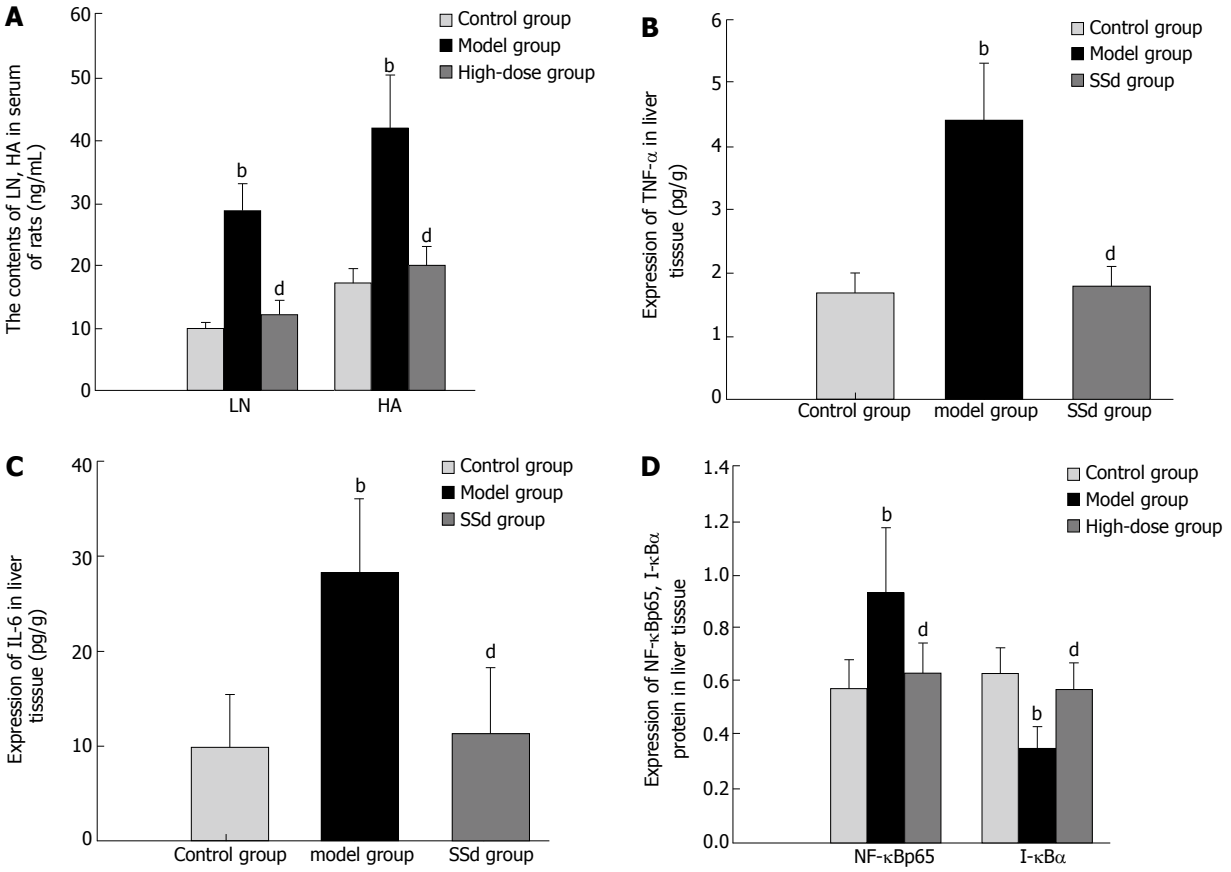

Figure 2 Analysis of serum LN and HA levels (**A**), expressions of TNF-α (**B**), IL-6 (**C**), and NF-κBp65 and I-κBα (**D**) in liver tissue after treatment with SSd. [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* model group.

disease, which is involved in the infiltration of inflammatory cells and releasing of proinflammatory cytokines, such as TNF-α and IL-6. As a result, hepatic stellate cells (HSCs) are transformed into myofibroblast cells to synthesize more collagen and proteoglycans, increasing deposition and altered composition of extracellular matrix (ECM) in liver[21-24]. Based on the current knowledge, a "three-step cascade theory of inflammation involving in liver fibrogenesis" including preinflammatory phases 1-3, has been proposed by Gressner[25], which implies that multiple inflammatory cell interactions with Kupffer cells, platelets, endothelial cells and hepatocytes mediated by various cytokines and growth factors (TNF-α, IL-6 and TGF-β) are involved in the mechanism of fibrogenesis. Therefore, suppressing the inflammatory response can prevent and reverse hepatic fibrosis. Our study showed that, 6 wk treatment with SSd, especially at the middle dose used, could decrease serum levels of ALT, TG, GLB and ALB in rats with hepatic injury caused by CCl_4. Histological examination also demonstrated that a large number of inflammatory cells infiltrated the intralobular and interlobular regions, more fibrous tissue was formed and the margin of liver was uneven in model group compared with the control group. In contrast, SSd especially its medium-dose could obviously attenuate the extent of necrosis and reduce the immigration of inflammatory cells compared with the model group, and no pseudoloculus could be observed. Moreover, SSd could decrease the scores of hepatic fibrosis grading (Table 1), indicating that SSd can significantly protect liver against fibrosis, which may be related to its inhibitory effects on inflammation. These findings are consistent with

previously reported results[26]. It has been demonstrated that SSd has marked inhibitory actions on the processes of inflammation, including capillary permeability, releasing of inflammation mediators, leukoplasia and desmoplasia[15,27,28]. In addition, SSd can increase serum concentrations of adrenocorticotropic hormone and corticosterone[29] as well as corticotropin-releasing factor (mRNA) level in the hypothalamus[16].

HA and LN levels in serum and HYP in liver are the important indices reflecting the degree of hepatic fibrosis[30-32]. In this study, the contents of HA and LN in serum and HYP in liver were much higher than those in the controls, but markedly lower in treatment groups ($P < 0.01$), indicating that SSd can prevent hepatic fibrosis due to chronic liver injury, thus delaying the development of cirrhosis.

Recent studies have identified NF-κB as a critical component to bridge inflammation by producing proinflammatory cytokines (such as TNF-α and IL-6) and more ECM in liver, thus further boosting inflammatory processes and activating HSCs[21-23,33-36]. It was also reported that TNF-α released from activating macrophages can turn up NF-κB activity both in target tissue cells and in macrophages themselves[37-40].

NF-κB is a transcription factor consisting of p65 and p50 subunits of the Rel protein family[41]. In most cells, it binds to its inhibitory counterpart I-κBα and other IκB proteins to form P65-P50-IκB trimer which is located in the cytoplasm as an inactive complex. Following I-κBα degradation by a complex signaling cascade initiated on the cell surface, the activated NF-κBp65 disassociates from I-κBα and shifts into nuclei where it binds to specific DNA motifs to regulate transcriptional activity of its target genes involved in HSC activation[42], releasing of proinflammatory cytokines including IL-6 and TNF-α. Thus, inhabiting IκBα phosphorylation is an indispensable step to activate the NF-κB signaling pathway[43-46]. In the present study, NF-κBp65 expression increased significantly in model group compared with normal control group, whereas it was markedly decreased in all SSd treatment groups, especial in the high-dose group. There was no difference in the expression of NF-κBp65 between SSd treatment groups and control group. On the contrary, its inhibitory I-κBα was significantly lower in model group but higher in SSd treatment group, suggesting that SSd can significantly inhibit the activation of NF-κBp65, which may be associated with a reduction in I-κBα degradation.

In addition, Spearman's correlation analysis showed that NF-κBp65 was highly correlated with the expression of TNF-α protein ($r = 0.823$, $P < 0.01$) and both of them had a strong positive correlation with the serum levels of HA induced by CCl_4 ($r = 0.862$, $P < 0.01$; $r = 0.928$, $P < 0.01$, respectively).

In conclusion, SSd has beneficial effects on hepatic fibrosis. Down-regulation of TNF-α, IL-6 and NF-κBp65 expression and increased I-κBα activity of SSd in rat liver may play an important role in the improvement of hepatic fibrosis induced by CCl_4. Since hepatic fibrogenesis is a very complicated process, the underlying mechanisms of SSd remain to be further explored.

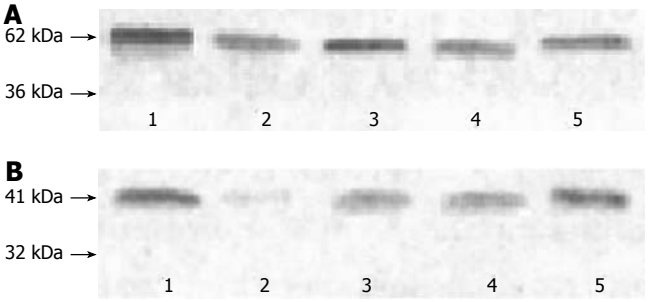

Figure 3 Images of Western blotting of NF-κBp65 (**A**) and I-κBα (**B**) in liver tissue of rats. Lane 1: NF-κBp65 and I-κBα protein in control group; lane 2: NF-κBp65 and I-κBα in model group; lanes 3-5: NF-κBp65 and I-κBα in SSd group (from low to high-dose group).

COMMENTS

Background

Hepatic fibrosis is a chronic inflammation-associated disease, which is involved in the infiltration of inflammatory cells and releasing of proinflammatory cytokines, such as NF-κB, TNF-α and IL-6. Recently, more and more clinical and experimental observations have demonstrated that SSd, a traditional Chinese medicine, is of some preventive and therapeutic values against liver fibrosis, whereas, the molecular mechanism involved in therapeutic effects of SSd on hepatic fibrosis has not been completely elucidated. Therefore, the aim of our study was to further evaluate the anti-hepatic fibrosis effect of SSd in rats and to study its relationship with the expression of NF-κBp65, TNF -α and IL-6.

Research frontiers

NF-κB as a critical component to bridge inflammation, can produce proinflammatory cytokines such as TNF-α and IL-6, which are involved in the process of fibrogenesis. Our study aimed at investigating the suppressive effect of SSd on hepatic fibrosis in rats induced by CCl_4 from the level of cytokine and its relationship with the expression of NF-κBp65, TNF-α and IL-6.

Innovations and breakthroughs

SSd has beneficial effects on hepatic fibrosis, and the down-regulation of TNF-α, IL-6 and NF-κBp65 expression and increased I-κBα activity of SSd in rat liver may play an important role in the improvement of hepatic fibrosis induced by CCl_4.

Applications

SSd may play a role in antifibrotic therapy. It protects liver cells against fibrosis and inhibits collagen fiber deposition in liver, and therefore can be used in the treatment of cirrhosis in clinic practice.

Terminology

Nuclear factor-κB (NF-κB) is a transcription factor consisting of p65 and p50 subunits of the Rel protein family. In most cells, it binds to its inhibitory counterpart I-κBα and other I-κB proteins to form P65-P50-IκB trimer that is located in the cytoplasm as an inactive complex. Following I-κBα degradation by a complex signaling cascade initiated at the cell surface, the activated NF-κBp65 disassociates from I-κBα and shifts into nuclei where it binds to specific DNA motifs to regulate transcriptional activity of its target genes involved in HSC activation, releasing of proinflammatory cytokines.

Peer review

In this study, rats with liver fibrosis were treated with CCl_4 in combination with ethanol, high fat and low protein diet. Rats receiving SSd in combination with CCl_4 injection developed less liver fibrosis. The effect was associated with less liver damage indicated by lower transaminase and higher albumin levels. Additionally, less activation of NF-κB was observed in the liver of treated rats, suggesting that SSd could attenuate CCl_4-induced liver fibrosis by down-regulating the inflammatory response in the liver. The study addresses an interesting issue, but the value of this study in its current form is limited. The data provided are preliminary but do not sufficiently support the conclusions drawn by the authors.

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BASIC RESEARCH

Protection of *Veratrum nigrum L. var. ussuriense Nakai alkaloids* against ischemia-reperfusion injury of the rat liver

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Abstract

AIM: To investigate the protective effects and possible mechanisms of *Veratrum nigrum L.var. ussuriense Nakai alkaloids* (VnA) on hepatic ischemia/reperfusion (I/R) injury in rats.

METHODS: Forty male Wistar rats were randomly divided into four experimental groups (n = 10 in each): (A) Control group (the sham operation group); (B) I/R group (pretreated with normal saline); (C) Small-dose (10 μg/kg) VnA pretreatment group; (D) Large-dose (20 μg/kg) VnA pretreatment group. Hepatic ischemia/reperfusion (Hepatic I/R) was induced by occlusion of the portal vein and the hepatic artery for 90 min, followed by reperfusion for 240 min. The pretreatment groups were administered with VnA intraperitoneally, 30 min before surgery, while the control group and I/R group were given equal volumes of normal saline. Superoxide dismutase (SOD) activity, myeloperoxidase (MPO) activity and nitric oxide (NO) content in the liver tissue at the end of reperfusion were determined and liver function was measured. The expression of intercellular adhesion molecule-1 (ICAM-1) and E-selectin (ES) were detected by immunohistochemical examinations and Western blot analyses.

RESULTS: The results showed that hepatic I/R elicited a significant increase in the plasma levels of alanine aminotransferase (ALT: 74.53 ± 2.58 IU/L *vs* 1512.54 ± 200.76 IU/L, $P < 0.01$) and lactic dehydrogenase (LDH: 473.48 ± 52.17 IU/L *vs* 5821.53 ± 163.69 IU/L, $P < 0.01$), as well as the levels of MPO (1.97 ± 0.11 U/g *vs* 2.57 ± 0.13 U/g, $P < 0.01$) and NO (69.37 ± 1.52 μmol/g protein *vs* 78.39 ± 2.28 μmol/g protein, $P < 0.01$) in the liver tissue, all of which were reduced by pretreatment with VnA, respectively (ALT: 1512.54 ± 200.76 IU/L *vs* 977.93 ± 89.62 IU/L, 909.81 ± 132.76 IU/L, $P < 0.01$, $P < 0.01$; LDH: 5821.53 ± 163.69 IU/L *vs* 3015.44 ± 253.01 IU/L, 2448.75 ± 169.4 IU/L, $P < 0.01$, $P < 0.01$; MPO: 2.57 ± 0.13 U/g *vs* 2.13 ± 0.13 U/g, 2.07 ± 0.05 U/g, $P < 0.01$, $P < 0.01$; NO: 78.39 ± 2.28 μmol/g protein *vs* 71.11 ± 1.73 μmol/g protein, 68.58 ± 1.95 μmol/g protein, $P < 0.05$, $P < 0.01$). The activity of SOD (361.75 ± 16.22 U/mg protein *vs* 263.19 ± 12.10 U/mg protein, $P < 0.01$) in the liver tissue was decreased after I/R, which was enhanced by VnA pretreatment (263.19 ± 12.10 U/mg protein *vs* 299.40 ± 10.80 U/mg protein, 302.09 ± 14.80 U/mg protein, $P < 0.05$, $P < 0.05$). Simultaneously, the histological evidence of liver hemorrhage, polymorphonuclear neutrophil infiltration and the overexpression of ICAM-1 and E-selectin in the liver tissue were observed, all of which were attenuated in the VnA pretreated groups.

CONCLUSION: The results demonstrate that VnA pretreatment exerts significant protection against hepatic I/R injury in rats. The protective effects are possibly associated with enhancement of antioxidant capacity, reduction of inflammatory responses and suppressed expression of ICAM-1 and E-selectin.

Key words: *Veratrum nigrum L.var. ussuriense Nakai alkaloids*; Hepatic Ischemia/Reperfusion Injury; Intracellular adhesion molecule-1; E-selectin

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INTRODUCTION

Re-establishment of blood flow to the ischemic tissue is the therapeutic goal in the treatment of arterial occlusion.

However, surgically restoring the blood flow often leads to continued or accelerated local tissue injury and systemic toxicity[1]. Hepatic ischemia/reperfusion (Hepatic I/R) injury is an important non-immunologic problem and a limitating factor in liver transplantation and it may result in liver dysfunction, liver failure, and even death[2]. Hepatic I/R injury is one of the major complications of hepatic resection, hemorrhagic shock with fluid resuscitation, and liver transplantation, particularly with grafts from marginal donors[3]. The mechanisms underlying ischemia/reperfusion induced organ damage are likely multifactorial and interdependent. Previous studies have identified many mediators involved in the pathogenesis of hepatic I/R injury. Among them, reactive oxygen species (ROS)[4] released by active Kupffer cells, and proinflammatory cytokines[5-7] are central to this process. Expression of the adhesion molecules is up-regulated by cytokines and mediates the recruitment of neutrophils to the liver resulting in hepatic injury[8-9]. In addition, selectins are also up-regulated during this process[10].

It has been shown that short episodes of liver ischemia confer protection against longer ischemic insults and subsequent I/R injury. This phenomenon is called ischemic or mechanical preconditioning[11-12]. Pharmacological preconditioning can be an effective alternative to ischemic preconditioning. With pharmacological preconditioning, liver protection is achieved by the administration of substances before or at the early phase of the I/R process.

Veratrum nigrum L.var. ussuriense Nakai alkaloids (VnA) is the total alkaloid extracted from the root of *Veratrum nigrum L. var. ussuriense Nakai* of Mount Qian in Liaoning Province, China. VnA contains at least eleven alkaloids featuring an ester-type isosteroidal structure identified by modern analytical techniques such as HPLC-MS, NMR, *etc*. The principal components of VnA include verussurien, verabenzoamine, verazine, germidine, jervine, germerine, 15-O-(methylbuty-royl)-germine, verussurinine, neogermbudine, zygadenine and echinuline[13-15]. Some researchers have demonstrated that VnA has powerful inhibitory effects against both arterial and venous thrombosis, and it could produce beneficial effects on brain I/R injury[16-18]. In this study, we investigated whether VnA had a protective effect against liver injury induced by hepatic I/R and explored its putative protective mechanism.

MATERIALS AND METHODS

Reagents

The VnA hydrochloride injection (100 mg/L) was prepared by the Department of Pharmaceutical Engineering, Dalian University of Science and Technology. It was diluted to the required concentrations with sterile saline solution prior to use.

Animals

Male Wistar rats (Experimental Animal Center of Dalian Medical University, Dalian, China) weighing 220 ± 20 g were used in this study. All rats were fed with standard laboratory chow and water, and housed in accordance with institutional animal care guidelines.

Experimental design

Male Wistar rats were fasted overnight but had free access to tap water. The rats were assigned randomly into four experimental groups (n = 10 in each): (A) Control group (the sham operation group); (B) I/R group (pretreated with normal saline); (C) Small-dose (10 μg/kg) VnA pretreatment group; (D) Large-dose (20 μg/kg) VnA pretreatment group. The model of hepatic I/R injury was established by the clamping and unclamping of the vessels supplying the left lateral and median hepatic lobes, which account for 70% of the rat liver mass, according to the published method[12]. We reproduced a lobar rather than a total hepatic I/R injury model to induce a severe hepatic ischemic insult without mesenteric venous congestion to avoid the development of intestinal congestion and leakage of bacteria or bacterial products into the circulation[6,19]. Briefly, 30 min before operation, the rats received intraperitoneally VnA (10 μg/kg, 20 μg/kg) or equal volumes of normal saline solution. A midline laparotomy was performed and a microvascular clip was placed to interrupt the arterial and portal venous blood flow to the left and middle lobes of the liver. Reflow was initiated after 90 min of hepatic ischemia by removing the clamp. The doses of VnA administration were determined according to previous studies, combined with our preliminary experiments[17-18]. All rats were killed at 240 min of reperfusion, and blood and liver samples were obtained for the following analyses.

Histopathological assessment

Liver samples were fixed in 10% formalin and embedded in paraffin. Five-micrometer sections of liver tissue were stained with hematoxylin and eosin according to standard procedures. Light microscopy was used to assess the degree of liver damage.

Measurement of plasma alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) level

The abdominal aorta was punctured and 5 mL of blood was taken and put into heparinized tubes. The blood sample was centrifuged at 3000 r/min for 15 min at a room temperature to separate plasma for analyses. The plasma concentrations of ALT (a specific marker for hepatic parenchymal injury), and LDH were measured with an OLYMPUS AU5400 automatic analyzer. Both values were expressed as U/L.

Liver superoxide dismutase (SOD), myeloperoxidase (MPO) and nitric oxide (NO) assay

Liver samples were homogenized on ice in 5 volumes of normal saline and centrifuged at 3000 r/min for 15 min. Liver SOD, MPO and NO levels were measured using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The levels of SOD, MPO and NO were expressed as U/mgprot, U/g and μmol/gprot, respectively.

Immunohistochemical analyses

Formalin-fixed, paraffin-embedded liver specimens were stained by streptavidin/peroxidase immunohistochemistry

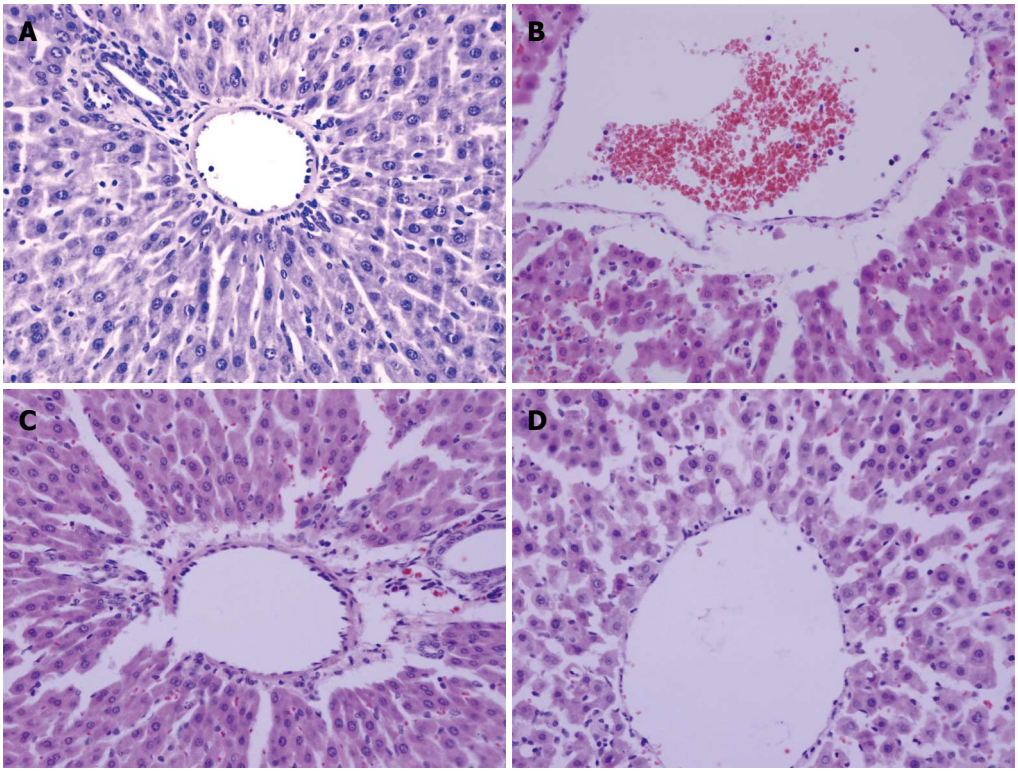

Figure 1 Changes of histology in the liver tissue 90 min after ischemia and 240 min after reperfusion in rats (× 400). Five μm sections of liver tissue were stained with hematoxylin and eosin according to standard procedures. For all groups, n = 10. **A**: Control group: Normal appearance of hepatocytes and sinusoids; **B**: I/R group: Histological edema, hemorrhage, partial exfoliation of blood vessel endothelium and infiltration with inflammatory cells; **C**: I/R + VnA (10 μg/kg) group: A significant amelioration of histological edema, hemorrhage, and exfoliation of blood vessel endothelium; **D**: I/R + VnA (20 μg/kg) group: Slight inflammatory cell infiltration with most hepatocytes in normal appearance.

technique for intercellular adhesion molecule-1 (ICAM-1) and E-selectin (ES) detection. Five-micrometer sections were treated with 0.3% H_2O_2 in methanol to block endogenous peroxide activity and then incubated with the polyclonal rabbit anti-rat ICAM-1 and E-selectin antibody (Wuhan Boster Biological Technology Co., Ltd, Wuhan, China, both 1:500 dilution). Biotinylated anti-rabbit immunoglobulin was added as a secondary antibody. The horseradish peroxidase labeled streptomycin-avidin complex was then used to detect the second antibody. Finally, slides were stained with 3,3'-diaminobenzidine, which was used as a chromagen, and the sections were counterstained with hematoxylin before being examined under a light microscope. The brown or dark brown stained cells were considered as positive. The results were evaluated semi-quantitatively according to the percentage of positive cells in 5 high power fields at 400 multiple signal magnification: 0, less than 5%; 1, from 6% to 25%; 2, from 26% to 50%; 3, from 51% to 75%; 4, more than 75%[20].

ICAM-1 and E-selectin Western blot analysis

Frozen liver tissue was homogenized with PBS (pH = 7.2) and centrifuged at 4℃, 10 000 g for 10 min. After precipitation the insoluble fraction was discarded, and the protein concentration in the supernatant was determined by a spectrophotometer. Aliquots (20 μg) of protein from each sample were loaded into each lane of 10% SDS-PAGE gel electrophoresis and then electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were then probed with the rabbit antibody against rat ICAM-1(intercellular adhesion molecule-1) or E-selectin (Boster Biological Technology Co., Ltd, Wuhan, China, both 1:1000 dilution) and biotin-conjugated anti-rabbit IgG (Fuzhou Maixin Biological Technology Co., Ltd, Fuzhou, China) according to the manufacturer's recommendations. The signals were visualized by a DAB assay kit (Fuzhou Maixin Biological Technology Co., Ltd, Fuzhou, China) and analyzed with a gel imaging system (Kodak system EDAS120, Japan).

Statistical analyses

All data were presented as mean ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) with the SPSS 11.5 statistical software package. The differences between means were analyzed using Student-Newman-Keuls (SNK) test for multiple comparisons. P values less than 0.05 were considered statistically significant.

RESULTS

Pathological alterations of liver tissue

The histological structure of cells was normal in the control group. After 90 min of hepatic ischemia followed by 240 min of reperfusion, the occluded liver tissue appeared as dark color with an obtuse fringe. Compared with the control group, the liver tissue from the I/R group was markedly damaged characterized by edema, hemorrhage, partial exfoliation of vascular endothelium and infiltration with inflammatory cells under the microscope. Pretreatment of rats with 10 or 20 μg/kg VnA resulted in a significant amelioration of hepatic injury (Figure 1).

Levels of plasma liver enzymes

Liver function was tested by measuring plasma levels of ALT and LDH. Hepatic I/R led to a marked elevation of plasma ALT and LDH activity (ALT: 74.53 ± 2.58 IU/L *vs* 1512.54 ± 200.76 IU/L, $P < 0.01$; LDH: 473.48 ± 52.17

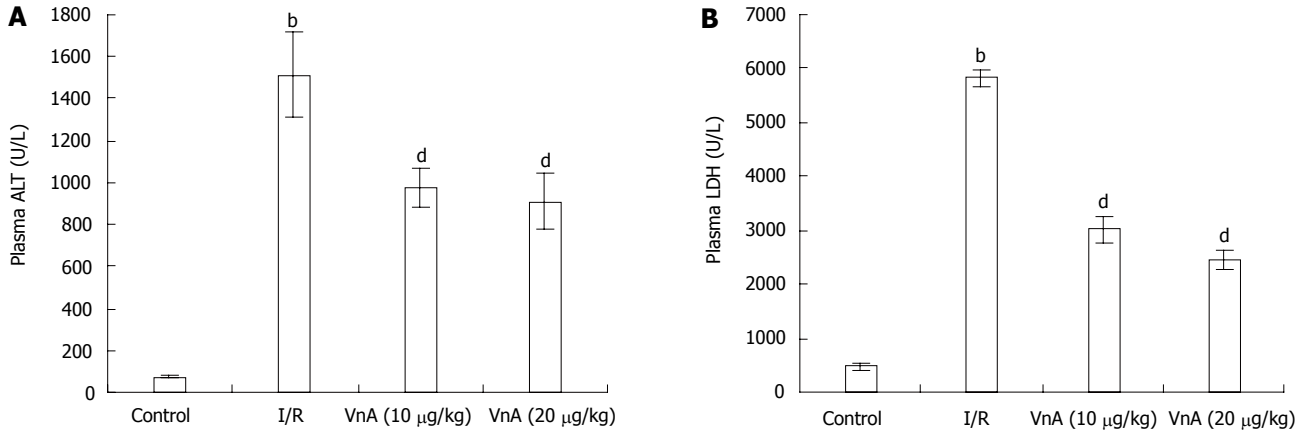

Figure 2 Plasma aminotransferase (ALT) (**A**) and acetic dehydrogenase (LDH) (**B**) levels in different groups (mean ± SD, $n = 10$). After 90 min of hepatic ischemia and 4 h of reperfusion, plasma levels of ALT and LDH were determined with an OLYMPUS AU5400 automatic analyzer. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* I/R group.

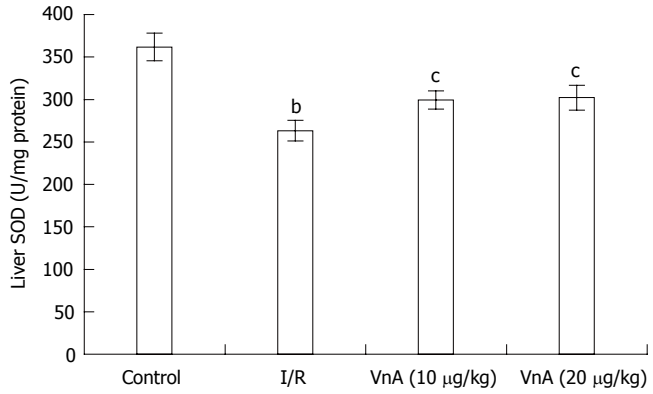

Figure 3 Activity of superoxide dismutase (SOD) in the liver tissue in different groups (mean ± SD, $n = 10$). After 90 min of ischemia and 4 h of reperfusion, the liver tissue was homogenized and assayed for SOD levels with an SOD assay kit as the index of hepatic oxidative stress. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* I/R group.

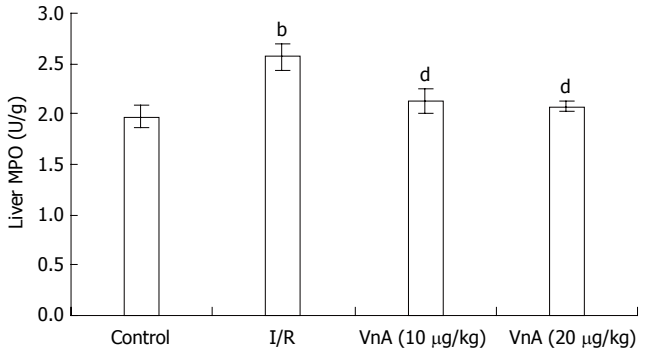

Figure 4 Activity of myeloperoxidase (MPO) in the liver tissue in different groups (mean ± SD, $n = 10$). MPO contents in liver tissue were analyzed with an MPO assay kit as the index of neutrophil recruitment. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* I/R group.

IU/L *vs* 5821.53 ± 163.69 IU/L, $P < 0.01$, Figure 2). The increase in plasma ALT activity elicited by hepatic I/R was significantly attenuated by administration of VnA at doses of 10 and 20 μg/kg by about 35.35% and 39.85%, respectively (1512.54 ± 200.76 IU/L *vs* 977.93 ± 89.62 IU/L, 909.81 ± 132.76 IU/L, $P < 0.01$, $P < 0.01$); and the activity of LDH was reduced by administration of VnA at doses of 10 and 20 μg/kg by about 48.20% and 57.94%, respectively (5821.53 ± 163.69 IU/L *vs* 3015.44 ± 253.01 IU/L, 2448.75 ± 169.4 IU/L, $P < 0.01$, $P < 0.01$). This result demonstrated the dose-dependent protective effects of VnA on liver injury.

SOD activity in liver tissue

Compared with the control group, the level of liver SOD in the I/R group reduced significantly (361.75 ± 16.22 U/mg protein *vs* 263.19 ± 12.10 U/mg protein, $P < 0.01$). After administration of VnA at doses of 10 and 20 μg/kg, respectively, the liver SOD activity was elevated significantly (263.19 ± 12.10 U/mg protein *vs* 299.40 ± 10.80 U/mg protein, 302.09 ± 14.80 U/mg protein, $P < 0.05$, $P < 0.05$, Figure 3).

MPO activity in liver tissue

Hepatic neutrophil recruitment was determined by liver MPO content. Hepatic I/R caused a marked increase in liver MPO content compared with the control group (1.97 ± 0.11 U/g *vs* 2.57 ± 0.13 U/g, $P < 0.01$). Administration of VnA at doses of 10 and 20 μg/kg resulted in a marked reduction of MPO in a dose-dependent manner (2.57 ± 0.13 U/g *vs* 2.13 ± 0.13 U/g, 2.07 ± 0.05 U/g, $P < 0.01$, $P < 0.01$), suggesting that VnA prevents leukocyte recruitment to the liver tissue (Figure 4).

NO content in liver tissue

The level of NO in the liver tissue was significantly higher in the I/R group compared with the control group (69.37 ± 1.52 μmol/g protein *vs* 78.39 ± 2.28 μmol/g protein, $P < 0.01$). Pretreatment with VnA at both 10 and 20 μg/kg, however, caused an obvious reduction in a dose-dependent manner when compared with the I/R group (78.39 ± 2.28 μmol/g protein *vs* 71.11 ± 1.73 μmol/g protein, 68.58 ± 1.95 μmol/g protein, $P < 0.05$, $P < 0.01$, Figure 5).

Immunohistochemical analysis for liver ICAM-1 and E-selectin

The expression of ICAM-1 and E-selectin in the control

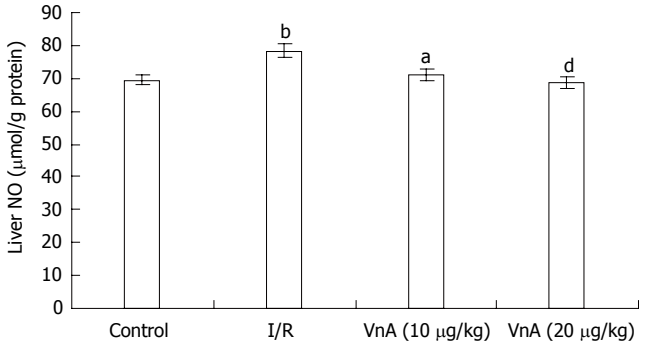

Figure 5 Content of nitric oxide (NO) in the liver tissue in different groups (mean ± SD, n = 10). After 90 min of ischemia and 4 h of reperfusion, the liver tissue was homogenized and assayed for NO levels with an NO assay kit as the index of hepatic oxidative stress. $^{b}P < 0.01$ *vs* control group. $^{a}P < 0.05$, $^{d}P < 0.01$ *vs* I/R group.

group showed as light brown immunostaining. However, their expression was highly up-regulated after 90 min of ischemia and 240 min of reperfusion ($P < 0.01$). Compared with the I/R group, the increase of ICAM-1 and E-selectin expression was significantly suppressed by VnA in a dose-dependent manner ($P < 0.05$, $P < 0.01$; $P < 0.05$, $P < 0.05$ respectively) (Figure 6, Figure 7 and Figure 8).

Western blot analysis for liver ICAM-1 and E-selectin

Western blot showed weak positive ICAM-1 and E-selectin signals in the control group. In contrast, a marked increase in ICAM-1 and E-selectin protein expression in the liver tissue was found in the I/R group. Compared with the I/R group, the signals were weakened obviously in the VnA pretreated group (Figure 9).

DISCUSSION

Liver I/R injury occurs in a number of clinical settings including liver surgery, transplantation, and hemorrhagic shock. A major disadvantage of this event is an acute inflammatory response that may cause significant organ damage or dysfunction. Experimental evidence demonstrates that kupffer cells play an important part in mediating hepatic I/R[21-22]. Kuppfer cell activation leads to structural changes, formation of vascular ROS and production of proinflammatory cytokines, which in turn induce the expression of adhesion molecules in vascular endothelial cells and stimulate the production and release of neutrophil-attracting chemokines. Neutrophil recruitment in the liver tissue causes direct hepatocellular damage through exhaustion of hepatic microcirculation by blocking the capillary perfusion and releasing ROS and proteases[23-24].

VnA is a well known herbal plant widely distributed in the northeast region of China and has various pharmacological effects including antithrombotic and antihypertensive properties[15,17]. Some researchers have also demonstrated that VnA exerted beneficial effects on brain I/R injury by inhibiting oxidation and leukocyte priming and expression of inflammatory mediators[18]. In the present study, we demonstrated that VnA pretreatment at doses of 10 and 20 μg/kg could attenuate hepatic I/R injury, indicated by improved alteration in liver tissue pathology and liver function, by an enhanced antioxidant capacity with augmentation of free radical scavengers and reduced polymorphonuclear neutrophil (PMN) infiltration, as well as by suppressed overexpression of adhesion molecules and selectins.

Reperfusion of the ischemic liver in rats resulted in hepatic damage with histological evidence of liver hemorrhage, edema, accumulation of adherent leukocytes in sinusoids and terminal hepatic vein (THV), as well as partial exfoliation of blood vessel endothelia. Furthermore, hepatic I/R caused the release of liver enzymes into the blood stream, thereby eliciting a significant increase in plasma levels of ALT and LDH. VnA pretreatment abated liver pathologic injury and reduced plasma ALT and LDH activity, thus liver function was ameliorated.

SOD catalyses the dismutation of the superoxide anion ($O_2^{\cdot}$) into H_2O_2, which can be transformed into H_2O and O_2 by catalase (CAT). In this study, we found that I/R impaired SOD activity, as indicated by the markedly lowered activity compared with the control group. But in the VnA pretreated group, the decrease of SOD activity was significantly counteracted. In addition, the hepatic I/R increased the levels of nitric oxide (NO), an important ROS product. This result is consistent with a previous report[25]. NO may combine with superoxide radicals to form peroxynitrite, a substance extremely toxic to cells[26]. VnA pretreatment significantly decreased liver NO content, resulting in a significant reduction in liver damage when compared with the control group. These data indicated that VnA might confer protection on the liver during I/R injury in part by improving activity of the endogenous antioxidant enzyme, which scavenges ROS and reduces their effects.

Because MPO is an enzyme restricted mainly to PMNs, the increase in MPO activity reflects neutrophil tissue infiltration. The significant increase in MPO activity in the liver tissue after hepatic I/R in the present study is consistent with another study[27]. However, VnA significantly blunted the increase of liver MPO activity compared with the I/R group. That is to say, VnA reduced infiltration of leukocytes into the inflammatory sites.

ICAM-1 is a member of the immunoglobulin superfamily that mediates firm adhesion and emigration of activated leukocytes in postcapillary venules, which process may be one of the important steps in the development of tissue injury and organ dysfunction[28]. Previous studies have shown an upregulation of ICAM-1 expression following endothelial cell activation with cytokines or LPS, accompanied by an increased binding of neutrophils and lymphocytes to endothelial cells. An anti-ICAM-1 monoclonal antibody inhibited neutrophil infiltration into pericentral sinusoids and improved and restored liver integrity in I/R injury[29]. In this study we detected overexpression of ICAM-1 after hepatic I/R, which was significantly decreased in the VnA pretreated groups. This indicated that VnA could suppress leukocyte adhesion to endothelia.

Murine E-selectin is a 110 kDa, type-1 transmembrane glycoprotein expressed only in endothelial cells after cytokine activation[30]. E-selectin participates in leukocyte

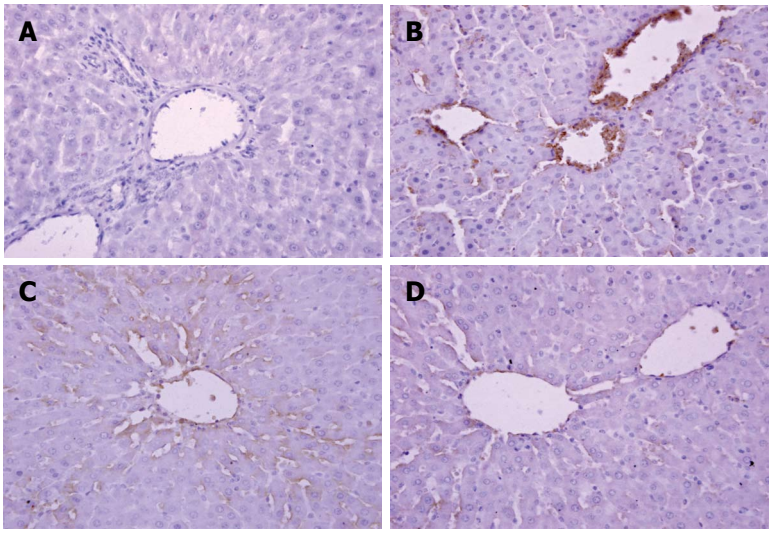

Figure 6 Immunohistochemical staining of adhesion molecule-1 (ICAM-1) expression in the liver tissue after 90 min of ischemia and reperfusion for 240 min in rats (× 400). Formalin-fixed, paraffin-embedded liver specimens were stained by streptavidin/peroxidase immunohistochemistry technique. For all groups, *n* = 10. **A**: Control group; **B**: I/R group; **C**: I/R + VnA (10 μg/kg) group; **D**: I/R + VnA (20 μg/kg) group.

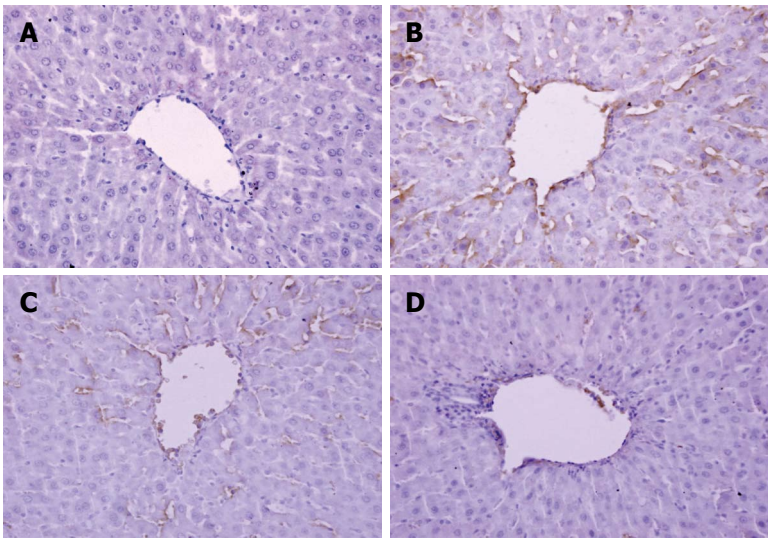

Figure 7 Immunohistochemical staining of E-selectin in liver tissue after ischemia for 90 min and reperfusion for 240 min in rats (× 400). Formalin-fixed, paraffin-embedded liver specimens were stained by the streptavidin/peroxidase immunohistochemistry technique. For all groups, *n* = 10. **A**: Control group; **B**: I/R group; **C**: I/R + VnA (10 μg/kg) group; **D**: I/R + VnA (20 μg/kg) group.

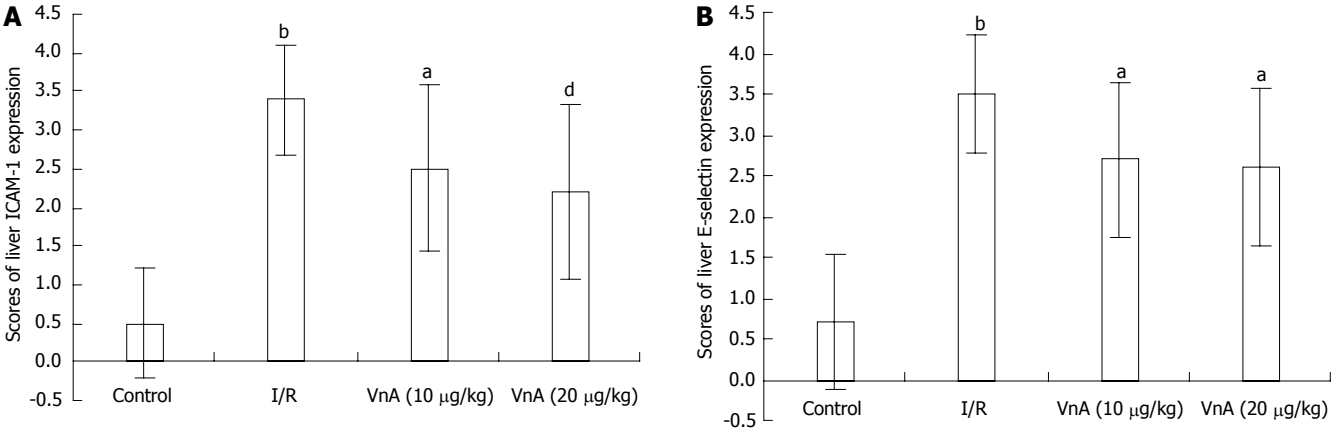

Figure 8 Immunohistochemical results (semi-quantitative analysis) of adhesion molecule-1 (ICAM-1) (**A**) and E-selectin (**B**) in liver tissue in different groups. Data were presented as mean ± SD. $^{b}P < 0.01$ *vs* Control group; $^{a}P < 0.05$, $^{d}P < 0.01$ *vs* I/R group.

rolling and firm adhesion *in vitro*[31] and *in vivo*[32]. It is down-regulated by re-internalization and by shedding from the endothelial surface into the plasma[33]. Previous studies revealed that blockade of E-selectin protected from severe acute renal failure induced by ischemia-reperfusion[34]. In our study, E-selectin was also found to be overexpressed after hepatic I/R, however, this was attenuated in the VnA pretreated groups as compared with the I/R group.

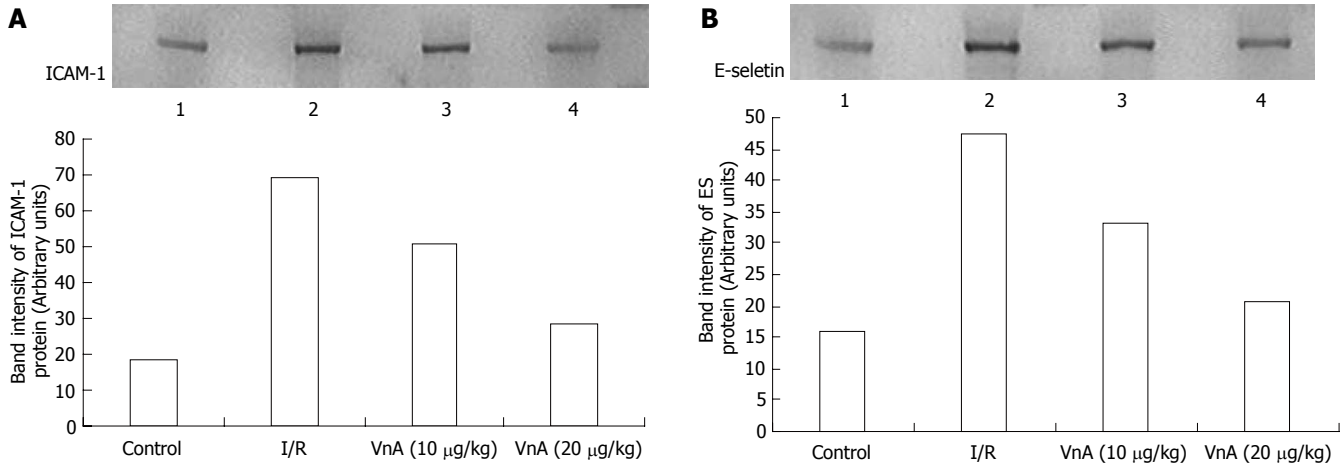

Figure 9 Liver adhesion molecule-1 (ICAM-1) (**A**) and E-selectin (ES) (**B**) protein signals of Western blot analyzed with a gel imaging system (Kodak system EDAS120, Japan). For all groups, *n* = 10. L1: Control group; L2: I/R group; L3: VnA (10 μg/kg) group; L4: VnA (20 μg/kg) group. Compared with the control group, the signals in I/R group increased and weakened in the VnA pretreated group in a dose-dependent manner.

The above results suggested that ICAM-1 and E-selectin dependent neutrophil recruitment into the liver tissue was responsible partially for hepatic I/R. The dose-dependent protective effects of VnA were likely related to the suppression of ICAM-1 and E-selectin expression. This suppression reduced the neutrophil recruitment associated with the hepatic I/R, and effectively protected the liver against ischemia/reperfusion.

In conclusion, VnA has protective effects against liver injury induced by hepatic I/R. The protective effects are probably associated with enhancement of antioxidant capacities, reduction of inflammatory responses and suppressed expression of ICAM-1 and E-selectin. These findings may be useful for clinical management to reduce I/R related hepatic damage. However, the precise mechanisms of VnA protecting against hepatic I/R still need to be clarified in further studies.

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CLINICAL RESEARCH

Gastrointestinal symptoms in a Japanese population: A health diary study

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Abstract

AIM: To investigate the incidence of gastrointestinal symptoms and the nature of consequent utilization of health care services in a Japanese population.

METHODS: Using self-report, we conducted a prospective cohort study of a nationally representative sample of the Japanese population over a one-month period to determine the incidence of gastrointestinal symptoms of all kinds and resultant health care utilization. Both information on visits to physicians and use of complementary and alternative medicine therapies were collected.

RESULTS: From a total of 3568 in the recruitment sample, 3477 participants completed a health diary (response rate 97%). The data of 112 participants with baseline active gastrointestinal diseases were excluded from the analysis, leaving 3365 participants in the study. The incidence of gastrointestinal symptoms was 25% and the mean number of symptomatic episodes was 0.66 in a month. Abdominal pain, diarrhea, nausea, constipation and dyspepsia were the most frequent symptoms. Female gender, younger age, and low baseline quality of life were risk factors for developing these symptoms. The participants were more likely to treat themselves, using dietary, complementary or alternative medicines, than to visit physicians, except in the case of vomiting.

CONCLUSION: Gastrointestinal symptoms are common in the Japanese population, with an incidence of 25%. Abdominal pain, diarrhea, nausea, constipation and dyspepsia are the most frequent symptoms. Risk factors for developing these symptoms include female gender, younger age, and low baseline quality of life.

Key words: Gastrointestinal diseases; Abdominal Pain; Diarrhea; Nausea; Constipation; Dyspepsia

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INTRODUCTION

Although the prevalence of gastrointestinal disease in the Japanese population is known to be high, its epidemiology, incidence, and the consequent utilization of health care services are not well described[1-3]. An accurate analysis of the incidence of the various symptoms and of the health care services utilized in relation to them would clarify the public health consequences of gastrointestinal symptoms and assist in the setting of priorities in the allocation of health care services and future research funding. There is also little information on the use of complementary and alternative medicine as compared with conventional medicine in the treatment of the various symptoms of gastrointestinal disease. This prospective cohort study was designed to ascertain the incidence of gastrointestinal symptoms in the Japanese general population and to document the subsequent use of health care, as recorded by the participants in a health diary.

MATERIALS AND METHODS

Participants

This analysis of the incidence of gastrointestinal symptoms and subsequent health care practices in Japan is drawn from a prospective cohort study, using participants' health diaries established for the ecological analysis of

medical care in Japanese communities[4]. A population-weighted random sample of households was selected by controlling for the size of cities, towns and villages. Participants who had baseline active gastrointestinal diseases were excluded from the analysis. Because of the national policy of universal health insurance coverage in Japan, all households sampled were covered by health insurance. Prior ethical approval from the Research Ethics Committee of Kyoto University Graduate School of Medicine was obtained.

There are advantages to using health diaries when investigating individual health and related behavior[4-8]. Health diaries can provide an immediate and continuous record of daily health events and behaviors, and minimize recall bias[4], without the intervention of direct observational measures[7,9]. The methodology of this health diary study is described in detail elsewhere[4].

Data collection

For the purposes of the study, the independent variables were baseline demographic and clinical data. The dependent variables were self-reported gastrointestinal symptoms, which were categorized and coded based on the ICPC-2 (International Classification of Primary Care second edition). They included diffuse abdominal pain, upper abdominal pain, diarrhea, nausea, constipation, dyspepsia, vomiting, abdominal fullness, heartburn, lower abdominal pain, hematemesis, and hematochezia.

The health diary procedure required the keeping of a daily record for one month, from October 1 to October 31, 2003, of all health-related events, including gastrointestinal symptoms, health care accessed, and anything else of relevance. The health diary format specifically sought responses to the following questions: (1) Did you have any pain or other health symptoms that caused you discomfort? (2) If so, what kind of symptoms did you have? (3) If the answer to the first question was yes, did you consult a physician? Did you use dietary supplements such as nutritional drinks, vitamins, and calcium? Did you undergo any physical remedy, such as acupressure, acupuncture, or massage? Subjects younger than 15 years old were also included in this study. The parents of these children were requested to ask the questionnaires and record them accordingly. We did not include the use of over-the-counter-medications as utilizations of health care services in this study.

Data was extracted on the number of days in which symptom-related visits to a physician occurred during the study period, whether to a primary care physician, a community hospital, a university hospital, or an emergency department. Data was also collected on the number of days complementary and alternative medicines were used, whether dietary supplements or physical remedies. The use of complementary and alternative medicine was divided into two categories: (1) Dietary complementary and alternative medicine, such as nutritional drinks, herbs, kampo, supplements, vitamins, minerals, and other dietary substances; (2) Physical complementary and alternative medicine, such as massage, acupuncture, acupressure, Judo-seifuku, moxibustion, chiropractic, and similar physical manipulations.

Baseline data, including demographic, health-related, and socioeconomic information, was also collected. The SF-8 instrument was used to measure baseline health-related quality of life. The SF-8 generates a health profile consisting of eight scales and two summary measures: a physical component summary (PCS8) and a mental component summary (MCS8)[10]. The SF-8 is scored by assigning the mean SF-36 scale score for the Japanese population as measured in 2002 to each response category of the SF-8 measuring the same concept. A higher or lower individual score indicates a better or worse health status than the mean, respectively[11]. We also included the baseline number for comorbidity as a covariate. The number of co-morbidities was calculated by counting the number of diseases present with no weights[12].

Data on a number of characteristics was collected as socioeconomic baseline measurements. Annual household income was divided into 6 categories. Employment status was recorded as one of 6 categories: student, homemaker, jobless or not able to work, retiree, part-time employee, and full-time employee or self-employed worker. Self-reported educational attainment was also classified at 6 levels: junior high school or below, high school graduate, vocational college, 1-2 years college, college degree, graduate school degree or higher.

Statistical analysis

The incidence (proportions) and the number of episodes (days with the symptom) of individual gastrointestinal symptoms were calculated during the one-month study period. Multivariable adjusted Poisson regression models were constructed to obtain adjusted rate ratios for the number of episodes of the various gastrointestinal symptoms in relation to baseline demographic and clinical factors. Health care utilization for one month of participants who developed gastrointestinal symptoms was also calculated. A two-tailed *P*-value of 0.05 was regarded as statistically significant. The STATA software version 8.2 (College Station, Texas, USA) was used for all statistical analyses.

RESULTS

From a total of 3568 in the study recruitment sample, 3477 participants completed the diary (97.4%). Of these, 112 with baseline active gastrointestinal diseases were excluded and the remaining 3365 participants were enrolled in the study (Table 1). 1573 (46%) were men. The mean age was 34 years (range 0-96 years). 17% of the 3365 participants lived in large cities, 24% in medium-sized cities, 38% in small cities, and 21% in rural areas. Table 1 shows the demographic, socioeconomic and clinical characteristics of the participants in two groups: those who developed any gastrointestinal symptom and those who recorded none. A univariate analysis showed no significant differences in socioeconomic characteristics between the two groups. A trend test yielded $P = 0.212$ for annual household income, $P = 0.143$ for occupational status, and $P = 0.719$ for educational attainment. Chi-square tests showed significant differences between the two groups on the variables of gender ($P < 0.001$), past history of gastrointestinal disease

Table 1 Demographic, socioeconomic and clinical characteristics of the participants

Variable	All participants (n = 3365)		Developed GI symptoms (n = 856)		No GI symptoms (n = 2509)	
	n	%	n	%	n	%
Demographics						
Gender						
Male	1573	45.7	328	38.3	1245	49.6
Female	1792	53.3	528	61.7	1264	50.4
Age (yr)						
0-9	651	19.3	155	18.1	496	19.8
10-19	431	12.8	104	12.1	327	13.0
20-29	416	12.4	133	15.5	283	11.3
30-39	476	14.1	139	16.2	337	13.4
40-49	490	14.6	134	15.7	356	14.2
50-59	345	10.3	76	8.9	269	10.7
60-69	331	9.8	61	7.1	270	10.8
70-79	188	5.6	45	5.3	143	5.7
⩾ 80	37	1.1	9	1.1	28	1.1
Socioeconomic characteristics						
Annual household income						
< 3 000 000 Japanese yen	424	12.6	112	13.1	312	12.4
3 000 000 to < 5 000 000	652	19.4	189	22.1	463	18.5
5 000 000 to < 7 000 000	522	15.5	127	14.8	395	15.7
7 000 000 to < 10 000 000	426	12.7	111	13.0	315	12.6
10 000 000 to < 12 000 000	167	5.0	42	4.9	125	5.0
⩾ 12 000 000	82	2.4	16	1.9	66	2.6
N/A	1092	32.5	259	30.3	833	33.2
Employment status						
Full-time employee/self-employed	1086	32.3	274	32.0	812	32.4
Part-time employee	374	11.1	100	11.7	274	10.9
Retiree	129	3.8	26	3.0	103	4.1
Jobless or unable to work	91	2.7	24	2.8	67	2.7
Homemaker	490	14.6	153	17.9	337	13.4
Student	89	2.6	18	2.1	71	2.8
N/A	1106	32.9	261	30.5	845	33.7
Educational attainment						
Junior high school or lower	84	2.5	16	1.9	68	2.7
High school graduate	471	14.0	122	14.3	349	13.9
Vocational college	135	4.0	38	4.4	97	3.9
1-3 yr of college	156	4.6	50	5.8	106	4.2
College degree	336	10.0	85	9.9	251	10.0
Graduate school degree	23	0.7	8	0.9	15	0.6
N/A	2160	64.2	537	62.7	1623	64.7
Baseline clinical characteristics						
Previous GI diseases						
Yes	326	9.7	117	13.7	209	8.3
No	2956	87.8	722	84.3	2234	89.0
N/A	83	2.5	17	2.0	66	2.6
No. of comorbidities						
None	2548	75.7	646	75.5	1902	75.8
One	578	17.2	157	18.3	421	16.8
Two or more	239	7.1	53	6.2	186	7.4
PCS8 score						
⩾ 50	1930	57.4	458	53.5	1472	58.7
< 50	1304	38.8	363	42.4	941	37.5
N/A	131	3.9	35	4.1	96	3.8
MCS8 score						
⩾ 50	1704	50.6	368	43.0	1336	53.2
< 50	1530	45.5	453	52.9	1077	42.9
N/A	131	3.9	35	4.1	96	3.8

N/A: indicates data not available; GI: gastrointestinal; PCS8: physical component of SF8; MCS8: mental component of SF8.

Table 2 Incidence and the number of episodes of gastrointestinal symptoms (n = 3365)

Symptom	Incidence per month		Episodes in a month	
	n	(% of total)	mean	SD
Any gastrointestinal symtoms	856	(25.44)	0.656	1.794
Diffuse abdominal pain	401	(11.92)	0.214	0.799
Upper abdominal pain	179	(5.32)	0.114	0.801
Diarrhea	169	(5.02)	0.095	0.651
Nausea	148	(4.40)	0.067	0.393
Constipation	70	(2.08)	0.064	0.689
Dyspepsia	86	(2.56)	0.051	0.423
Vomiting	44	(1.31)	0.016	0.164
Abdominal fullness	34	(1.01)	0.015	0.187
Heartburn	18	(0.53)	0.010	0.173
Lower abdominal pain	16	(0.48)	0.009	0.158
Hematemesis	1	(0.03)	0.000	0.017

SD: standard deviation. There were no participants with hematochezia.

($P < 0.001$), MCS8 ($P < 0.001$), and PCS8 ($P = 0.008$).

Table 2 shows the incidence per month for 3365 participants and the number of episodes of gastrointestinal symptoms of these participants during the one month study period. 856 (25%) developed one or more gastrointestinal symptoms. The symptoms of high incidence ($\geqslant$ 1%) were diffuse abdominal pain (12%), upper abdominal pain (5%), diarrhea (5%), nausea (4%), dyspepsia (3%), constipation (2%), vomiting (1%), and abdominal fullness (1%). The mean number of episodes of gastrointestinal symptoms of any kind was 0.66 in the one-month period. The symptoms with a high number of episodes were diffuse abdominal pain (0.21), upper abdominal pain (0.11), diarrhea (0.10), nausea (0.07), constipation (0.06), dyspepsia (0.05), vomiting (0.02), and abdominal fullness (0.01).

Table 3 shows rate ratios based on multivariable adjusted Poisson regression analyses. Age and the number of comorbidity are treated as continuous variables in this Table. Gastrointestinal symptoms were reported more commonly by women than men. Symptoms with a significantly higher number of episodes in women were diffuse abdominal pain, upper abdominal pain, nausea, and constipation, and symptoms with a significantly higher number of episodes in men were diarrhea and heartburn.

Gastrointestinal symptoms were reported more often in younger than in older age groups. Symptoms associated with older age were upper abdominal pain, dyspepsia, constipation, abdominal fullness, and heartburn, while symptoms associated with younger age were diffuse abdominal pain, diarrhea, nausea, and vomiting. The symptoms that featured in comorbidity were nausea and constipation.

Gastrointestinal symptoms were reported more often by participants with poor baseline quality of life scores. Diffuse abdominal pain, diarrhea and dyspepsia were symptoms associated with a poor baseline score on the physical component of the health-related quality of life test. Symptoms associated with a poor baseline score on the mental component of the health-related quality of life test were diffuse abdominal pain, upper abdominal pain, nausea, constipation, and abdominal fullness.

Table 4 shows the health care utilization characteristics of participants with one or more gastrointestinal symptoms in the survey month. Overall, use of dietary complementary and alternative medicine was more frequent than visiting a physician (Bonferroni pair-wise comparison, $P < 0.001$), but visiting a physician was more frequent than use of physical forms of complementary and alternative medicine (Bonferroni pair-wise comparison, $P < 0.001$). However, visiting a physician was more frequent than use of dietary complementary and alternative medicine in those whose symptom was vomiting. Among those with dyspepsia and heartburn, use of both dietary and physical complementary and alternative medicine was more frequent than visiting a physician.

DISCUSSION

Our results indicate that gastrointestinal symptoms are of common occurrence in the Japanese general population, with about a quarter developing a gastrointestinal symptom of some kind in a month. Abdominal pain, diarrhea, nausea, constipation and dyspepsia were the most frequent gastrointestinal symptoms in our sample. Risks for developing these symptoms differ in relation to the baseline factors of gender, age, and quality of life. Japanese who develop gastrointestinal symptoms are more likely to treat themselves with dietary forms of complementary and alternative medicine than to visit physicians, except in the case of vomiting.

Gastrointestinal symptoms with a high incidence were, in order, diffuse and upper abdominal pain, diarrhea, nausea, constipation, and dyspepsia. These findings are consistent with those of one previous study[2], while another found that diarrhea was more common than abdominal pain[3]. It may be that self-reporting of diarrhea underestimates its actual incidence[13]. A study that asks about diarrhea and loose stools separately obtains a lower incidence of diarrhea than one in which participants include loose stool in their definition of diarrhea[3]. Thus differences in participants' definitions could account for differences in the estimated incidence of diarrhea between previous studies[14].

Our study found that women were more likely to develop diffuse and upper abdominal pain, nausea, and constipation than men. Previous studies suggest that the incidence of many gastrointestinal symptoms is higher in women than in men[1,3,15-18], and many studies indicate that abdominal pain, specifically, is more common in women[1,3,15-18], although two studies have shown no gender difference[19,20]. Abdominal fullness, also, is more common in women than in men[21]. A higher prevalence of occult irritable bowel syndrome in women could account for the higher incidence and prevalence of such symptoms as abdominal pain, nausea, and constipation[3,22]. Alternatively, a higher sensitivity in the perception of such symptoms in women could also contribute to the difference[3].

Our study found that men were more likely to develop diarrhea and heartburn than women. This finding differs from a number of studies indicating no gender difference for diarrhea[3,16,18], while a recent international study found

Table 3 Rate ratios based on multivariable adjusted poisson regression analyses

Variable symptom	Female gender	Older age	Previous GI disease	No. of comorbidity	Better PCS8	Better MCS8
Any gastrointestinal symtoms	1.412	0.994	1.894	1.055	0.738	0.645
	(< 0.01)	(< 0.01)	(< 0.01)	NS	(< 0.01)	(< 0.01)
Diffuse abdominal pain	1.455	0.980	1.385	0.886	0.545	0.548
	(< 0.01)	(< 0.01)	(< 0.01)	NS	(< 0.01)	(< 0.01)
Upper abdominal pain	1.485	1.016	2.504	0.855	0.975	0.426
	(< 0.01)	(< 0.01)	(< 0.01)	(< 0.05)	NS	(< 0.01)
Diarrhea	0.765	0.972	1.485	0.981	0.636	0.986
	(< 0.05)	(< 0.01)	(< 0.05)	NS	(< 0.01)	NS
Nausea	2.828	0.983	2.364	1.259	1.032	0.575
	(< 0.01)	(< 0.01)	(< 0.01)	(< 0.01)	NS	(< 0.01)
Dyspepsia	1.171	1.033	4.035	0.888	0.462	1.068
	NS	(< 0.01)	(< 0.01)	NS	(< 0.01)	NS
Constipation	2.877	1.014	1.198	1.427	1.234	0.364
	(< 0.01)	(< 0.01)	NS	(< 0.01)	NS	(< 0.01)
Vomiting	0.847	0.940	1.617	1.141	0.903	1.421
	NS	(< 0.01)	NS	NS	NS	NS
Abdominal fullness	1.833	1.020	1.871	0.936	1.305	0.476
	NS	(< 0.05)	NS	NS	NS	(< 0.05)
Heartburn	0.286	1.053	2.653	1.027	1.324	1.227
	(< 0.01)	(< 0.01)	(< 0.05)	NS	NS	NS
Lower abdominal pain	1.555	1.005	0.844	1.023	0.699	0.515
	NS	NS	NS	NS	NS	NS

GI: gastrointestinal; PCS8: physical component of SF8; MCS8: mental component of SF8. Adjusted for all covariates shown above. The numbers of parehtheses indicate statistically significant *P*-values. Age and No. of comorbidity were treated as continuous variables. PCS8 and MCS8 were treated as binary variables with cutoff pint of 50. Hematemesis and hematochezia could not be analyzed becaused of few incidence.

Table 4 Health care utilization in a month among the participants with gastrointestinal symptoms

	Visits to a physician		Dietary CAM uses		Physical CAM uses	
Symptom	mean (d)	SD	mean (d)	SD	mean (d)	SD
Any gastrointestinal symptoms	0.67	1.502	1.82	5.614	0.12	0.891
Diffuse abdominal pain	0.58	1.518	1.48	5.075	0.10	1.133
Upper abdominal pain	0.45	1.040	2.76	6.823	0.32	1.791
Diarrhea	0.81	1.300	1.39	4.747	0.07	0.431
Nausea	0.76	1.274	1.70	4.738	0.25	1.851
Dyspepsia	0.50	1.344	4.47	8.023	0.52	2.533
Constipation	0.90	1.746	2.43	6.333	0.20	0.651
Vomiting	1.64	2.354	0.89	2.442	0.00	0.00
Abdominal fullness	0.32	0.638	2.91	6.440	0.06	0.343
Heartburn	0.44	0.784	2.11	3.692	0.50	1.465
Lower abdominal pain	0.63	1.628	2.06	7.206	0.19	0.750
Hematemesis	0.00	0.000	0.00	0.000	0.00	0.000

CAM: complementary and alternative medicine; SD: standard deviation. Hematochezia could not be analyzed becaused of few incidence.

that the incidence of diarrhea was higher in women in a number of countries, including Australia, Canada, Ireland, and the United States[23]. Gastroesophageal reflux disease symptoms, such as heartburn and acid regurgitation, showed no gender difference in an earlier Japanese study[24], and studies in Sweden and Belgium also found no gender difference in the prevalence of heartburn[1,25]. These conflicting results suggest the need for further investigation.

Overall, gastrointestinal symptoms were reported more commonly in younger than in older participants in the current study. Previous studies have shown a significantly higher prevalence of abdominal symptoms in young women, which decreases with age[1,3,26]. Nevertheless the current study found that older individuals are more likely to suffer upper abdominal pain, dyspepsia, constipation, abdominal fullness, and heartburn than the young. The young are more likely to report diffuse abdominal pain, diarrhea, nausea, and vomiting than the old. This difference between the old and the young in the incidence of many gastrointestinal symptoms may derive from an age-associated change in visceral sensitivity[3], but the

source of the difference has not yet been established.

Our study found that those with a poor baseline score on the physical component of quality of life had a higher likelihood than those with an average score of developing diffuse abdominal pain, diarrhea and dyspepsia, while those with a poor baseline score on the mental component of quality of life were more likely than those with an average score to report diffuse and upper abdominal pain, nausea, constipation, and abdominal fullness. To our knowledge, this study is the first prospective cohort study to analyze baseline quality of life scores as predictors of gastrointestinal symptoms. A possible higher prevalence of occult irritable bowel syndrome in those with a poor score on the mental component of the quality of life measure may explain their higher incidence and prevalence of such symptoms as abdominal pain, nausea, constipation, and abdominal fullness[3,22]. This finding requires further study for confirmation.

The current study may be the first prospective cohort study to describe health care utilization in individuals in response to gastrointestinal symptoms. Our results indicate that self-treatment with dietary complementary and alternative medicine is more frequent than visiting a physician regarding all symptoms except vomiting. Recourse to physical as well as dietary complementary and alternative therapies was more frequent than visiting a physician in the case of dyspepsia and heartburn.

Complementary and alternative therapies, some of which had their origins in Japan, are increasingly used by the general population in industrialized countries[27-29]. This is true of a substantial proportion of the Japanese population, who use them frequently at a high cost to personal income[30]. Patients with functional and general gastrointestinal disorders are likely to turn to complementary and alternative medicine when conventional therapies fail to relieve their symptoms[31]. Therefore physicians need to keep their knowledge up to date on the regulations, side effects, and possible benefits of specific herbal products used by patients[32]. Studies of the effectiveness of complementary and alternative therapies for functional gastrointestinal disorders have, however, often been limited by study designs[33].

It should be borne in mind that the health diaries in this study were self-reports and therefore subjective. A further limitation is that information on the severity of symptoms was not requested. As symptoms were not classified as less or more severe, there was no means of determining whether severity differentially influenced decisions to note them in the diaries or to seek different forms of treatment. This may have resulted in misclassification biases[14].

In summary, gastrointestinal symptoms are of common occurrence in the Japanese population. Overall, the mean number of episodes of gastrointestinal symptoms was 0.66 in a month. About a quarter of respondents developed at least one gastrointestinal symptom in the course of the month. Abdominal pain, diarrhea, nausea, constipation and dyspepsia were the most frequent gastrointestinal symptoms. Female gender, younger age, and low baseline quality of life are risk factors for developing gastrointestinal symptoms. Japanese with gastrointestinal symptoms other than vomiting are more likely to resort to dietary forms of complementary and alternative medicine than to visit physicians.

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COMMENTS

Background

The incidence of gastrointestinal symptoms and the nature of consequent utilization of health care services in the Japanese population are not well documented. Our aim was to provide better description in this epidemiology of gastrointestinal symptoms.

Research frontiers

The prevalence of gastrointestinal disease in the Japanese population is known to be high. However, its epidemiology, its incidence, and the consequent utilization of health care services are not well described. There is also little information on the use of complementary and alternative medicine as compared with conventional medicine in the treatment of the various symptoms of gastrointestinal disease.

Innovations and breakthroughs

In this study of Japan, the incidence of gastrointestinal symptoms was 25% and the mean number of the symptomatic episodes was 0.66 in a month. Abdominal pain, diarrhea, nausea, constipation and dyspepsia were the most frequent symptoms. Female gender, younger age, and low baseline quality of life were risk factors for developing these symptoms. The participants were more likely to treat themselves, using dietary complementary or alternative medicines, than to visit physicians, except in the case of vomiting.

Applications

Gastrointestinal symptoms are very common in the Japanese general population. The most frequent symptoms include abdominal pain, diarrhea, nausea, constipation and dyspepsia. Risk factors for developing these symptoms are female gender, younger age, and low baseline quality of life. These results may help to understand the public health consequences of gastrointestinal symptoms and to assist in the setting of priorities in the allocation of health care services and of future research funding.

Terminology

Health diary: a daily record of daily health events and behaviors. This research methodology can provide an immediate and continuous record of daily health events and behaviors and minimize recall bias without the intervention of direct observational measures.

Peer review

This article documented about epidemiology of gastrointestinal symptom among Japanese. Study period is relatively short. Generally, it is well designed and clarified the incidence of gastrointestinal symptom among Japanese. The study also revealed Japanese actions to gastrointestinal symptoms.

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RAPID COMMUNICATION

Recent Ⅳ-drug users with chronic hepatitis C can be efficiently treated with daily high dose induction therapy using consensus interferon: An open-label pilot study

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Abstract

AIM: To investigate the use of high dose consensus-interferon in combination with ribavirin in former iv drug users infected with hepatitis C.

METHODS: We started, before pegylated (PEG)-interferons were available, an open-label study to investigate the efficacy and tolerability of high dose induction therapy with consensus interferon (CIFN) and ribavirin in treatment of naiive patients with chronic hepatitis C. Fifty-eight patients who were former iv drug users, were enrolled receiving 18 μg of CIFN daily for 8 wk, followed by 9 μg daily for up to wk 24 or 48 and 800 mg of ribavirin daily. End point of the study was tolerability and eradication of the virus at wk 48 and sustained virological response at wk 72.

RESULTS: More than 62% of patients responded to the treatment with CIFN at wk 24 or 48, respectively, showing a negative qualitative PCR [genotype 1 fourteen patients (56%), genotype 2 five (50%), genotype 3 thirteen (87%), genotype 4 four (50%)]. Forty-eight percent of genotype 1 patients showed sustained virological response (SVR) six months after the treatment.

CONCLUSION: CIFN on a daily basis is well tolerated and side effects like leuko- and thrombocytopenia are moderate. End of therapy (EOT) rates are slightly lower than the newer standard therapy with pegylated interferons. CIFN on a daily basis might be a favourable therapy regimen for patients with GT1 and high viral load or for non-responders after failure of standard therapy.

INTRODUCTION

Under physiological conditions, interferon-α (IFN-α) is a key cytokine produced by virtually all cells in the mammalian organism in response to a variety of bacterial and viral stimuli. In response to viral infection, IFN-α produced by the infected target cells induces a number of cellular genes involved in inhibition of viral replication. In addition, IFN-α is secreted by stimulated NK-cells and T-cells and exerts a multitude of immune stimulatory effects of innate and adaptive immunity[1].

The current standard of treating patients with chronic hepatitis C infection is using IFN-α with or without ribavirin and great advances have been achieved[2]. So far two allelic α-2 species, interferon α-2a and interferon α-2b, have been used. Introduction of pegylated IFN in 2001 showed a slight increase in the overall sustained virological response rates (approximately 55%) compared to conventional IFN-α[3,4]. However, recent studies showed that these response rates depend on several factors, including HCV genotype, baseline viral load, ethnicity, body weight and presence of advanced liver disease[3]. More than 75% of patients in western Europe are infected with genotype 1 often showing a high viral load and these patients are so called "difficult to treat" and therefore remain at risk not to respond to standard HCV treatments[5].

Before pegylated IFN was available, we introduced a study using IFN-alfacon-1, a second-generation cytokine that was engineered to contain the most frequently occurring amino acids among the non-allelic IFN-α subtypes in humans[6]. *In vitro* studies showed that IFN-alfacon-1 induces a more dramatic decrease of HCV-

RNA compared to IFN-2b[7] and shows a 10-time higher antiviral efficacy[6]. The rationale for daily dosing in our study was the fact that serum levels of IFN-α given three times a week were dropping almost below the detection limit every other day and therefore reducing the antiviral capability. High initial dosing would reduce the viral load even further and early virological response (EVR) would lead to a higher SVR than 9 μg daily[8]. Taking these results into account, the aim of this study was to look at the efficacy, tolerability and safety of high dose IFN-alfacon-1 plus ribavirin combination therapy in patients with chronic hepatitis C.

MATERIALS AND METHODS

Patient population

Patients aged 18 years and older with a serological and histological diagnosis of chronic HCV infection were asked to participate in the study. All patients were naïve to antiviral treatment and were recent iv drug users referred by a clinic with a detoxification program. Each individual had to be off drugs for at least 4 to 6 mo, and replacement medication (e.g. buprenorphine) was allowed. The local ethics committee approved the study and informed consent of patients was obtained prior to serological and histological testing and antiviral therapy. The study protocol was in accordance to the 1975 Declaration of Helsinki.

Inclusion criteria required that patients had detectable serum HCV-RNA and liver biopsy compatible with a diagnosis of chronic HCV infection. Exclusion criteria included decompensated liver disease, hemoglobin < 12 g/dL for men and women, white blood cell count < 3000/μL, neutrophil count < 1500/μL, and platelet count < 70 000/μL. Patients with hepatitis B virus (HBV) or HIV infection were excluded. Similarly, patients with antinuclear antibody ⩾ 160 or diagnosis of other chronic liver diseases (hemochromatosis, alpha1-antitrypsin deficiency, Wilson's disease, or other chronic liver diseases), or who had prior organ transplantation or hyper- or hypothyroidism were also excluded. A history of major depression or ongoing alcohol or drug abuse within the previous 6 mo, renal insufficiency, hemophilia, poorly controlled diabetes, cardiac disease, immunologically mediated diseases, active seizure disorders or brain injury requiring medication for stabilization and pregnancy were further criteria for not being included in the study. Eleven patients were on replacement medication including methadone and codeine.

Study design

The study was a mono-center clinical trial with consecutive and prospective enrolment. All patients were naïve to antiviral therapy including IFN and ribavirin. All patients received a high induction therapy with IFN-alfacon-1, 18 μg (Inferax®; Yamanouchi Pharma GmbH, Heidelberg, now Astellas Pharma GmbH, Munich) subcutaneously daily for eight weeks, followed by 9 μg subcutaneously daily until the end of treatment. Ribavirin (Meduna Pharma GmbH, Isernhagen, Germany), 800 mg daily, was administered bid over the whole treatment period. If HCV RNA levels were detectable after 24 wk, treatment was considered as failure and stopped; if HCV RNA was undetectable at wk 24, treatment was continued for a total of 48 wk in HCV genotypes 1 and 4. Treatment of genotypes 2 and 3 was stopped after 24 wk in general. After therapy was ended, patients were followed up for an additional 24 wk.

It was initially planned to treat 100 patients. However, after 58 subjects were enrolled, recruitment was suspended because of ethical concern following the release of pegylated IFN α-2b which became the standard of treatment.

Identification of genotype

HCV genotyping was performed using the INNO-LiPA HCV Ⅱ kit assay (Innogenetics, Gent, Belgium).

Serum HCV RNA

Reverse-transcription polymerase chain reaction was performed using the Cobas Amplicor hepatitis C monitor test (v2.0, Roche Diagnostics, Grenzach-Wyhlen, Germany). The results of HCV RNA are expressed as international units per millilitre based on published formulas where 2 000 000 copies are equivalent to 800.000 IU/mL[9].

Assessment of efficacy

The primary end point of the study was assessment of sustained virological response rate defined as loss of detectable HCV RNA by RT-PCR at wk 72 [24 wk after end of therapy (EOT)]. A secondary end point was assessment of the sustained biochemical response (SBR), defined as normalization of serum alanine aminotransferase (ALT) at wk 72. No second histological end point was sought. Early virological response (EVR) at wk 4 was not determined.

Assessment of safety and tolerability

Safety and tolerability assessments were performed at 0, 2, 4, 8, 12, 24, and 48 wk of therapy and then 12 and 24 wk post-treatment. All adverse events, laboratory test-results, discontinuation or withdrawal due to adverse events, and dose reduction were recorded and evaluated.

Statistical analysis

Analysis was based on the intent to treat 48 patients enrolled in the study. Data were described by rates, means with standard deviation, medians, and ranges. In addition, multivariate step-wise logistic regression was used to identify independent predictors from baseline characteristics which were associated with SVR in univariate analysis. All *P*-values reported were two-sided and *P*-values below 5% were considered statistically significant.

RESULTS

Patients

Between July 2000 and May 2003, 58 patients with chronic

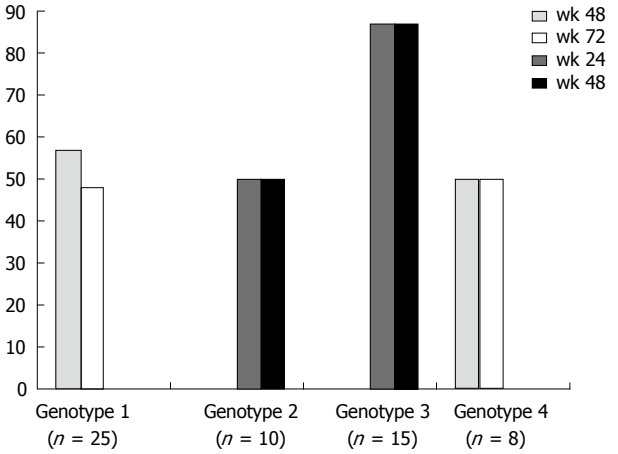

Figure 1 Response rates (in percent) after end of therapy (EOT) at wk 24 or 48 and sustained virological response (SVR) after 48 or 72 wk after beginning of antiviral therapy. Two patients with genotype 1 showed a relapse of the hepatitis C virus during 6 mo of follow at wk 72. In two patients genotype was not evaluated prior to therapy. Patients got antiviral treatment for 48 wk and both achieved SVR at wk 72.

Table 1 Demographic and clinical characteristics of 58 patients, *n* (%)

Characteristics	
Mean age, years, (range)	36 (22–62)
Sex	
Male	33
Female	25
Race	
Caucasian	48 (100)
ALT, IU/L (range)	57 (9-263)
HCV-RNA level, > 800.000 IU/mL	23 (40)
Mean level, IU/mL	615.000
HCV genotype	
1, 1a, 1b, 4	32 (55)
2a, 2b, 3	24 (42)
Not available	2 (3)
HCV genotype 1 and HCV-RNA > 800.000 IU/mL	19 (33)
Liver biopsy (available in 56 out of 58 patients)	
No chronic hepatitis	10 (17)
Chronic hepatitis	43 (74)
Cirrhosis	3 (5)
Co-medication	
Methadone	4 (7)
Codeine	7 (12)

Table 2 Sustained viralogical response by HCV genotype, gender and sex (wk 72), *n* (%)

All genotypes	
< 800.000 IU/mL	23/33 (70)
⩾ 800.000 IU/mL	13/25 (52)
Genotype 1	
< 800.000 IU/mL	5/6 (83)
⩾ 800.000 IU/mL	9/19 (47)
Non-genotype 1	
< 800.000 IU/mL	18/27 (67)
⩾ 800.000 IU/mL	4/6 (67)
Sex	
Male	16/33 (49)
Female	14/25 (56)

Sixteen patients who failed to follow up, dropped off the study due to side effects or died and were taken as non-responders, *n* = 58.

HCV infection were enrolled at our site in Luebeck. Of these patients, 57% were men and 43% women. All patients were of Caucasian origin and the mean age was 36 years. Ninety percent of the patients showed a histological diagnosis of chronic hepatitis and only 2% showed histologically cirrhosis that was deemed clinically compensated. The mean serum HCV RNA concentration was 617.000 IU/mL. Fifty-two percent of the patients were infected with genotype 1. Genotypes 2 and 3 were present in 39% of the patients and 4% of the patients showed genotype 4. The baseline characteristics are shown in Table 1.

Efficacy

Overall, 62% of the patients showed a sustained virological response (SVR) at the end of therapy, whereas 16 individuals did not respond to the therapy. ALT levels dropped significantly from initially elevated levels during the first 12 wk of therapy, whereas AST and bilirubin were stable in the normal laboratory range. Fifty-six percent and 50% of the patients with genotypes 1 and 4, respectively, showed a virological response at the end of treatment, while 48% and 48% respectively, reached a sustained virological response at wk 72. In the group of patients with easy to treat genotypes 2 and 3, 50% and 87% respectively, achieved a negative HCV-PCR at the end of treatment and no relapse occurred in these 19 patients until wk 48 (Figure 1). The patients with a low viral load (< 800.000 IU/mL) initially had a slightly greater chance of achieving SVR compared to those with a high viral load (48% *vs* 40%) irrespective of their genotype (Table 2). Women responded slightly better to therapy than men. The drop in viral load was accompanied with a significant decrease in ALT levels throughout the first three months of therapy (57 U/L to 22 U/L). AST levels were in their normal range at the beginning of treatment and there was only little if any further normalisation (25 U/L to 17 U/L). In some patients bilirubin was slightly elevated as well but showed normal levels at the end of therapy (data not shown). There was no positive correlation between either genotype or ALT or SVR (data not shown).

Neither consensus interferon (CIFN) nor ribavirin dosing had to be modified throughout the therapy in any patient.

Co-medication with either methadone or codeine did not play a role in treating patients with HCV. Neither dosage nor application of replacement medicine had to be adjusted. The patients on replacement medication did not succeed in regard to SVR compared to those without methadone or codeine (data not shown). Ninety-two percent of the patients, who did achieve sustained virological response, took more than 80% of medication throughout the whole treatment period, demonstrating a remarkably high adherence to antiviral therapy.

However, only 2 out of all patients (all genotype 1) with negative PCR at wk 48 (EOT) had a virological relapse during the follow-up period between wk 48 and 72.

Table 3 Frequency of laboratory abnormalities (n = 58)

Event	n
Neutropenia	
< 1500	21
< 1000	10
< 500	1
Anemia	
< 10.5 g	1
< 8.5 g	0
Platelets	
< 100.000	4
< 50.000	0

Tolerablity

Seven patients showed a relapse of their intravenous drug abuse prior to the end of therapy and one 28-year old male patient died due to a heroin overdose. Six patients stoped therapy due to treatment-related side-effects (bleeding due to low platelets). Two patients did not show up for follow-up visits during antiviral treatment. No patient showed evidence of hypocalcemia, previously reported for daily dosing of CIFN[10]. Liver function in all patients was stable as measured clinically and by testing of coagulation function and serum albumin (data not shown).

Safety and adverse events

There was one serious adverse event 8 wk after beginning of therapy, leading to a death due to an overdose of intravenous heroin abuse. Six patients showed a restart of their iv drug abuse while on combination therapy. One patient developed a major depression, one female patient showed a significant drop in platelets (43.000/μL), one showed recurrent epistaxis (89.000/μL) and one male patient showed a long- lasting episode of arthritis and suicidal thoughts. Otherwise dose modifications due to adverse events were not necessary in any patient.

The spectrum of side effects of daily high dose induction therapy with CIFN was similar to previous trials with IFN and ribavirin. All the 58 patients were included in safety analysis. All the patients experienced flu-like symptoms (100%). Most of them experienced fatigue (93%), headache (91%), cough (72%), and mood disorders (87%). Two patients experienced bleeding disorders due to low platelets [e.g. bleeding gingivitis (101/nL), epistaxis (89/nL)] and one patient was taken off antiviral medication due to a significant drop of WBC (Table 3). One patient did not receive further treatment at wk 8 due to impaired vision, myalgia, and suicidal thoughts. None of the patient experiencing mood disorders required any psychopharmacological co-medication.

In patients with significant neutropenia, this side effect occurred mostly during wk 6 and 8 of therapy, after being treated with high dose CIFN for the first 8 wk. GMCSF was not administered since low neutrophil count always returned to almost normal level in all patients by reducing daily CIFN dosing to 9 μg daily according to the study protocol. Neither platelets nor blood transfusion was given in patients with low platelets due to CIFN or anemia due to ribavirin.

One severe adverse event occurred due to the relapse of intravenous drug use followed by a lethal overdose injection. Five other patients took intravenous drugs as well and were immediately taken off the antiviral study drugs.

Table 4 Questionnaire (Int. Quality of Life Assessment SF-36). Listed are those answers presenting the majority of possible answers (%)

	wk 0	wk 12
Personal feeling	Very good (76)	Good (63)
Personal feeling compared to last week	Good (77)	Better (55)
Impaired physical activity	Never (90)	Slightly (77)
Impaired physical activity compared to last week	Never (88)	Slightly (73)
Emotional problems	Never (93)	Never (48)
Pain	None (90)	None (30)
Pain last week	None (83)	None (54)
Feeling depressed	Never (88)	Seldom (45)
Feeling happy	Often (85)	Sometimes (50)
Feeling tired	Seldom (77)	Sometimes (51)
Impaired personal contact to relatives and friends due to disease	Never (88)	Seldom (71)
"I'm feeling fit like others"	Mostly (94)	No (37)
"I'm feeling healthy"	Mostly (78)	No (65)
"I expect not to feel healthy"	Do not know (44)	No (32)

International quality of life assessment

Because personal mental and physical components of health of a patient undergoing such an antiviral therapy is important, each patient was requested to answer a widely accepted questionnaire (IQOLA SF-36, German Acute version 1.0) with respect to the quality of life at wk 0 and 12.

There was a decrease in well being after 12 wk of therapy, but a significant number felt better after finishing the high induction treatment period. The physical activity in most patients was slightly impaired 3 mo after therapy (Table 4). There was a significant increase in emotional distress at wk 12 compared to wk 0, possibly due to CIFN. More patients experienced physical pain (70%), depression (50%), unhappiness (50%) and tiredness (51%) at wk 12, while personal contact to friends and relatives was not influenced by therapy (29%). Due to the side effects, fewer people (35%) felt healthy three months after the beginning of treatment and 68% did not feel healthy during the upcoming treatment phase.

DISCUSSION

Chronic hepatitis C virus infection is responsible for an increase in morbidity and mortality. This is the first study on the treatment of naïve patients with chronic HCV infection with high-dosing of IFN-alfacon-1 in combination with ribavirin. Recent intravenous drug addicts can be successfully treated for HCV and the response rates are as good as in patients without iv drug history.

The goal of our study was to address the issue of efficacy, tolerability and safety of daily high dose induction therapy with consensus-interferon (CIFN) and ribavirin in naïve HCV-infected patients. However, the usually recommended dosing of CIFN is 9 μg, three times a week

at the beginning of the trial. Ribavirin was given on a daily basis at 800 mg, irrespective of the genotyping. Weight-based dosing of ribavirin for therapy in HCV genotypes 1 and 4 has been introduced after several studies were conducted in 2001[3].

All patients received a higher dosage of interferon (18 μg of CIFN subcutaneously daily for up to 8 wk followed by 9 μg daily) in combination with ribavirin for 24 or 48 wk. The patients were then carefully followed up for an additional 24 wk.

The overall response rate was 62 % showing a sustained virological response (SVR) 24 wk after the EOT. There was a slight difference ($P < 0.05$) in response rates in regard to the initial viral load. The patients with a low viral load (< 800.000 IU/mL) responded better (70% *vs* 52%) than those with a high viral load. These results are comparable to data (54%) obtained with pegylated α-2b/ribavirin[3].

There was a significant difference ($P < 0.01$) in response rates for the patients with a high viral load. With respect to genotyping, the patients with non-genotype 1 showed a SVR rate of 67% compared to 47% in the genotype 1 patients, even though the number of patients in the later group was quite small. In contrast, different response rates were obtained for HCV-infected patients presenting a low viral load initially. The non-genotype 1 patients showed a 64% SVR rate compared to 83% for the genotype 1-infected individuals. This fact might be explained by the small number of patients presenting genotype 1 and low-viral load. Overall, the patients with genotype 1 and high viral load showed an acceptable response rate, which is in accordance to Sjögren *et al*[11], showing a higher response rate for this population treated with CIFN and ribavirin instead of pegylated interferon α-2b and ribavirin (46% *vs* 14%).

In this treatment-naïve patient population, neither baseline ALT nor genotype was significantly associated with SVR, similar to other reports comparing treatment of non-responders with either CIFN or pegylated interferon α-2b in combination with ribavirin[15].

Adherence to therapy is another important issue for success of treatment[12]. Therapy with a wide range of side effects is less favourable and therefore less successful in achieving SVR. In our study, the overall adherence was good and comparable to previous studies[13]. It is known that HCV-1-infected patients maintained on > 80% of their interferon or peginterferon α-2b and ribavirin dosage for the duration of treatment have enhanced sustained response rates. In addition, some results suggest that adherence enhances the likelihood of achieving an initial virologic response[12].

However, the rate of reuse of intravenous drugs in almost 10% of patients (6 out of 58 patients) is substantial and accounts partly for the high drop-out rate in our study even though former iv drug users had to be off active drug use for at least 6 mo prior to study enrolment.

The increased side effects in patients treated with CIFN at a high induction rate (18 μg *vs* 9 μg daily) can result in a higher drop out rate[14,15]. The initial rapid decline in viral load at wk 4 is counteracted by increased side effects, resulting in a lesser SVR rate, at least in non responders[15].

In conclusion, the safety and tolerability profile of this treatment is reasonable in a certain subset of patients, but might be difficult to tolerate in some individuals, resulting in discontinuation of therapy. However, these data suggest that CIFN may be safely combined with ribavirin and enhance the sustained response rate when close monitoring of side-effects and laboratory results are obeyed[10]. Patients with chronic hepatitis C and genotype 2 or 3 should be treated with pegylated interferon α-2a or 2b in combination with ribavirin for up to 24 wk[16], respectively. In difficult-to-treat patients with genotype 1 and high viral load, a daily therapy regimen with consensus interferon in combination with ribavirin might be of choice as well as in non-responders to combination therapy[15,17].

This is the first study showing that recent iv drug users can be safely and successfully treated for HCV if they are closely monitored.

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RAPID COMMUNICATION

Elastic band ligation of hemorrhoids: Flexible gastroscope or rigid proctoscope?

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Abstract

AIM: To compare rigid proctoscope and flexible endoscope for elastic band ligation of internal hemorrhoids.

METHODS: Patients between 18 and 80 years old, with chronic complaints (blood loss, pain, itching or prolapse) of internal hemorrhoids of grade I-Ⅲ, were randomized to elastic band ligation by rigid proctoscope or flexible endoscope (preloaded with 7 bands). Patients were re-treated every 6 wk until the cessation of complaints. Evaluation by three-dimensional anal endosonography was performed.

RESULTS: Forty-one patients were included (median age 52.0, range 27-79 years, 20 men). Nineteen patients were treated with a rigid proctoscope and twenty two with a flexible endoscope. Twenty-nine patients had grade Ⅰ hemorrhoids, 9 patients had grade Ⅱ hemorrhoids and 3 patients had grade Ⅲ hemorrhoids. All patients needed a minimum of 1 treatment and a maximum of 3 treatments. A median of 4.0 bands was used in the rigid proctoscope group and a median of 6.0 bands was used in the flexible endoscope group ($P < 0.05$). Pain after ligation tended to be more frequent in patients treated with the flexible endoscope (first treatment: 3 *vs* 10 patients, $P < 0.05$). Three-dimensional endosonography showed no sphincter defects or alterations in submucosal thickness.

CONCLUSION: Both techniques are easy to perform, well tolerated and have a good and fast effect. It is easier to perform more ligations with the flexible endoscope. Additional advantages of the flexible scope are the maneuverability and photographic documentation. However, treatment with the flexible endoscope might be more painful and is more expensive.

INTRODUCTION

The initial treatment of symptomatic internal hemorrhoids is conservative and consists of a fiber enriched diet, increased fluid intake, prevention of straining and local hygiene, which may be combined with local anesthetic and antiphlogistic medication[1,2]. Over 90% of patients with symptomatic hemorrhoids can be treated conservatively or by rubber band ligation. Infrared coagulation and cryotherapy are also options, but are no longer commonly applied[3]. Surgery is reserved for the most severe cases. Since the early sixties the treatment of choice for persisting internal hemorrhoids is elastic band ligation by means of a rigid proctoscope (Barron ligation)[4,5]. Wrobleski *et al*[6] reported results of long term follow up (mean of 60 mo) of 266 patients; 80% had fewer symptoms and 69% had no symptoms. The procedure is usually safe and can be easily repeated[7]. Some studies evaluated the use of a flexible endoscope equipped with a ligation cap, normally used for ligation of esophageal varices, in treating hemorrhoids[8-11]. A flexible endoscope could have some advantages such as more maneuverability, a wider view and photographic documentation. The present prospective randomized trial was performed to compare both techniques in effectiveness. Additionally we used a three-dimensional endosonography to evaluate changes in the submucosa and sphincter defects.

MATERIALS AND METHODS

Patients between 18 and 80 years old, with chronic complaints (blood loss, pain, itching or prolapse) of internal hemorrhoids grade I-Ⅲ, were randomized by computer software to elastic band ligation by rigid proctoscope or gastroscope (Olympus Excera®). Patients were re-treated every 6 wk till the cessation of complaints.

The rigid proctoscope was gently introduced and then

Table 1 Patient characteristics and results

	Proctoscope	Gastroscope
n	19	22
Age [median (range)]	53 (27-75)	50.5 (33-79)
Male/Female	7/12	13/9
Hemorrhoids grade I	15	14
Hemorrhoids grade Ⅱ	4	5
Hemorrhoids grade Ⅲ	0	3
No of treatments [median (range)]	1.0 (1-3)	1.0 (1-3)
No of total ligations [median (range)]	4.0 (2-10)	6.0 (1-15)[a]
Pain after treatment	3	10[a]
Cost	3 euro	150 euro

[a]$P < 0.05$ *vs* proctoscope.

elastic bands were applied using a standard elastic band applicator in anterograde fashion. For endoscopic ligation, a single-use multiband ligator device (Sevenshooter®, Boston Scientific, USA) was attached to the end of a gastroscope. The device can only be attached to a gastroscope. The Sevenshooter is provided with 7 bands. The hemorrhoids were suctioned into the ligation cap in either retrograde or antegrade fashion. The number of bands applied depended on the amount of hemorrhoidal tissue present. All treatments were performed in an outpatient setting.

A three-dimensional anal endosonography was made before the first treatment and 6 wk after the last treatment. Endosonography was performed, using a three dimensional diagnostic ultrasound system (Hawk type 2102, B-K Medical) with a 16 MHz rotating endoprobe (type 1850, focal range 2 to 4.5 cm) covered by a hard sonolucent cone (diameter 1.7 cm) filled with water, producing a 360° view. Three dimensional anal endosonography was performed according to a standard procedure. The endoprobe was covered with a lubricated condom which was filled with ultrasound gel. The probe was then introduced into the rectum and a recording was made of the distal part of the rectum, the puborectalis muscle and the anal canal. After the endosonography, images were reconstructed to three dimensional images by computer software. The Medical Ethical Committee granted approval for this study.

Statistical analysis

Data are presented as a median with range. Differences between groups were assessed with a Mann-Whitney test, Chi square test or Fischer's exact test when appropriate. $P < 0.05$ was taken as significant.

RESULTS

Detailed demographic and clinical data are shown in Table 1. Forty-one patients (median age 52, range 27-79 years, 20 men) with recurrent rectal bleeding, pain, itching or prolapse underwent ligation. Nobody had been treated previously. All procedures were easy to perform. Sometimes, in patients treated with the flexible endoscope, a rectal enema was needed when the rectum was not clean.

Nineteen patients were treated with a rigid proctoscope and twenty two with a flexible endoscope. Twenty-nine, 9, and 3 patients had grade Ⅰ, Ⅱ and Ⅲ hemorrhoids, respectively.

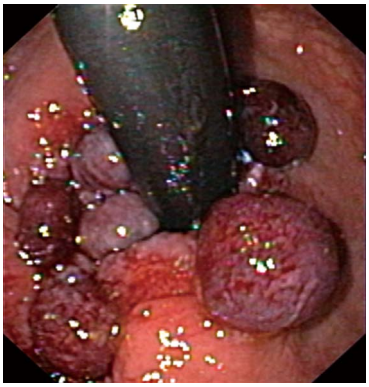

Figure 1 Picture showing the retroflexed endoscopic view after ligation.

Patients treated with a proctoscope needed a median of 1 treatment (range 1-3) and patients treated with an endoscope also needed a median of 1 treatment (range 1-3, $P = 0.664$).

A median of 4 bands (range 2-10) was used in the rigid proctoscope group and a median of 6 bands (range 1-15) was used in the flexible endoscope group ($P < 0.05$) (Figure 1).

In the group treated with a rigid proctoscope, 5 patients needed 1 re-treatment and 1 patient needed 2 re-treatments. In the group treated with a flexible endocope, 2 patients needed 1 re-treatment and 1 patient needed 2 re-treatments.

Two patients were excluded from follow up: One patient had a rupture of an abdominal aortic aneurysm and 1 patient developed an anal fissure.

Pain after ligation was more frequent in patients treated with the flexible endoscope (first treatment: 3 *vs* 10 patients, $P < 0.05$). The single-use ligation device (Sevenshooter®) costs approximately 150 euro for one set (2005). One elastic band for the rigid proctoscope costs approximately 0.50 euro. Further costs (cleaning of the gastroscope and proctoscope, personnel costs) did not substantially differ between both techniques.

With three-dimensional endosonography 30 patients were evaluated before and after treatment. Hemorrhoidal tissue was clearly visible in all patients. No sphincter defects were found before and after treatment and there was no significant change in submucosa thickness after endoscopic/proctoscopic treatment (Figure 2). After one year follow up, 5 patients needed re-treatment (2 proctoscope, 3 gastroscope).

DISCUSSION

Hemorrhoids are a major health problem; the community-wide prevalence in the Western World is reported to be around 4%. Adequate intake of fluids and fiber, use of suppositories, stool softeners and topical creams are important in the treatment of hemorrhoids, especially in grade I disease. When conservative treatment fails, there are still options, including surgical therapy.

Rubber band ligation with a proctoscope is very effective and inexpensive. Since 2005, it has been the treatment of choice. Studies have been undertaken investigating effectiveness and new techniques[12-14].

There have been 4 studies that analyzed the use of

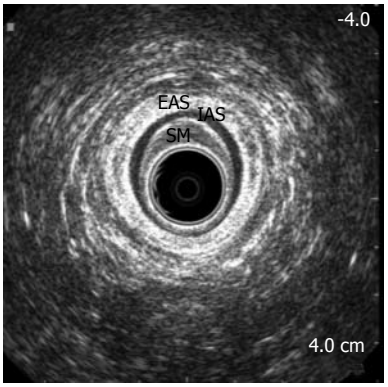

Figure 2 Endosonographic image showing thickening of the submucosa. IAS: Internal anal sphincter; EAS: External anal sphincter; SM: Submucosa (hypoechogenic area is hemorrhoidal).

a flexible endoscope for hemorrhoidal elastic band ligation[8-11]. These studies conclude that it is a safe and efficient method with some advantages, although costs are still a major drawback. Only one study compared endoscopic band ligation (reusable) with the conventional rubber band ligation through a rigid proctoscope[10].

In our pilot study we found that both techniques were easy to perform, well tolerated and had a good and fast effect. It was easier to perform more ligations with the flexible endoscope. No serious adverse events were reported. Additional advantages of the flexible scope were the maneuverability and photographic documentation.

However, treatment with the flexible endoscope seemed to be more painful and was more expensive. More pain sensation can be explained by the learning curve we had to deal with and that more bands could be applied. In contrast, Wehrmann *et al*[10] found no significant difference in pain and reported that the total number of bands applied was significantly lower in the group treated with the endoscope.

So far as we know, this is the first study that evaluated with three-dimensional endosonography the presence of possible sphincter defects and changes in the submucosa. Poen *et al*[15] already found, with two-dimensional endosonography, no difference in appearance of the anal configuration after treatment with either rubber band ligation or infrared coagulation. In our study no significant alterations or sphincter defects were found and these endosonographic findings confirmed that band ligation is a safe technique.

When costs can be reduced the endoscopic treatment of hemorrhoids can be a good alternative for ligation with the rigid proctoscope.

In summary, endoscopic ligation is an effective, safe treatment and is comparable with proctoscopic ligation. However, the treatment is more expensive.

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RAPID COMMUNICATION

Immunohistochemical analysis of p53, cyclinD1, RB1, c-fos and N-ras gene expression in hepatocellular carcinoma in Iran

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Abstract

AIM: To study the effect of some genes especially those involved in cell cycle regulation on hepatocellular carcinoma.

METHODS: Paraffin-embedded tissue samples of 25 patients (18 males and 7 females) with hepatocellular carcinoma were collected from 22 pathology centers in Tehran during 2000-2001, and stained using immunohistochemistry method (avidin-biotin-peroxidase) for detection of p53, cyclinD1, RB1, c-fos and N-ras proteins.

RESULTS: Six (24%), 5 (20%), 12 (48%) and 2 samples (8%) were positive for p53, cyclinD1, C-fos and N-ras expression, respectively. Twenty-two (88%) samples had alterations in the G1 cell-cycle checkpoint protein expression (RB1 or cyclinD1). P53 positive samples showed a higher (9 times) risk of being positive for RB1 protein than p53 negative samples. Loss of expression of RB1 in association with p53 over-expression was observed in 4 (66.7%) of 6 samples. Loss of expression of RB1 was seen in all cyclinD1 positive, 20 (90.9%) N-ras negative, and 11 (50%) C-fos positive samples, respectively. CyclinD1 positive samples showed a higher (2.85 and 4.75 times) risk of being positive for c-fos and N-ras expression than cyclinD1 negative samples.

CONCLUSION: The expression of p53, RB1 and c-fos genes appears to have a key role in the pathogenesis of hepatocellular carcinoma in Iran. Simultaneous overexpression of these genes is significantly associated with their loss of expression during development of hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and the fourth most common cause of cancer-related death in the world[1]. Male predominance is, however, more obvious in populations at high risk of developing this tumor (mean ratio 3.7:1.0) than in those at low or intermediate risk (2.4:1.0)[2].

HCC has a heterogenous geographical distribution. Countries or regions with the highest incidence (50-120 cases per 100 000 population per year) include China, Taiwan, Korea and other Southeast Asian countries, as well as Sub-Saharan Africa. HCC is linked to environmental, dietary, and lifestyle factors, so that its incidence and distribution vary widely among ethnic groups, geographic regions, and the two sexes[3]. Tumor suppressor genes such as RB1 and p53 may play a significant part in hepatocarcinogensis[3]. As a favorable background for neoplastic transformation, cirrhosis is expected to harbor early genetic changes, but very few studies have been conducted thus far to address this issue. Ashida *et al*[4] have reported in both HCC and adjacent cirrhosis a 60% rate of loss of heterozygosity (LOH) at 13q, the site of the RB1 gene. The loss of heterozygosity and abnormalities in structure and function of the p53 gene are also frequently found in HCC patients[5]. A specific p53 mutation is found in more than 50% of HCC patients from India, China and South Africa, where dietary aflatoxins are suspected to be the major liver specific carcinogens[5-11]. However, it occurs less frequently in Western countries[3,5]. Activation of oncogenes of the "ras" family and others has been detected during chemically induced HCC in rodents, but there is little evidence of such activation in human tumors[12]. CyclinD1 over-expression may be an early event in hepatocarcinogenesis and plays a role in tumor differentiation[13]. Yuen *et al*[14] reported that the expression of c-fos is significantly higher in tumor tissue than in non-

tumor tissue. Specific mutations of the p53, cyclinD1, RB1, c-fos, and N-ras genes and their expression in HCC have been reported from several parts of the world, but to the authors' knowledge to date, the expression status of these genes has not been studied in HCC patients in Iran, where the frequency of chronic hepatitis B and C virus infection as well as exposure to dietary aflatoxin is very high[15].

MATERIALS AND METHODS

Sample collection

Formalin-fixed and paraffin-embedded tissue samples of 25 patients (18 males and 7 females) with documented HCC (surgically resected material or biopsy) were provided for analysis. The samples were collected from 22 pathology centers in Tehran during 2000-2001. The study was approved by the Medical Ethics Committee, Ministry of Health, Iran, as conforming to the ethical guidelines of the 1975 Declaration of Helsinki. Hospital records were used to verify age, sex and other demographic items.

Tissue preparation

These samples were sectioned and stained with hematoxylin and eosin (HE). Diagnosis of HCC was confirmed and the grade of tumor was determined according to the criteria proposed by the World Health Organization by the collaborating pathologist in Research Center for Gastroenterology and Liver Disease.

HCC was considered to be adequate for immunohistochemical study only if the block was of adequate size (surface area of section > 4 cm^2 and > 10% of the surface area of the block was occupied by the tumor).

Immunostaining

The technique was based on avidin-biotin-peroxidase method using 10% formaldehyde-fixed and paraffin-embedded sections. The selected paraffin blocks were cut into 5 μm-thick sections. The sections were applied to precoated glass slides to avoid becoming detached, then dried at 37℃ overnight followed by drying at 56℃ for 60 min, deparaffinized with xylene and rehydrated through graded concentrations of alcohol. Antigen retrieval was performed by 3 × 5-min cycles of microwave oven heating (750W) at 100℃ in 0.01 mol/L citrate buffer at pH 6. After washing and rinsing with Tris-buffered saline (TBS, 0.05 M, pH 7.2-7.6), endogenous-peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature and then rinsed with distilled water. Subsequently, sections were treated for 10 min with 10% bovine serum albumin (BSA) at room temperature for blocking nonspecific background staining. Afterward, primary monoclonal mouse antihuman-p53 protein, clone DO-7, isotype IgG2bkappa (DAKO, Lot 108) at dilution 1:100, primary monoclonal mouse antihuman-RB1 gene product, clone RB1, isotype IgG1kappa (DAKO, Lot 019) at dilution 1:50, primary monoclonal mouse antihuman-cyclinD1, clone DCS-6, isotype IgG2a kappa (DAKO, Lot 012) at dilution 1:50, primary monoclonal mouse antihuman-c-fos, clone D-1, isotype IgG2b (Santa Cruz Biotechnology Inc., Lot J 251) at dilution 1:100 and primary monoclonal mouse antihuman-N-ras, clone F155, isotype IgG1 (Santa Cruz Biotechnology Inc., Lot I 251) at dilution 1:100 were added and incubated in a moist chamber overnight at 4℃. The sections were again washed three times in TBS for 5 min using the DAKO LSAB2 system (Universal, HRP, Lot 10106). Goat anti-mouse and anti-rabbit biotinylated IgG (diluted in PBS containing carrier protein and 0.015 mol/L sodium azide) and preincubated streptavidin conjugated to horseradish peroxidase (diluted in PBS containing carrier protein and anti-microbial agents) were added for 30 min at room temperature. The sections were washed in TBS as before and then developed in prepared 3-amino-9-ethylcarbazole substrate chromogen (AEC/H_2O_2) for p53 and RB1 and in prepared 3,3'-diaminobenzidine chromogen solution (DAB/H_2O_2) for cyclinD1, c-fos and N-ras for 10 min at room temperature. The sections were then washed in water, counterstained with Mayer's hematoxylin for 2-5 min at room temperature, dehydrated, cleared with 37 mmol/L ammonia water, rinsed in a bath of distilled water for 2-5 min, finally mounted and coverslipped with Faramount aqueous-based mounting medium (DAKO, Lot 00029). A section of the same tumor incubated in BSA instead of the primary antibody was included as the negative control. We used one standard p53 positive section of human SCC (DAKO, Lot 071-1), one known RB1 negative retinoblastoma section, one known cyclin D1 positive breast tumor section, one known c-fos positive astrocytoma section and one known N-ras positive lymphoma section as a positive control for each staining.

Assessment of immunostaining

Staining of p53, RB1, cyclinD1, c-fos and N-ras genes was examined at high power fields (× 400) under a standard light microscope. Nuclear staining was regarded as positive if there was homogeneous staining or > 10% of the cancer cells were heterogeneously stained.

Statistical analysis

The results were expressed as frequency for gene expression changes and odds ratio for association between expression changes of these five genes. All statistical tests were performed with the Program Statistical Package for the Social Sciences (SPSS version 11, Chicago, IL).

RESULTS

The mean ± SD age of our patients was 60.56 ± 12.52 years and the highest frequency (44%) was seen in the sixth decade of life. Male to female ratio was 2.57 (18 males and 7 females) and mean ± SD age of patients in each sex was 62.72 ± 10.43 years and 55 ± 16.4 years, respectively.

Histopathology

All the 25 samples were well differentiated (grade Ⅰ).

Accumulation of p53, RB1, cyclinD1, c-fos and N-ras proteins

Intense immunostaining of p53, RB1, cyclinD1, c-fos and

Table 1 Expression of p53 gene in relation to the expression of RB1, cyclinD1, c-fos and N-ras genes, *n* (%)

Gene expression	RB1		Cyclin-D1		c-fos		N-ras	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
P53 positive	2 (33.3)	4 (66.7)	2 (33.3)	4 (66.7)	4 (66.7)	2 (33.3)	1 (16.75)	5 (83.3)
P53 negative	1 (5.3)	18 (94.7)	3 (15.8)	16 (84.2)	8 (42.1)	11 (57.9)	1 (5.3)	18 (94.7)
	OR: 0.1, 95% CI: 0.0-1.5		OR: 2.6, 95% CI: 0.3-21.7		OR: 2.7, 95% CI: 0.4-18.8		OR: 3.6, 95% CI: 0.1-68.3	

Table 2 RB1 gene expression in relation to the expression of c-fos and N-ras genes, *n* (%)

Gene expression	c-fos		N-ras	
	Positive	Negative	Positive	Negative
RB1 positive	1 (33.3)	2 (66.7)	0	3 (100)
RB1 negative	11 (50)	11 (50)	2 (9.1)	20 (90.9)
	OR: 0.5, 95% CI: 0.0-6.3		OR: 1.1, 95% CI : 0.9-1.2	

Table 3 G1 checkpoint protein expression in relation to p53, *n* (%)

Expression pattern of G1 checkpoint proteins	P53 (+)	P53 (-)	*P*
Rb+/cyclinD1+	Zero	Zero	
Rb-/cyclinD1+	2 (40)	3 (60)	
Rb-/cyclinD1-	2 (11.8)	15 (88.2)	0.02
Subtotal	4 (66.6)	18 (94.7)	
Rb+/cyclin D1-	2 (66.7)	1 (33.3)	
Total	6 (24)	19 (76)	

N-ras proteins was observed in the cell nuclei of tissues. Overall, six (24%) samples showed nuclear accumulation of p53 protein in varying proportions of tumor cells, the rest of the samples (76%) were negative. Twenty-two (88%) samples showed complete loss of RB1 protein expression in the primary tumor, the rest of the tumors (12%) displayed variable proportions of RB1 protein positive tumor cells. The intensity and subcellular location of the staining in the tumor were similar to those observed in the normal epithelia. We detected high levels of cyclinD1 protein in 5 (20%) samples while 20 (80%) samples were negative for cyclinD1 expression. The frequency of c-fos and N-ras positive staining was 48% (12 samples) and 8% (2 samples), respectively. The expression of p53 gene in relation to the expression of RB1, cyclinD1, c-fos and N-ras genes is depicted in Table 1. The RB1 gene expression in relation to the expression of c-fos and N-ras genes is shown in Table 2. Of those samples positive for the c-fos gene, 1 (8.3%) was N-ras positive and 11 (91.7%) were N-ras negative. On the other hand, among the c-fos negative samples, 1 (7.7%) was N-ras positive and 12 (92.3%) were N-ras negative.

When overexpression of p53 was seen, loss of expression of RB1 was found in 4 (66.7%) samples. Loss of expression of RB1 was observed in all those with positive cyclinD1 (5 samples), while expression of RB1 was found in 17 (85%) with negative cyclinD1, and in 3 (15%) samples with positive RB1. CyclinD1 positive samples showed a higher risk of being positive for C-fos and N-ras (2.85 and 4.75 times, respectively) than cyclinD1 negative samples. Finally, loss of expression of RB1 was detected in 2 samples with overexpression of N-ras. On the other hand, among the samples with loss of expression of RB1, overexpression of c-fos was found in 11 (50%).

Overall, 22/25 (88%) samples had alterations in the G1 cell-cycle checkpoint proteins, as assessed by means of cyclinD1 and RB1 expression (Table 3). These occurred in 4 (66.6%) of 6 p53-positive samples and in 18 (94.7%) of 19 p53-negative samples. P53-negative samples showed absence of the RB1 protein more frequently. P53 positive samples showed a higher (9 times) risk of being positive for RB1 than p53 negative samples, being 3.6, 2.75, and 2.66 for N-ras, c-fos, and cyclinD1, respectively. In samples with cyclinD1 positive staining, the risk of being positive for N-ras was 4.75 times higher in samples with cyclinD1 positive staining than in samples with negative cyclinD1 staining for this protein.

DISCUSSION

Several oncogenic pathways have been implicated in malignant transformation of liver cells. Inactivation of the p53 tumor suppressor gene by mutations and allelic deletions in about 30% of HCC cases has been associated predominantly with exposure to aflatoxin B1 and hepatitis B virus infection[16]. Activation of cyclinD1, c-fos and N-ras and disruption of the RB1 pathway are also commonly involved in liver tumorigenesis. New major challenges include the identification of candidate genes located in frequently altered chromosomal regions and oncogenic pathways driven by different risk factors. Deranged expression of cell cycle modulators has been reported to contribute to the development and progression of HCC[17]. In human HCC, high frequencies of aberration have been detected in the p53 and RB1 genes[14]. Mutations of the p53 tumor suppressor gene have been reported to occur with varying frequency in different geographic regions, which might be a different etiology for HCC[18]. In our study, nuclear accumulation of p53 protein was seen in 24% of samples. Mutations of this gene have been identified in 30%-50% of HCC patients in some geographic areas[19]. An *et al*[20] reported that there is histological heterogeneity in established HCC, which is accompanied with increased proliferative activity and p53 overexpression. Overexpression of p53 has identified in 37.5% of Japanese HCC patients and 62.5% of Indonesian HCC patients[18]. Recently, Ming *et al*[21] also showed that the frequency of mutation of p53 gene is much higher in high prevalent HCC area than in the low-risk HCC area in China. More than 95% cancer specimens

exhibit strong intranuclear accumulation of p53 protein, which can be detected by immunohistology. However, Biersing *et al*[22] and Vesey[23] have found little or no point mutations of p53 gene in human hepatocarcinoma in Swedish and Australian patients. Therefore overexpression of p53 protein in hepatocarcinoma specimens can be used as the mutant p53 biopathological marker in tumor tissues. Qin *et al*[8] reported that accumulation of p53 is a valuable marker for predicting the prognosis of HCC patients. Lin GY *et al*[7] reported that inactivation of the tumor suppressor genes p53 and RB1 has been demonstrated in different forms, and implies the pathogenesis of human malignant diseases. The study of Kondoh *et al*[24] supports the idea that deletion or inactivation of tumor suppressors including RB1, p53 and other candidate genes seems to be common events in HCC development. Abnormalities of the RB1 tumor suppressor gene have been found in 20%-25% of HCCs, including 80%-86% of HCCs with p53 mutations[19]. Nishida *et al*[25] reported that RB1 protein is positive in 85.6% of HCC cases but is not related to any clinicopathological parameters. Positive immunostaining for RB1 and mutant p53 protein is detectable in 58% and 37% of HCCs, respectively[26]. Loss of expression of RB1 in HCC has been reported in several studies[21,27]. In this study, loss of expression of RB1 gene was found in 88% of samples. The proto-oncogene c-fos is involved in cell cycle progression and cellular proliferation[14]. Abuthnot *et al*[28] reported that c-myc and c-fos mRNA, as well as their protein products, are increased in human liver cancers. Wang *et al*[29] have also found an apparently higher expression of N-ras and c-fos in human hepatoma than in its adjacent liver tissue. Recently, Feng *et al*[30,31] reported that the positive rates and signal intensity of c-fos and some other proteins in HCC are significantly higher than those in pericarcinomatous tissues. Yuen *et al*[14] found that the expression of c-fos was significantly higher in tumor tissue than in nontumor tissue (91% *vs* 0%, $P < 0.0001$). C-fos primarily induces cyclinD1 up-regulation by a mediator called MAPK/ERK[32]. In our study, the expression of c-fos gene was detected in 48% of patients with documented HCC. There was no significant relationship between c-fos and Cyclin-D1 expressions. Aflatoxin B1 may evoke an intense and prolonged expression of c-fos, including persistent signals for regeneration, which in turn may activate the replication of immature cells[33]. CyclinD1 is frequently overexpressed in a variety of cancers, including HCC, as a result of gene amplification. Overexpression of cyclinD1 protein, through gene amplification, correlates with poor prognosis of several cancers, but its role in HCC is the subject of controversy. Increased expression of cyclinD1 may play an important role in the development of HCC owing to the perturbation of normal control of the cell cycle[34]. On the other hand, Azechi *et al*[35] reported that cyclinD1 is a known oncogene and a key regulator of cell cycle progression. Amplification of the cyclinD1 gene and its overexpression are associated with aggressive forms of HCC. Overexpression of cyclinD1 is sufficient to initiate hepatocellular carcinogenesis. Choi *et al*[17] and Deane *et al*[36] have found a positive relationship between cyclinD1 overexpression and advanced tumor stage and aberrant p53 expression in HCC ($P < 0.05$). Joo *et al*[13] reported that cyclinD1 overexpression may confer additional growth advantages to the tumor in addition to protein RB1 inactivation in HCC. On the contrary, Sato *et al*[37] have found no significant relationship between the expressions of cyclinD1 and p53. Ito *et al*[38] conducted a simultaneous immunohistochemical study with p53 and cyclinD1 antibody in the same series of HCC and revealed that 88% of the patients positive for cyclinD1 also expressed p53 and 91% of the patients negative for p53 did not express cyclinD1, suggesting that cyclinD1 is expressed later than the alteration of p53 in the progression of human HCC. In our study, cyclinD1 was positively related to aberrant p53 expression. In HCC, N-ras was first proved as one of the transforming genes[39], which belongs to the G protein family. When it is converted to an active oncogene by point mutation, chromosome rearrangement or gene amplification, the signal transmission of cell membranes may change, which drives cell division, leading to abnormal differentiation and formation of neoplasm. Cerruti[40] and Tada[41] reported that the mutagenesis of a proto-oncogene from "ras" family and p53 tumor suppressor gene might be the most important event in HCC. Tabor[19] reported that overexpression of oncogenes N-ras and c-fos has been found in high percentages of HCC patients. Imai *et al*[42] and Tamano *et al*[43] found that mutations of ras oncogene may be the early events, and the expression in tumor or non-tumor tissues can be detected with different rates. Luo *et al*[26] reported that N-ras and p53 genes might be involved in the carcinogenesis and development of HCC. They also showed that mutation of the tumor suppressor gene p53 can convert ras gene into oncogene. In their study, 38% of HCCs with N-ras gene mutation did not express p53 protein, indicating that some other genes or factors may participate in the carcinogenesis and development of HCC. In our study, 83% of p53 positive samples did not show N-ras mutation. Chao *et al*[9] suggested that activation of the ras gene might not be a major event in aflatoxin-related human hepatocarcinogenesis. This hypothesis is supported by another study[44] conducted in southern Africa on Blacks, where dietary exposure to aflatoxin is a risk factor.

In conclusion, as in other parts of the world, the change in expression pattern of these genes especially p53, RB1 and c-fos, appears to have a key role in the pathogenesis of HCC in Iran. There is likely a relation between the simultaneous changes in these genes during development of HCC. This research might shed some light on the carcinogenic role of the expression of p53, RB1, cyclinD1, c-fos and N-ras genes. Besides, in order to understand the exact role of these changes in development of HCC, further studies with a larger number of samples are essential.

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RAPID COMMUNICATION

Achalasia and thyroid disease

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Abstract

AIM: To investigate some possible etiologies of achalasia by screening patients with achalasia for some autoimmune diseases such as thyroid disease.

METHODS: We examined 30 known cases of achalasia (20 females, 10 males). Their age ranged 15-70 years. All of them were referred to our institute for treatment. Their sera were evaluated to detect some possible associations with rheumatoid disease, thyroid disease, inflammatory process, anemia, *etc*.

RESULTS: Seven out of 30 patients (23%) had thyroid disease including four patients with hypothyroidism (13.3%), two patients with hyperthyroidism (6.6%), and one had only thyroid nodule but was in euthyroid state (3.3%). Two of these hypothyroid patients had no related clinical symptoms (subclinical) and two had clinical manifestations of hypothyroidism**.** There were no correlations between the intensity of thyroid diseases and the severity of achalasia symptoms.

CONCLUSION: The etiology of achalasia is unknown although autoimmunity has been implicated and is supported by several studies. Thyroid disease presents concomitantly with achalasia in about one fourth of our patients who may have a common etiology.

Key words: Achalasia; Thyroid disease; Hypothyroidism; Esophageal motility; Etiology

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INTRODUCTION

Achalasia is a common and well characterized primary motility disorder of the esophageal body and lower esophageal sphincter (LES), causing impaired progressive peristalsis in the esophageal body, incomplete relaxation of LES during swallowing, and sometimes increased abnormal relaxation of the LES pressure. Previous studies evaluating esophagomyotomy and esophageal resection specimens have shown that the presence of myenteric inflammation is a consistent and early pathologic change in patients with achalasia[1,2]. The disorder is observed in both genders primarily in the fifth and sixth decades of life, although achalasia can present at any age[3,4]. The cause of the degeneration of neurons in achalasia is not known. The observations that achalasia is associated with HLA-DQw1 and that affected patients often have circulating antibodies to enteric neurons suggest that achalasia may be an autoimmune disorder[5-7]. Nonspecific degeneration of smooth muscle cells and a loss of small nerve fibers have been reported[8-10]. Another controversial issue is the role of inflammatory infiltrates in the pathogenesis of the disease. A study has suggested that complement activation is involved in the autoimmune pathogenesis of achalasia[11]. Achalasia is associated with extra esophageal autonomic nervous dysfunction that involves cardiovascular and papillary function as well as regulation of mesenteric arterial blood flow[12]. Some investigators have proposed that achalasia may result from chronic infections with herpes zoster or measles viruses, but modern studies have not confirmed an association between achalasia and any recognized viral disease[13,14]. However, a recent study also showed that there is no association between the most common viruses and achalasia[14]. As myenteric neurons synthesizing nitric oxide are responsible for the inhibitory component of esophageal peristalsis and LES relaxation, it is considered likely that these neurons are involved in this disease[15,16].

Since there is no consistent single abnormality in achalasia, this study was designed to evaluate the relationship between achalasia and some of the commonest autoimmune diseases.

MATERIALS AND METHODS

Patients

All 42 newly diagnosed patients referred between 2001 and 2005 to our research center (Poursina Hakim Research Institution, Gastroenterology Division, Isfahan, Iran) were examined prospectively and enrolled in the study. Poursina

Hakim Institution is a reference tertiary care center that services a population of about 4 000 000 individuals in the central area of Iran. Of the 42 enrolled cases in the study based on clinical and radiological findings, 6 patients with pseudoachalasia but without endoscopic features of achalasia were excluded due to non-compliance (one case) or some other reasons such as loss of follow-up due to changing their address or phone number, immigration to other provinces and admission by other physicians far from our institution. Finally 30 cases were included. Their symptoms, laboratory results, and historical and functional data were recorded. Achalasia was defined by clinical criteria, namely prolonged (more than 1 year) intermittent or progressive dysphagia to liquids and solids in addition to some other minor symptoms such as regurgitation, chest pain and nocturnal cough plus barium swallow with a typical beak like appearance. We obtained posterior-anterior chest x ray radiographies at 1, 5, and 20 min after a single barium meal to assess barium column and measure its heights and widths as well as the delay time of esophageal clearance (timed barium swallow). Upper gastrointestinal endoscopy was done to confirm the diagnosis and to rule out secondary achalasia (such as esophageal tumors of cardia and fundus, submucosal tumor of distal esophagus, peptic stricture and other mucosal or infiltrative lesions of the esophagus or gastric cardia). Endoscopic findings suggestive of achalasia may be seen and are very helpful if endoscopy is done carefully by experts in this field. Some of the suggestive criteria for achalasia are as following: esophageal dilation, esophageal dysmotility or aperistalsis in response to air insufflations, abnormal LES opening and high pressure LES during scope passage. Manometery although very helpful especially in early stages of the disease, was not available in our province at the time of the study, so it was not done for most of them.

Exclusion criteria included those found to have an underlying malignancy during the study, any types of generalized neuropathies, diabetes mellitus, non-compliance or unwillingness of patients to continue the study, and finally those lost to follow up by any reason.

The patients' history was taken using a structured questionnaire. We interviewed, examined and completed a questionnaire designed to detect possibility of connective tissue and autoimmune diseases, other gastrointestinal motility disorders, diabetes mellitus, use of immunosuppressive medications, chronic viral illnesses, thyroid problems, usage of a regular immunosuppressive medication and also habits and addictions for all patients. Informed consent was obtained from all patients. None of the patients underwent surgical therapy.

Laboratory methods

After diagnosis of achalasia, all patients were screened for a set of selected autoimmune disease and other markers as shown in Table 1. Serum factors were evaluated by biochemical techniques. A double check of laboratory tests was conducted by a second laboratory to assure validity of abnormal results. Equivocal laboratory results were rechecked by a reference laboratory.

Symptoms

Clinical data regarding symptoms of dysphagia, regurgitation, and chest pain, nocturnal cough and pyrosis were collected by means of a validated questionnaire. Dysphagia was described as difficulty in swallowing, and regurgitation as the sensation of stomach contents going up the esophagus. The frequency of each symptom was graded on a scale ranging from 0 to 5 (0 = none, 1 = once per month or less, 2 = once per week up to three to four times a month, 3 = two to four times per week, 4 = once per day, 5 = several times per day). This scoring system is similar, but with some modifications, to that used by Eckardt *et al*[17] and modified by Vaezi *et al*[18].

Table 1 Test and methods used in this study

Test title	Kit	Manufactured country
Anti nuclear anti body (ANA)	Biostems	Spain
Antineutrophil cytoplasm antibody (c-ANCA)	Binding site	England
Calcium	Man	Iran
Phosphorus	Man	Iran
Alkaline phosphatase	Man	Iran
Thyroid stimulating hormone(TSH)	Isfahan pharmacology faculty	Iran
T3 Resin uptake	kavoshiar	Iran
T3, T4	Isfahan pharmacology faculty	Iran
Albumin	Man	Iran
Globulin	Man	Iran
Rapid plasmin reagin (RPR)	Anison	Iran
Urine analysis	Combi	Germany
Complete blood count	-	Germany

All tests except for urine analysis were performed on blood or serum samples.

Statistical analysis

For comparison between patients with achalasia and normal population, Student's *t*-test assuming equal variance was used. In addition, we analyzed the symptoms using the non-parametric Mann-Whitney test, because the data were not normally distributed.

RESULTS

Of the 42 newly suspected achalasia patients referred to our institution, 36 (11 men and 25 women) fulfilled the participating criteria in the study, but only 30 (20 females, 10 males) finished the study. The remaining six patients were statistically similar by age and gender to the 30 cases and they had no unusual reason for their loss of follow-up.

Thyroid disease

Of the 30 patients, 7 had thyroid disease (23.7%), 4 had hypothyroidism (13.3%), 2 had hyperthyroidism (6.7%) and one (3.3%) had only a thyroid nodule in euthyroid state. Two of these hypothyroidism patients had no related clinical symptoms (subclinical) and two had clinical manifestations of hypothyroidism. Both patients with subclinical hypothyroidism were diagnosed in our

Table 2 Average score of all patients and those suffering from thyroid disease respectively (mean ± SD)

	Patients with hypothyroidism	Patients with benign thyroid diseases[1]	Patients without thyroid problems	Mean score of all patients
Age	39.5 ± 14.3[a]	35.8[b] ± 11.9[b]	38.5 ± 18.1	37.9 ± 16.7
Dysphagia	5[c]	4.5 ± 1.1[d]	4.8 ± 0.45	4.8 ± 0.66
Regurgitation	5[e]	4.7 ± 0.75[f]	3.5 ± 1.9	3.8 ± 1.8
Nocturnal cough	2.5 ± 2.8[g]	2.5 ± 2.5[h]	2.5 ± 2.2	2.5 ± 2.2
Chest discomfort	3.75 ± 1.5[i]	2.2 ± 2.1[j]	1.86 ± 2.7	1.96 ± 2
Weight loss	0.5 ± 0.5[k]	0.4 ± 0.5[l]	0.65 ± 4.08	0.6 ± 0.49

Scoring system: 0 = none; 1 = once per month or less; 2 = three to four times a month; 3 = two to four times per week; 4 = once per day; 5 = several times per day, [1]Composed of thyroid nodules, hypothyroidism and hyperthyroidism. No significant differences between groups were found by independent *t*-test, Mann-Witney test and chi-square test. *P* values of differences between thyroid disease groups and other patients with achalasia: [a]: 0.7; [b]: 0.7; [c]: 0.5; [d]: 0.6; [e]: 0.09; [f]: 0.1; [g]: 1; [h]: 0.97; [i]: 0.08; [j]: 0.57; [k]: 0.6; [l]: 0.3. (the first and second columns were compared with the third column).

Table 3 Overall and specific ethnicity and gender of all patients and those suffering from thyroid disease respectively

	Hypothyroid patients		Benign thyroid diseases[1]		Without thyroid problems		Total	
	n	%	*n*	%	*n*	%	*n*	%
Number	4	13.3	7	23.7	23	76.6	30	100
Gender	♂1 ♀3	♂25 ♀75	♂2 ♀5	♂28 ♀72	♂8 ♀15	♂34 ♀66	♂10 ♀20	♂33 ♀67
Ethnicity[2]	F: 3	F: 75	F: 5	F: 71	F: 16	F: 69	F: 21	F: 70
	B: 1	B: 25	B: 2	B: 28	B: 5	B: 21	B: 7	B: 23

[1]Composed of thyroid nodules, hypothyroidism and hyperthyroidism. [2]Composed of the most common ethnicity in patients, F: Fars; B: Bakhtiari.

screening. One patient experienced hypothyroid shock after balloon dilation therapy (cool skin, hypothermia, hypotension and bradicardia not responsive to volume expansion and intravenous atropine injection). She had clinical symptoms of hypothyroidism which was not detected before balloon dilation therapy. The results of laboratory tests are listed in Table 2.

Patients' ethnicity

There were 21 (70%) Fars (the major Persian ethnicity), 7 Bakhtiari, an Iranian tribe, living in west of Isfahan Province) (23%), one Gilaki (another group of Iranians originating from northern Iran) (3.3%), and one patient was Turkish inhabitant in the central area of Iran (3.3. 0%).

Age and symptoms

The median age of onset of symptoms was 37 years (range 15-80 years). The mean age of patients with thyroid disease and other patients with achalasia was 35.8 and 38.5 years (*P*: 0.9). All patients complained of dysphagia to both liquids and solids. Regurgitation, weight loss, chest discomfort and heartburn were reported in 28 (93.3%), 18 (60%), 15 (50%) and 8 (26.6%) patients, respectively.

The symptoms of patients were compared (Table 2). Correlations between the intensity of thyroid disease and the severity of achalasia symptoms were statistically insignificant by Mann-Whitney test.

Prevalence and incidence of achalasia

In 2002 -2005, 42 cases of suspected achalasia were identified in or referred to our center. Of these, 19 were residents of Isfahan during the period of the study. The population of Isfahan was about 4 million in 2006. The overall ethnic- and gender-specific incidences of achalasia in our cases are shown in Table 3. No strong history of rheumatic disease or neuropathy in patients and their family was detected in our study.

Serological study

All serum samples were evaluated with sensitive tests for autoimmune diseases but we did not find any positive test except for 2 positive anti nuclear antibody tests (6.6%). Evaluation for vasculitis, some electrolyte disturbances, and anemia did not show any important finding (Table 4).

DISCUSSION

Although achalasia is a historically recognized clinical entity, its fundamental pathophysiology remains poorly understood. Primary achalasia accounts for the majority of cases. The etiology of primary achalasia is not entirely clear and probably multidimensional[19] including genetic predisposition (supported by the concordance for the disease in monozygotic twins[20] and associations with some HLA loci[7]), inflammation (myopathy of the smooth muscle cells[21], neuropathy[22,23] and inflammatory changes in esophageal specimens[24]), infections (virus[14], Chagas' disease, poliomyelitis[25,26], varicella zoster virus[27] and Helicobacter[28]), ischemia, toxicity and autoimmune disease[19]. Some other diseases postulate these etiologies such as concomitant appearance of achalasia and Guillain-Barré syndrome [29], Parkinson's disease[30], triple A syndrome[31], *etc.*

Table 4 Final result of laboratory evaluation in all patients

Test	Normal (%)	Abnormal (%)
Thyroid function tests	80	20
Calcium and phosphorus	100	-
Alkaline phosphatase	100	-
Albumin	100	-
Globulin	100	-
ANA	93.4	6.6
C_ANCA	100	-
RPR (VDRL)	100	-
Complete Blood Count	100	-
Urine analysis	100	-

All tests except for urine analysis were performed on blood or serum samples.

There are some reports about thyroid and esophageal problems in animals[32] but we have not found any reports about such problems in humans in English articles. Although it has been reported that there is a correlation between achalasia and autoimmune diseases, this is the first cross sectional study evaluating the relative prevalence of these problems (rheumatic disease, thyroid disease, inflammatory disease, *etc*) in a consecutive series of patients affected by achalasia which led us to a few interesting points. First, the results of serological tests obtained from this study confirmed some literature data that predict a potential autoimmune etiology of achalasia. Second, an association was found between thyroid problems and achalasia. Third, the frequency of thyroid disease was higher in patients with achalasia than in normal population.

It was reported that the prevalence of hypothyroidism is 4.6% (0.3% clinical and 4.3% subclinical) in the United States of America[33]. Whickham showed that the prevalence of spontaneous hypothyroidism in community is 1.5% in females and less than 0.1% in males [34]. These prevalence rates are similar to those reported in Finland[35], Japan[36], and in another US survey[37-39]. Heydarian and Azizi reported that the prevalence of hyperthyroidism, overt and subclinical hypothyroidism is 0.45%, 0.35% and 2.2% in Iran, respectively[40]. The prevalence of hypothyroidism and benign thyroid diseases (hypothyroidism, hyperthyroidism and thyroid nodules) in our patients was significantly higher than that reported in latter study (*P* value and confidence interval for each group were 0.025 and 0.000, 1.3-43.4 and 2.9-76.5 respectively).

The mean age of our patients is one -two decades lower than reported in other studies[3,4]. It may have a role in these findings. We found about a 5-fold rise in the prevalence of thyroid dysfunction in this study. Since our center is the only center offering balloon dilation therapy in Isfahan, we can assume that the overall population with achalasia in this area is very similar to that registered in our center and therefore these data are repeatable in our future study, but they may not be generalized in all cases of achalasia in other societies. Hence we suggest a similar study in all centers working in this subject. We should direct our future studies to find the possible genetic and environmental risk factors for earlier disease in this geographic area.

The reason why there is such a relatively strong association between these diseases can be explained by the literature describing most thyroid problems as probable autoimmune diseases[41-44]. Autoimmune etiology of achalasia is also supported by the presence of circulating autoantibodies against the myenteric plexus and inflammatory T-cell infiltrates in the myenteric plexus, as well as the increased prevalence of HLA class Ⅱ antigens. Possibly the initiating event may be an unknown environmental insult due to a viral infection resulting in inflammation of the myenteric plexus in susceptible patients. Not all affected individuals develop achalasia[45]. However, autommunity at least has a concomitant or susceptible role in pathogenesis.

Is there a symptomatic mimicry between achalasia and thyroid diseases? There is some evidence in animal model that a mega esophagus may occur in hypothyroidism which is a criterion of achalasia[32]. We had one clinical hypothyroid patient and one clinical hyperthyroid patient detected before balloon dilation therapy. We treated these two patients with levothyroxine and methimazole respectively for 6 mo after achievement of euthyroid state. No clinical or radiological improvements were obtained in symptomatology and radiological findings of achalasia after treatment, suggesting that what we found is actually a true association rather than a symptomatic mimicry or a transient neurological dysfunction. We also found a 6% positive ANA in this series which is similar to that in general population[46]. These findings may give some stronger evidence for certain autoimmune bases of achalasia. However, more and larger groups are necessary and a larger spectrum of autoimmune markers must be tested to make it possible to establish the utility of the issue in the early diagnosis of achalasia and identification of different etiologic mechanisms, which would allow us to think about novel outline of management since the existing treatments are only partially palliative.

We also found a hypothyroid shock after balloon dilation therapy which was fortunately well managed because we were alert to the problem. Therefore we recommend clinical evaluation of thyroid disease for all patients and thyroid function test for suspicious cases to control thyroid function before any invasive therapy. Clinical severity was statistically similar in both groups of patients with normal and abnormal thyroid function tests. Therefore we cannot predict the possibility of thyroid disease on the basis of severity indexes.

In conclusion, achalasia may be associated with thyroid diseases (hypothyroidism, hyperthyroidism and thyroid nodules), and this association should be kept in mind when a new case of achalasia is diagnosed. More accurate and extensive immunopathological studies should be performed to assess the possibility of a common immunopathogenesis or a cause and effect relationship in these diseases.

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RAPID COMMUNICATION

Analysis of immune responses against *H pylori* in rabbits

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Abstract

AIM: To investigate the immunogenicity of *H pylori* proteins, to evaluate the production rate of anti *H pylori* IgG antibodies in relation to time and to demonstrate the fidelity of newly optimized in-house enzyme-linked immunosorbent assay (ELISA) technique as an alternative for *H pylori* infection assay.

METHODS: In the present study, 100 μg of formalin-fixed *H pylori* whole cell antigens was injected into an experimental animal (New Zealand white female rabbit) intramuscularly on d 0, 16, 27 and 36. The first two doses were injected with adjuvants. On d 0, a serum sample was collected from the rabbit before immunization and this pre-immunized serum was used as a negative control for the whole study. To evaluate the immunogenic responses of the injected antigen, serum samples were collected from the rabbit at regular intervals up to d 42. The sera were analyzed using in-house ELISA and Western blot techniques.

RESULTS: The production of anti *H pylori* IgG antibodies in the rabbit in response to the injected antigen increased almost exponentially up to d 14 and after that it was maintained at the same level until the last day (d 42). By analyzing the immune profiles of immunized sera, 11 proteins were identified to be immunogenic, among them 2 (approximately 100 kDa and 85 kDa) were most prominent.

CONCLUSION: Analysis of the immune responses against pathogenic microorganisms like *H pylori* is necessary for the development of various diagnostic and preventive approaches. The results of this experiment reveal that the formalin-fixed *H pylori* whole cell antigens injected into the rabbit are highly immunogenic. These prominent proteins (approximately 100 kDa and 85 kDa) might have higher immunogenic effects among humans infected with *H pylori* and some of these immunogenic proteins can be included in diagnostic approaches based on serology and also for vaccine formulation. The in-house ELISA is a promising alternative compared to invasive techniques.

Key words: *H pylori*; Whole cell antigen; Immunogenicity; Rabbit; Serum antibody kinetics; In-house enzyme-linked immunosorbent assay

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INTRODUCTION

In the early 1980's, Barry Marshall and Robin Warren of Australia discovered the bacterium *H pylori* in the stomach lining of patients with chronic gastritis and peptic ulcers[1]. The discovery of the infective organism *H pylori* and its involvement in these diseases has changed our views on how to diagnose and treat these diseases. Strains carrying the genes encoding the cytotoxin-associated protein (Cag-A) cause chronic active gastritis[2]. Gastric infection with *H pylori* is one of the common chronic infections in humans, causing substantial morbidity and some mortality[3]. Before an active protective response occurs, the gut must first be exposed to *H pylori*, which is a slowly growing microaerophilic, highly motile, Gram-negative spiral organism whose most striking biochemical characteristic is the abundant production of urease[4]. Colonization of *H pylori* in the gastric epithelium leads to a chronic inflammatory reaction[5-7]. Such a reaction may involve specific IgG and/or IgA antibody responses against the bacterium both in the peripheral blood and in the gastric mucosa. However, despite the production of such antibodies, the microorganism usually persists and gastritis progresses chronically through unknown mechanisms[8].

H pylori infection and peptic ulcer disease are more common in developing countries than in developed countries. Until the mid 1980s, it was felt that one or more of these factors working together could lead to the development of gastritis and ulcers. Since then, evidence has been mounting that *H pylori* has a major role in causing these diseases. Today the standard triple antibiotic

therapy is amoxicillin, clarithromycin and proton pump inhibitors such as omeprazole. Unfortunately, an increasing number of infected individuals are found to harbour bacteria resistant to first-line antibiotics. This results in initial treatment failure and requires additional rounds of antibiotic therapy[9]. One of the promising recent developments in medicine is the concept that chronic afflictions, such as peptic ulcer disease and cancer, can be controlled through immunization like classic infectious diseases. One approach has been the oral administration of purified recombinant subunit proteins of *H pylori* and a mucosal adjuvant, the labile toxin (LT) of Escherichia coli[10,11]. As a single-component vaccine, urease protein has shown some prophylactic and therapeutic activity in animal models and partial therapeutic activity in humans[12]. Another research was directed at the comparison of adjuvants and vaccine delivery systems and toward the immunologic mechanisms mediating protection[13].

Serological methods for detection of *H pylori* infection have reached sufficient accuracy and can be used as screening tests before endoscopy or for seroepidemiological surveys[4]. A number of different serological techniques have been used to detect antibodies, including haemagglutination, complement fixation, coagglutination, indirect immunofluorescence and latex agglutination[14]. Antibodies developed in rabbits against *H pylori* antigen can easily be detected by slide agglutination test. However, immunoblotting and enzyme-linked immunosorbent assay (ELISA) have emerged as the most frequently used techniques. A combination of immunoblotting and ELISA is the most efficient means of detecting serum antibodies to *H pylori* antigens and can be applied to the screening of rabbit sera for *H pylori*-specific antibodies[15]. These two techniques can be used in analysis of immune responses against *H pylori* in rabbits.

It is difficult to eradicate *H pylori* by antibiotic therapy and to date no vaccine is available for use in humans[16]. An effective vaccine would be a desirable way to control *H pylori*-induced gastric disease. Initial studies in animal models have demonstrated the feasibility of immunization, thus leading to high hopes for a human vaccine. In the mouse model, immunological approaches have to date not brought a satisfactory explanation for the mechanisms of protection against this largely luminal pathogen. In the present study, we used a rabbit model with whole cell extract from *H pylori* as antigen to analyze the immune responses.

MATERIALS AND METHODS

Animals

In this study, two healthy New Zealand white female rabbits aged 2 mo (weighing 2 kg) were used for serum antibody response. Although other strains of rabbits (e.g., Californian, Giant blank, Beveren etc) were available, this strain could easily adapt to the tropical area like Bangladesh (studied area). A great care was taken during the study with proper feeding and supplying adequate amount of fresh water daily. Rabbit house was cleaned daily. During the study, the climate was fine. Hygiene condition was maintained properly.

Preparation of whole cell antigen

Bacterial cells were grown and harvested from agar plates. Bacterial cells were suspended in phosphate buffered saline (PBS) containing 1% (w/v) formalin and kept at 4℃ for 1 h. The cells were then centrifuged at 12 500 × *g* for 5 min and the pellet was resuspended in 1 mL of PBS. The cells were washed 4 times in PBS to remove the formalin. Finally, a suspension of 1 mg/mL cells in PBS was made.

Preparation of antigen adjuvant mixture

H pylori antigens were administered in combination with incomplete Freund's adjuvant (which does not contain killed mycobacteria) to enhance the response to the first two doses. Freund's adjuvant is a water-in-oil emulsion consisting primarily of mineral oil. The oil acts as a repository, which releases the immunogen. The mixture was prepared by taking 250 μL of *H pylori* whole cell antigen (formalin fixed, 1 mg/mL, stored at -20℃) with 250 μL of incomplete Freund's adjuvant and then 200 μL of antigen-adjuvant mixture was injected intramuscularly.

Immunization of rabbits

Using a 22G needle, rabbits were immunized intramuscularly with whole cell antigen adjuvant mixture on d 0 and 16. Subsequent doses without adjuvant were administered on d 27 and 36. However, before immunization 1.5 mL blood was collected from the marginal vein of the ear for collecting pre-immunization sera. This was used as a negative control.

Blood collection

After immunization, rabbit blood were collected from marginal ear vein on d 0, 7, 17, 21, 27 and 35. Blood was also collected by cardiac puncture on d 42. Serum was separated from these blood samples and analyzed for antibody response by in-house ELISA and immunoblot techniques.

In-house ELISA

An aliquot of formalin-fixed bacteria was diluted in coating buffer to a final concentration of 1 μg/100 μL. One hundred μL of antigen preparation was added to all the wells except wells A1 and B1, which were used to calibrate the ELISA reader. The plates were covered with plate sealer and incubated at 4℃ overnight. On the following day, the plates were washed 3 times with PBS containing 0.05% Tween-20 (PBS-Tween 20). The wells were blocked with 200 μL of 1% (w/v) bovine serum albumin (BSA) in PBS, and then plates were incubated at 37℃ for 30 min. The PBS-BSA was discarded and the plates were washed 3 times with PBS-Tween 20. Then, 100 μL of diluted serum samples (neat, 1:50, 1:100, 1:200, 1:400 and 1:800 dilution in PBS) collected on d 7, 14, 21, 27, 35 and 42 was added into each of two consecutive wells, i.e. duplicate wells were used for each sample and each dilution. To the wells A1 and B1, 100 μL of PBS was added. The plate was covered with the plate sealer and incubated at room temperature for 2 h. The plate was

then washed three times as described above and 100 μL of diluted secondary antibody conjugate (1:3000 in PBS) was added (goat anti-rabbit polyvalent antibody conjugated with alkaline phosphatase; A-3937, Sigma Chemical Co, Ltd. UK), and incubated at room temperature for 2 h. The plate was again washed three times with PBS Tween-20 and 200 μL of 1 mg/mL substrate (p-nitrophenyl phosphate in diethanolamine buffer) was added to each well. The plate was placed in dark and an optical density (A_{405}) was measured after exactly 25 min. In this study, a sample was considered positive for antibodies to *H pylori* if the absorbance of the reaction was ⩾ 1. Any value < 1 was considered seronegative[17]. For the validation of the experiment, a positive serum sample from humans was tested by in-house ELISA using similar dilution (Neat, 1:50, 1:100, 1:200, 1:400, and 1:800). For ELISA, all the tests were done in duplicate well to minimize the handling error. The average of the two values from duplicate well was used for further data analysis.

Immunoblot

The 7 serum samples collected from rabbits on d 7, 14, 21, 35 and 42 were examined by immunoblot assay to check the immunological response of the pre-immunized polyclonal rabbit serum to the antigens from whole cell extract of *H pylori*.

Preparation of whole cell extracts for SDS-PAGE

After confluent growth, bacterial cells were harvested and taken into preweighed, screw-capped Eppendorf tubes. Bacteria were sedimented by centrifugation at 12500 × *g* for 2 min and suspended in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) solubilization buffer[18] to a cell concentration of 500 μg cells per 5 μL. The suspension was incubated at 100℃ for 5 min to denature bacterial proteins and DNA was disrupted by brief sonication (30 s). The suspension was reheated at 100℃ for 2 min and then diluted in SDS-PAGE solubilization buffer to produce a final concentration of 70 μg whole cell extract per 5 μL. The aliquots were stored at -20℃ for SDS-PAGE.

SDS-PAGE

The SDS-PAGE profile of whole cell extract was prepared as described previously[19]. Gels comprised a 4.5% (w/w) acrylamide stacking gel and a 12.5% (w/w) acrylamide separation gel. Samples were applied to gels alongside protein molecular weight standards (1610305; Bio-Rad, UK). Electrophoresis was performed using a minigel system (Consort, UK) with a constant current of 40 mA for 40 min. Gels were either stained with Coomassie blue[19] or used for immunoblotting.

Immunoblotting

H pylori serostatus in all serum samples was determined by immunoblot technique as described previously[20]. The SDS-PAGE protein profiles were transferred onto nitrocellulose sheets[21] using a semidry electrotransfer apparatus (Bio-Rad). Individual protein profiles were prepared by cutting nitrocellulose sheets into strips. Strips were incubated separately with different rabbit serum (1:200 dilution in 3% skimmed milk) samples (primary antibody). Antibody-antigen complexes were detected with a goat anti-rabbit polyvalent antibody conjugated with alkaline phosphatase, diluted 1:5000 in 3% skimmed milk. Color development was carried out in a polythene bag at 37℃ in the dark for 10 min. Individual serum antibodies binding to five or more protein bands were considered positive results and those binding to less than five protein bands were considered negative results[20].

Table 1 Serum antibody response in rabbit to *H pylori* whole cell antigen detected by in-house ELISA

		Dilution of sera				
Serum collection	Neat	1:50	1:100	1:200	1:400	1:800
d 0	0.998	0.847	0.620	0.063	0.024	Not done
d 7	2.777	2.562	2.029	1.835	1.245	1.028
d 14	2.003	3.136	2.792	2.241	0.408	1.404
d 21	3.004	3.267	2.857	3.303	2.973	1.974
d 27	2.805	2.894	2.021	2.074	2.323	1.004
d 35	2.62	2.958	3.128	2.947	3.112	2.857
d 42	3.002	2.945	2.763	2.719	2.999	3.303

RESULTS

The serum collected from two rabbits for six weeks was examined for antibodies specific for antigen using in-house ELISA. Absorbance values ⩾ 1 at 405 nm were considered seropositive (Table 1). The collected immunized sera from rabbits were evaluated and the result supported that the experimental animal (rabbit) was effectively immunized with *H pylori* whole cell antigen. For the validation of the experiment, a positive serum sample from humans was tested by using similar dilution (Neat, 1:50, 1:100, 1:200, 1:400, 1:800). The ELISA values (OD_{405}) from all dilutions showed presence of adequate amount of anti *H pylori* antibody. There was also a negative control serum collected from the rabbit on d 0 (before immunization with formalin-fixed *H pylori* whole cell antigen). The ELISA values from different dilutions showed that the negative control serum had no antibodies against *H pylori* antigen. Using ELISA values for 1:50 dilution on different days (0, 7, 14, 21, 27, 35, and 42) a plot of antibody titer versus time was drawn (Figure 1). Similar results were obtained from other dilutions (neat, 1:50, 1:100, 1:200, 1:400 and 1:800) (data not shown). The graph showed a significant increase in anti *H pylori* antibody production with time. After immunization, the production of anti *H pylori* antibody increased almost exponentially up to d 14 and after that, it maintained at the same level until the last day.

The serum samples collected from the rabbit were examined by immunoblot assay. A variable number of protein bands were observed among the immune profiles (Figure 2). Serum sample collected on d 0 represented pre-immunized serum and no visible protein band was observed, indicating absence of anti *H pylori* antibody. Several protein bands in other samples (collected on d 7, 14, 21, 27, 35 and 42) suggested that tested sera

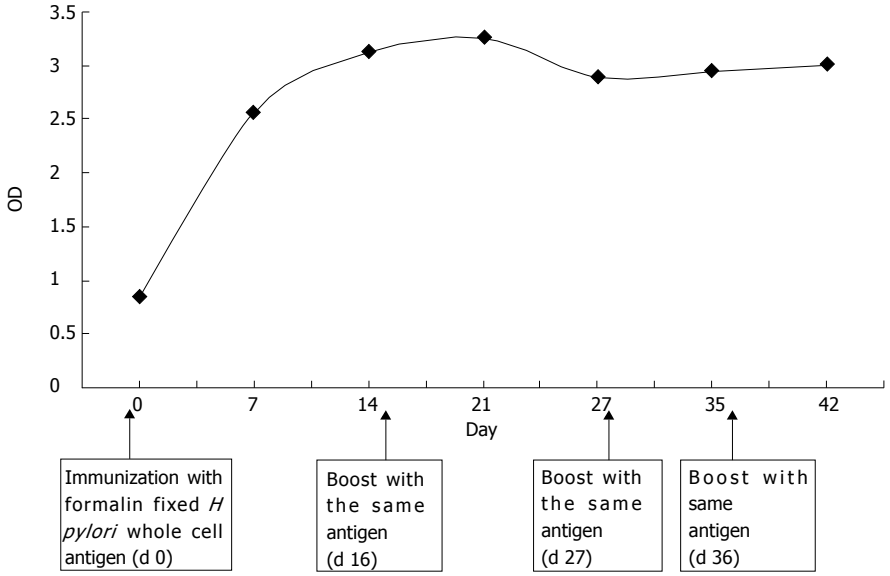

Figure 1 Kinetics of anti *H pylori* antibody responses in rabbits. Female rabbits were infected with formalin-fixed *H pylori* whole cell antigens and blood samples were collected at various time points after *H pylori* infection. Sera (1:50 dilution) were subjected to ELISA for antibody titer measurement (in terms of OD). The arrows indicate the time points when rabbits were challenged with antigens (booster dose).

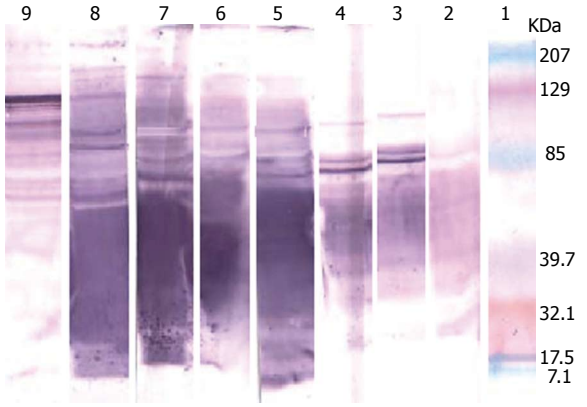

Figure 2 Western blot with whole cell extracts of *H pylori* against previously immunized rabbit sera and a human serum. Lane 1: molecular weight marker; lanes 2 to 8: serum collected on d 0, 7, 14, 21, 28, 35, and 42 respectively; lane 9: positive human serum.

had antibodies against *H pylori*. Immunoreactivity of the protein bands increased gradually from d 7 to d 42, suggesting that the production of antibody against *H pylori* increased with time in the rabbit. A positive serum sample from humans was also tested and found to have antibodies against that antigen.

The presence or absence of anti *H pylori* antibodies in pre-immunized and immunized rabbits was examined by in-house ELISA and immunoblotting. The findings from the two immunoassays showed a significant correlation ($P = 0.001$) (Table 2).

Table 2 Serum antibody responses of subjects to *H pylori* antigens detected by immunoblotting and in-house ELISA

Collection of serum samples	Sera with antibodies by immunoblotting[1]	Sera with antibodies by ELISA[2]
d 0	-	-
d 7	+	+
d 14	+	+
d 21	+	+
d 27	+	+
d 35	+	+
d 42	+	+

[1]In immunoblotting more than 4 protein bands indicated positive results; [2]When ELISA values were $\geqslant 1.0$, OD_{405} was considered positive results.

DISCUSSION

Current antibiotic regimens against *H pylori* infection may be effective, but complex dosing and development of resistance are always concerns. Animal studies and limited clinical trials of *H pylori* antigens have been conducted, with no final conclusive findings. A number of data now exist, supporting the potential for protection against *H pylori*. However, we are still at a preliminary stage in clinical development. The best immunogens, the best mode of presentation, the number of doses needed, optimal age at immunization, expected benefit, cost-effectiveness, and other factors involved in vaccine development require further study[13]. The present study employed rabbits to clarify the immunogenicity of *H pylori* proteins and to evaluate the production rate of anti *H pylori* IgG antibodies in relation to time.

For serological diagnosis of *H pylori* infection, immune responses against the relevant microorganism can be analyzed in experimental animal models. When endoscopy is not performed, the most commonly used diagnostic approach is the laboratory-based serological test. Enzyme-linked immunosorbent assay (ELISA) detects various classes of antibodies to *H pylori*, indicating current or past infection. Because *H pylori* infection is not known to spontaneously resolve, a positive serologic test suggests active infection in patients who have not undergone eradication therapy[22]. Although *H pylori* is not an invasive bacterium, it actively stimulates the immune system in its host by releasing lipopolysaccharides and immunogenic proteins. An immune response accompanies the presence of the bacterium in 98% of the cases[23]. The sensitivity and specificity of ELISA to identify infectious microorganisms are quite high. In the present study, since the antibody

response occurred a few days after a new infection or after a booster dose of antigen with or without adjuvant, the subject had to mount a strong immune response

IgG usually appears several days after *H pylori* infection in rabbits. But after eradication of *H pylori*, the drop in antibody titer is not significant until the 6th mo in humans[24]. In the present study, specific IgG antibodies to *H pylori* were detected in rabbit sera with the help of enzyme immunoassay. Analysis of serum samples by in-house ELISA technique is an alternative for *H pylori* infection assay. The present study evaluated the noninvasive methods to screen for *H pylori* infection in rabbits. The relationship between time course and magnitude of antibody response showed the kinetics of the development of specific antibody responses in the studied animals (Figure 1). A clear kinetics of the development of anti *H pylori* antibody response found in the rabbit showed a significant increase in anti *H pylori* IgG antibody production with time. After immunization, the production of anti *H pylori* antibody increased almost exponentially up to d 14 and after that, it maintained at the same level till the last day of serum collection. The ELISA values from different dilutions showed that the negative control serum had no antibodies against *H pylori* antigen. However, 1:400 dilution of serum (collected at d 14) showed a negative ELISA value, which could be due to the handling error.

A large number of different proteins present in single cells and may play a major antigenic role in infection. The complex pathogenesis of this infection[25,26], including the presence of antigens on *H pylori* in the host[27], demands better approaches to the identification of novel immunogens that would give substantial protection. The selection of defined and well-characterized antigens appears to be the most viable approach. The present in-house ELISA with formalin-fixed whole cell antigens had a high diagnostic value for studied animals. Antibody was raised against whole cell antigens which were at first confirmed by the slide agglutination test. The collected sera subjected to immunoblot analysis showed a number of antibody bands. Some bands were more prominent than others in relation to color intensity. Previous studies revealed that *H pylori* whole cell lysate contains protein bands like CagA (140-121 kDa), VacA (87 kDa), heat-shock protein (60 kDa), two urease subunits of 62 and 26 kDa and thiol peroxidase (18 kDa)[28], suggesting that the concentration of some proteins from the whole cells is high. These proteins might have immunogenic effects in the rabbit. By analyzing the immune profiles of immunized serum samples collected at regular intervals from the rabbit, 11 proteins were identified to be immunogenic, of which 2 were more prominent than others (approximately 100 kDa and 85 kDa). Haque *et al*[29] showed that approximately 61 kDa, 58 kDa and 24 kDa proteins from whole cell extract of *H pylori* are immunogenic. However, they could not prove it conclusively. The present study identified two such potential antigens which were proved most potent in eliciting protective immunity.

It is reasonable to assume that more than one protective component is needed in a vaccine[13]. Therefore the previously identified urease[8] or catalase[30] alone could not be used for final vaccine formulation. There is also concern about inducing an immune response to heat shock protein B (HspB) because this protein has homologies to the GroEL family of heat shock proteins[31]. In the present study, we demonstrated some potential immunogens which could give better protection and could be used for diagnosis purpose.

Adjuvant is an important component of any vaccine. It is responsible for stimulating immune system. In some previous trials[10,30], cholera toxin was used as adjuvant for immunization of mice. However this effort might raise multiple problems including safety. E. coli heat-labile toxin (LT) that was used as an oral adjuvant in humans does not show a significant decrease in gastric *H pylori* density but is associated with cramping and diarrhea[32]. Due to all these adverse effects, no suitable and safe adjuvants are currently available for use in humans[33]. However, in the present study an incomplete form of Freund's adjuvant not containing such a cytotoxic agent was used to enhance the immune response. Therefore, this adjuvant could be considered a substitute to cholera toxin or other toxic adjuvants.

The identified proteins might have higher immunogenic effects among humans infected with *H pylori* and some of these immunogenic proteins could be included in diagnostic approaches based on serology and also in vaccine preparation. However, further characterization of these antigens is required. Finally, in-house ELISA and immunoblot can be better applied in analysis of antigenic response in experimental animal model (rabbits).

COMMENTS

Background

Gastric infection with *H pylori* is one of the common chronic infections in humans, causing substantial morbidity and some mortality. Still there is still no effective vaccine against *H pylori,* a causative agent of gastric and peptic ulcer. We are still at a preliminary stage in clinical development. The best immunogens, the best mode of presentation, the number of doses needed, optimal age at immunization, expected benefit, cost-effectiveness, and other factors involved in vaccine development require further study. Initial studies in animal models have demonstrated the feasibility of immunization, thus leading to high hopes for a human vaccine. In the mouse model, immunological approaches have to date not brought a satisfactory explanation for the mechanisms of protection against this largely luminal pathogen. In the present study, we used a rabbit model with the whole cell extract antigens from *H pylori*.

Research frontiers

H pylori infection is a newly discovered stomach infection which was first reported by Barry Marshall and Robin Warren of Perth, Western Australia, in 1983, who were awarded the Nobel Prize in Medicine in 2005 for their work on *H pylori*. The Sydney gastroenterolgist Thomas Borody invented the first triple therapy in 1987. Such a therapy has revolutionized the treatment of gastric ulcer. Mode of infection, mechanism of pathogenesis, host immune response, chemotherapy and vaccine development are important areas of research.

Innovations and breakthroughs

Initial studies in animal models have demonstrated the feasibility of immunization, thus leading to high hopes for a human vaccine against *H pylori*. In the mouse model, immunological approaches have to date not brought a satisfactory explanation for the mechanisms of protection against this luminal pathogen. In the present study, we used a rabbit model with the whole cell extract from *H pylori* as antigen. The experiment identified two prominent proteins (approximately 100 kDa and 85 kDa) which have higher immunogenic effects among humans infected with

H pylori and some of these immunogenic proteins could be included in diagnostic approaches based on serology and also for vaccine formulation. Most of the previous studies used cholera toxin as adjuvants which have some side effects. In the present study, we used an incomplete form of Freund's adjuvant which does not contain cytotoxic agent. We established and optimized the low cost, non-invasive in-house ELISA technique for the detection of *H pylori* infection in Bangladeshi people.

Applications

Analysis of the immune responses against pathogenic microorganisms like *H pylori* is necessary for the development of various diagnostic and preventive approaches. The results of our experiment reveal that formalin-fixed *H pylori* whole cell antigens injected into the rabbit are highly immunogenic. These prominent proteins (approximately 100 kDa and 85 kDa) might have higher immunogenic effects among humans infected with *H pylori* and some of these immunogenic proteins could be included in diagnostic approaches based on serology. The in-house ELISA is a promising alternative in the developing countries compared to high cost invasive techniques.

Terminology

Freund's adjuvant is an antigen solution emulsified in mineral oil, and can be used as an immunopotentiator (booster of the immune system). The so-called complete form (FCA) is composed of inactivated and dried mycobacteria, usually Mycobacterium tuberculosis (the pathogenic agent of tuberculosis). The so-called incomplete form (FIA) is the same adjuvant without the mycobacterial components and is named after Jules T. Freund (1890-1960), a Hungarian-born American immunologist.

Peer review

It is an interesting study. The science seems good and the study is well performed. The results are also of interest.

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RAPID COMMUNICATION

Beneficial effects of *Foeniculum vulgare* on ethanol-induced acute gastric mucosal injury in rats

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Abstract

AIM: To examine the anti-ulcerogenic and antioxidant effects of aqueous extracts of *Foeniculum vulgare* (FVE) on ethanol-induced gastric lesions in rats.

METHODS: FVE was administered by gavage at doses of 75, 150 and 300 mg/kg, and famotidine was used at the dose of 20 mg/kg. Following a 60 min period, all the rats were given 1 mL of ethanol (80%) by gavage. One hour after the administration of ethanol, all groups were sacrificed, and the gastric ulcer index was calculated; whole blood malondialdehyde (MDA) and reduced glutathione (GSH), serum nitrate, nitrite, ascorbic acid, retinol and β-carotene levels were measured in all the groups.

RESULTS: It was found that pretreatment with FVE significantly reduced ethanol-induced gastric damage. This effect of FVE was highest and statistically significant in 300 mg/kg group compared with the control (4.18 ± 2.81 *vs* 13.15 ± 4.08, $P < 0.001$). Also, pretreatment with FVE significantly reduced the MDA levels, while significantly increased GSH, nitrite, nitrate, ascorbic acid, retinol and β-carotene levels.

CONCLUSION: FVE has clearly a protective effect against ethanol-induced gastric mucosal lesion, and this effect, at least in part, depends upon the reduction in lipid peroxidation and augmentation in the antioxidant activity.

Key words: *Foeniculum vulgare*; Ethanol; Rat; Ulcer; Antioxidant

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INTRODUCTION

Peptic ulcer is a common disorder of the gastrointestinal system and millions of people suffer from this disease in the world. The medical cost of treating peptic ulcer and its complications amounts to billions of dollars annually. The pathogenesis of peptic ulcer disease is multifactorial, including chronically using non-steroid anti- inflammatory drugs, cigarette smoking, alcohol, and reactive oxygen species (ROS). ROS are generated by cells in some physiological and pathological circumstances. Any derangement between pro-oxidants and antioxidants, in which pro-oxidants prevail is known as oxidative stress[1]. Insufficient antioxidant protection or excess production of ROS can result in this condition. ROS can react with all macromolecules, such as lipids, proteins, nucleic acids, and carbohydrates, particularly polyunsaturated fatty acids on cell membranes. After the beginning of an initial reaction with ROS, a continuing chain reaction is started and cell injury and, ultimately, cell death occur[2]. Peptic ulcer is produced by the imbalance between gastroduodenal mucosal defense mechanisms and offensive factors. Some studies have revealed that ROS and lipid peroxidation are implicated in the pathogenesis of ethanol-induced gastric lesions and gastrointestinal damage, and they attack and damage many biological molecules such as prostaglandins[3-5]. Therefore, treatment with antioxidants and free radical scavengers can decrease ethanol-induced gastric mucosal damage.

Foeniculum vulgare (FVE) is a well-known umbelliferous plant. For centuries, FVE fruits have been used as traditional herbal medicine in Europe and China. It is native to southern Europe and the Mediterranean area. The seeds of this plant have been known to be able to regulate menstruation, alleviate the symptoms of female climacteric syndrome, and increase libido[6]. FVE also

possesses emnenagague and galactagogue properties[7]. It has been reported that FVE could be used in the pediatric colic and some respiratory disorders due to its anti-spasmodic effects[8,9]. Seeds of it are used in folk remedies for treatment of dysmenorrhea. FVE (in Turkish "Rezene") is natively found in North and West regions of Turkey. It is cultivated for the herb as a spice (flavouring salads) and medicine in Turkey. Powders or tablets (0.5-1 g) of seeds, or its infusion forms (2%) are taken 2-3 times per day. As a medicinal plant, FVE has been used as an antispasmodic, carminative, diuretic, lactation stimulant, and as dressings for wounds in Turkish traditional medicine[10]. It contains 1%-3% of a volatile oil, which is composed of 50%-85% of anethole and about 20% of d-fenchone[11,12]. Other compunds present in FVE are d-a-pinene, d-a-phellandrene, dipentene, methyl chavicol, feniculun, anisaldehyde, and anisic acid[11,13].

The aim of this work was to assess the gastroprotective activity of FVE in rat models of experimentally ethanol-induced gastric lesions. In particular, we investigated the effects of aqueous extracts of FVE on gross mucosal lesions in the stomach, glutathione (GSH), nitrite, nitrate, ascorbic acid, retinol and β-carotene levels, and changes in lipid peroxidation determined by measuring malondialdehyde (MDA) levels in the blood.

MATERIALS AND METHODS

Plant material

The aerial parts of FVE were collected in June 2003 from Bursa. The plant was identified by the Department of Botany of Science and Arts Faculty, Atatürk University, Erzurum, Turkey, where a voucher specimen is kept.

Extraction and preparation of test samples

Air-dried FVE was pulverized with a blender. Obtained plant material (230 g) was mixed with boiling distilled water and stirred on the hot plate for 15 min. Subsequently, it was filtered over Whatman No.1 paper. Finally, the filtrate was frozen and lyophilized in a lyophilizator (Labconco, Freezone 1L, USA) at a 5 μmHg pressure and -50℃ (14.9 g).

Animals

Thirty-five Sprague-Dawley rats with a weight range of 190-225 g were used for the experimentation. The rats were fed with standard laboratory chow and water before the experiment. Rats were divided into 5 equal groups (n = 7) and housed in cages. Twenty-four hours before the experiment, the rats were fasted and allowed access to water *ad libitum*. The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and approval has been received from our institutional Animal Ethics Committee.

Chemicals

Chemicals used in this investigation, GSH, thiobarbituric acid, phosphate buffer, butylated hydroxytoluene, trichloroacetic acid, EDTA, [5,5-dithiobis-(2-nitrobenzoic acid)], phenylendiamine, sodium azide, 2,4-dinitrophe nylhydrazine, ethanol, hexane, sodium nitrite, sodium nitrate, sulfanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride and vanadium (Ⅲ) chloride were purchased from Sigma. All the other chemicals and reagents used in this study were of analytical grade.

Ulcer study

The anti-ulcerogenic effect of FVE was investigated with the ethanol-induced ulcer model. On the first day of the experiment, groups 1, 2 and 3 were administered with 75, 150 and 300 mg/kg FVE, group 4 was administered with 20 mg/kg famotidine, and group 5 was administered with saline solution. All of drugs were administered by gavage at the same volume (0.5 mL). Following a 60 min period, all the rats were given 1 mL of ethanol (80%) by gavage. One hour after the administration of ethanol, rats were injected with a high dose of ketamine (100 mg/kg), blood samples were taken by cardiac punctures, and stomachs were removed and opened along the greater curvature and washed in physiological saline solution. For measurement of the gross gastric mucosal lesions, freshly excised stomachs were laid flat and the mucosal lesions were traced on clear acetate paper. Gross mucosal lesions were recognised as hemorrhage or linear breaks (erosions) with damage to the mucosal surface. The area of stomach tissue and gross lesions were approximately calculated by planimetry using a simple magnifier. The results were translated to the term of "total ulcer area/total gastric area" and these were expressed as an ulcer index (%).

Biochemical analysis

Fasting blood samples were drawn into heparin-free tubes during routine blood sampling for biochemical analysis. After immediate centrifugation (1000 g for 10 min at 4℃), the serum was stored in polystyrene plastic tubes at -70℃ until analysis. Whole blood was collected into heparinized tubes and whole blood MDA and GSH levels were studied on the same day of admission.

Whole blood MDA (as an important indicator of lipid peroxidation) levels were measured according to a method of Jain *et al*[14]. The principle of the method was based on the spectrophotometric measurement of the color developed during the reaction of thiobarbituric acid with MDA. Concentrations of thiobarbituric acid reactive substances (TBARS) were calculated by the absorbance coefficient of malondialdehyde–thiobarbituric acid complex and expressed as nmol/mL. Whole blood GSH concentrations were also measured by the spectrophotometric method[15]. The concentrations of nitric oxide (nitrate and nitrite) were detected by the methods of Miranda *et al*[16]. Nitrite and nitrate calibration standards were prepared by diluting sodium nitrite and sodium nitrate in pure water. After loading the plate with samples (100 μL), addition of vanadium (Ⅲ) chloride (100 μL) to each well was rapidly followed by addition of the Griess reagents, sulfanilamide (50 μL) and N-(1-Naphthyl) ethylenediamine dihydrochloride (50 μL). The Griess solutions may also be premixed immediately prior to application to the plate. Nitrite mixed with Griess

Table 1 Effects of aqueous extracts of *Foe* FVE and famotidine on ethanol-induced gastric mucosal injury in rats

Groups (n = 7)	Ulcer index (%) (mean ± SD)	Inhibition (%)
Control (ethanol)	13.15 ± 4.08	-
75 mg/kg FVE + Ethanol	8.18 ± 2.66	37.8
150 mg/kg FVE + Ethanol	9.48 ± 3.78	27.9
300 mg/kg FVE + Ethanol	4.18 ± 2.81[b]	68.2
20 mg/kg Famotidine + Ethanol	8.68 ± 2.63[a]	34

[a]$P < 0.05$, [b]$P < 0.001$, *vs* ethanol.

reagents forms a chromophore from the diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines, such as N-1-(naphthyl) ethylenediamine. Sample blank values were obtained by substituting diluting medium for Griess reagent. Nitrite was measured in a similar manner except that samples and nitrite standards were only exposed to Griess reagents. The absorbance at 540 nm was read to assess the total level of nitrite and nitrate in all samples[16]. Serum vitamin C (ascorbic acid) level was determined after derivatization with 2,4-dinitr ophenylhydrazine[17]. The levels of β-carotene at 425 nm and vitamin A (retinol) at 325 nm were detected after the reaction of serum: ethanol: hexane at the ratio of 1:1:3, respectively[18].

Statistical analysis

All values were expressed as mean ± SD. Statistical analyses of data were performed using a one-way analysis of variance (ANOVA) and Tukey's posttest. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Ulcer study

Ulcer indices (UI) are shown in Table 1. Per-oral administration of 80% ethanol produced multiple mucosal lesions in the rat stomach. Pre-treatment with FVE and famotidine were found to inhibit ethanol-induced gastric mucosal injury. This inhibitor effect of FVE was highest and statistically significant in the 300 mg/kg group and higher than that of famotidine group. In 75 mg/kg and 150 mg/kg of FVE groups, the inhibitor effects on ethanol-induced gastric mucosal injury were similar to famotidine group, which were not significant statistically. Famotidine also significantly inhibited ethanol-induced gastric lesions compared with the control.

Biochemical analysis

MDA levels of whole blood are shown in Table 2. The administration of ethanol increased the MDA level in whole blood. In contrast, pretreatment with FVE significantly decrased the MDA levels at doses of 150 and 300 mg/kg, compared with ethanol administered alone. Additionally, famotidine was found to prevent the rise in MDA level.

GSH level in whole blood was decreased in the ethanol-administered group. In contrast, GSH levels significantly increased at doses of 150 and 300 mg/kg FVE and in famotidine groups (Table 2). Nitrite and nitrate levels in serum were decreased in the ethanol administered group, while increased in the FVE groups. This increase was significant only in 75 and 150 mg/kg FVE groups, but not in 300 mg/kg FVE or famotidine groups (Table 2). All doses of FVE and famotidine increased the serum ascorbic acid levels, whereas only 300 mg/kg of FVE induced a significant increase (Table 3). On the other hand, serum β-carotene and retinol levels in FVE groups were higher than that of control, while the difference was significant only in the 150 mg/kg FVE group (Table 3).

Table 2 Effects of aqueous extracts of FVE and famotidine on whole blood MDA and GSH, and serum nitrite and nitrate levels (mean ± SD) in rats

Groups (n = 7)	MDA (nml/mL)	GSH (mg/dL)	Nitrite (mg/L)	Nitrate (mg/L)
Control (ethanol)	5.29 ± 0.6	44.46 ± 3.1	1.33 ± 0.7	4.55 ± 2.6
75 mg/kg FVE + Ethanol	4.51 ± 1.0	48.87 ± 2.5	2.56 ± 0.9[a]	8.81 ± 2.7[a]
150 mg/kg FVE +Ethanol	4.05 ± 0.3[a]	56.79 ± 3.3[d]	2.73 ± 0.5[a]	8.88 ± 1.9[a]
300 mg/kg FVE + Ethanol	4.12 ± 0.6[a]	51.34 ± 3.1[b]	1.64 ± 0.5	5.52 ± 1.7
20 mg/kg Famotidine + Ethanol	3.98 ± 0.7[a]	51.68 ± 3.2[b]	2.10 ± 0.8	6.82 ± 2.1

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$, *vs* ethanol.

Table 3 Effects of aqueous extract of FVE and famotidine on serum antioxidant vitamins levels (mean ± SD) in rats

Groups (n = 7)	Ascorbic Acid (mg/dL)	β-Carotene (μg/dL)	Retinol (μg/dL)
Control (ethanol)	0.81 ± 0.2	26.09 ± 1.5	57.42 ± 3.4
75 mg/kg FVE + Ethanol	0.85 ± 0.2	27.48 ± 1.9	58.79 ± 4.6
150 mg/kg FVE + Ethanol	0.95 ± 0.2	31.13 ± 2.1[b]	68.45 ± 4.9[b]
300 mg/kg FVE + Ethanol	0.98 ± 0.2[a]	28.76 ± 1.8	63.61 ± 4.5
20 mg/kg Famotidine + Ethanol	0.97 ± 0.3	26.55 ± 1.6	58.57 ± 3.6

[a]$P < 0.05$, [b]$P < 0.01$, *vs* ethanol.

DISCUSSION

For a long time, peptic ulcer has been one of the important causes of morbidities and mortalities. Several factors such as increased vascular permeability, gastric motility and vagal activity, decreased gastric blood flow and protective prostaglandin levels play an important role in gastric ulcer pathogenesis. The treatment of peptic ulcers is still a big challenge and development of new drugs is urgent. There are a number of medicinal plants that have been shown to be effective against ulcer diseases in traditional medicine[19]. Because of the folkloric uses, these medicinal plants may be a good source for the development of potential drugs. In recent years, many efforts have been done to explore new anti-ulcer drugs from natural resources, and antiulcer activity of a variety of chemical compounds isolated from medicinal plants have been determined[20,21].

Ethanol is a commonly used ulcerogenic agent and when given by gavage to rats, it produces severe gastric hemorrhagic lesions. The mechanism of ethanol-induced gastric lesions is varied, including the depletion of gastric mucus content, damaged mucosal blood flow and mucosal cell injury. In addition, ethanol-induced gastric mucosal damage is associated with overproduction of free radicals, which lead to an increased lipid peroxidation[22]. Increase in lipid peroxide content and oxygen-derived free radicals results in marked changes in cellular levels and causes membrane damage, cell death, exfoliation and epithelial erosion. Accumulation of activated neutrophils in the gastric mucosa may be a source of free radicals. Several studies revealed that some antioxidant drugs such as melatonin and dantrolene have protective effects against ethanol-induced acute gastric injury in rats[23-25]. Results of the present study showed that all doses of FVE prevented gastric tissue damage against ethanol-induced stress, only significantly in the highest dose group. Furthermore, FVE decreased the lipid peroxidation and increased the non-enzymatic antioxidant. Antioxidative properties may, at least partially, be one of the possible mechanisms by which FVE ameliorated the ethanol-induced gastric lesions.

GSH is a well-known antioxidant, which is usually present as the most abundant low-molecular mass thiol in most organisms. It has various functions in the defense against oxidative stress and xenobiotic toxicity. It can act as an electron donor for glutathione peroxidase in animal cells, and also directly reacts with ROS. GSH is readily oxidized to glutathione disulfide (GSSG) by glutathione peroxidase, as well as by the reaction with ROS[26], which may subsequently cause the reduction in GSH levels.

Nitrate and nitrite [a marker of endogenous nitric oxide (NO) production], as a free radical, seems to be a potential antioxidant. It takes part in termination of lipid peroxidation (LPO) reactions. NO is an effective chain-breaking antioxidant in free radical-mediated LPO. It reacts rapidly with peroxyl radicals as a sacrificial chain-terminating antioxidant. The antioxidant effect of NO on LPO has been explained by terminating the radical chain reaction through the reaction of NO with lipid peroxy radical (ROO·) to form adducts by equation[27-29]. The protective effect of NO on LPO has also been shown[30,31].

$$4\ NO + 2\ ROO\cdot + H_2O \rightarrow 2\ ROONO + 2\ NO + H_2O \rightarrow RONO_2 + RONO + 2\ HNO_2$$

Aerobic organisms are protected against ROS by enzymatic antioxidant (superoxide dismutase, glutathione peroxidase and catalase) and non-enzymatic antioxidant (such as β-carotene, retinol, vitamin C and GSH) defense systems. Antioxidant vitamins, such as ascorbic acid, retinol and β-carotene play an important acute and chronic role in reducing or eliminating the oxidant damage produced by ROS[32]. In the present study, we also measured serum antioxidant vitamin capacity and, levels of all of the antioxidant vitamins were increased in FVE, but not in famotidine treated groups. Increase in vitamin levels in FVE groups may be related to vitamin content in FVE.

The preliminary phytochemical screening of FVE showed the presence of up to 8% volatile oil (including about 85% of anethole, up to 5% of estragole, and fenchone), flavonoids (rutin, quercetin and kaempferol glycosides), coumarins (bergapten, imperatorin, xanthotoxin and marmesin), sterols and sugars. These are also present in oil of FVE, d-a-pinene, d-a-phellandrene, dipentene, methyl chavicol, anisic acid, anisaldehyde and limonene[11-13,33]. Previous studies proved that anethole possesses significant antioxidant, anti-inflammatory and ulcer healing activity in experimental models[34]. Additionally, flavonoids, sterols, tannins and coumarins of some plants are also known to possess antiulcer activity[35-38]. Therefore, the presence of flavonoids content and other bioactive compounds in FVE may be associated with the ulcer preventing action.

In conclusion, our data show that FVE has an obvious gastroprotective effect and antioxidant properties. Although it is unclear about the exact mechanism underlying these actions, the effects on acute gastric lesions suggest a multifactorial mechanism, involving the antioxidant properties of FVE. FVE may be a new alternative for clinical management of gastric ulcer diseases and/or an antioxidant against oxidative stress. Further studies are required to clarify the anti-ulcer and antioxidant actions of FVE.

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RAPID COMMUNICATION

Distribution of trace metal concentrations in paired cancerous and non-cancerous human stomach tissues

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Abstract

AIM: To assess whether trace metal concentrations (which influence metabolism as both essential and non-essential elements) are increased or decreased in cancerous tissues and to understand the precise role of these metals in carcinogenesis.

METHODS: Concentrations of trace metals including Cd, Ni, Cu, Zn, Fe, Mg and Ca in both cancerous and non-cancerous stomach tissue samples were determined by atomic absorption spectrometry (AAS). Tissue samples were digested using microwave energy. Slotted tube atom trap was used to improve the sensitivity of copper and cadmium in flame AAS determinations.

RESULTS: From the obtained data in this study, the concentrations of nickel, copper and iron in the cancerous human stomach were found to be significantly higher than those in the non-cancerous tissues, by using *t*-test for the paired samples. Furthermore, the average calcium concentrations in the cancerous stomach tissue samples were found to be significantly lower than those in the non-cancerous stomach tissue samples by using *t*-test. Exceedingly high Zn concentrations (207-826 mg/kg) were found in two paired stomach tissue samples from both cancerous and non-cancerous parts.

CONCLUSION: In contrast to the literature data for Cu and Fe, the concentrations of copper, iron and nickel in cancerous tissue samples are higher than those in the non-cancerous samples. Furthermore, the Ca levels are lower in cancerous tissue samples than in non-cancerous tissue samples.

Key words: Cancer; Trace metals; Human stomach; Microwave energy; Atomic absorption

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INTRODUCTION

The importance of essential trace metals in health and disease is indisputable because of their essential role in specific concentration ranges and toxic role at relatively high levels. Essential trace elements have four major functions as stabilizers, elements of structure, essential elements for hormonal function and cofactors in enzymes. As a result, the lack of essential trace elements influences structure alone or alters function of structure through the lack of stabilization, change of charge properties or allosteric configuration[1]. It may be expected that deficiency of essential trace elements as cofactors of enzymes could severely impair the host's resistance against carcinogenic stress[2]. Among these elements, zinc is a component of more than 3000 zinc-associated transcription factors including DNA-binding proteins with zinc fingers, and more than 300 enzymes including Cu/Zn superoxide dismutase (CuZnSOD) (SOD is an important antioxidant enzyme for cellular protection against reactive oxygen species (ROS) and several proteins involved in DNA repair[3-5]. Metallothioneins, being intracellular polypeptides have a remarkable ability to bind to metallic ions including both essential and also toxic metals such as cadmium or lead. Copper is a component of more than 30 enzymes including caeruloplasmine, cytochrome oxidase, lysine oxidase, dopamine-hydroxylase, ascorbate oxidase and tyrosinase in human body, some of which are involved in collagen synthesis, as well as being necessary for the healthy development of connective tissue, nerve coverings and bone[6,7].

The role of metals in the development and inhibition of cancer has a complex character and raises many questions. In the past 25 years, some metals including cadmium, nickel, arsenic, beryllium and chromium (VI), have been recognized as human or animal carcinogens in addition to primary carcinogens such as radiation, viruses and other chemicals[7,8]. Their carcinogenic potential depend largely on factors such as oxidation states and chemical species[9]. It is supposed that oxidative DNA lesions play an important role in various diseases including cancer and premature aging. The increase in oxidative

DNA lesions are frequently described as being attributable to metal exposure. Metal carcinogenesis is mediated either by the increased generation of highly ROS on the basis of ESR spin trapping studies[10] and/or by interference with DNA repair processes[11]. Almost all metals are able to generate ROS, which can explain a great part of both their carcinogenicity and their aptitude in the treatment of cancer. Induction of oxidative DNA damage and interaction with DNA repair processes can lead to an enhancement of genotoxicity in combination with a variety of DNA-damaging agents. Nucleotide excision repair (NER) which is the major repair system, is inhibited at low levels as well as at non-cytotoxic concentrations of Ni (Ⅱ), Cd (Ⅱ), Co (Ⅱ) and As (Ⅲ). The repair of oxidative DNA base modifications is disturbed by Ni (Ⅱ) and Cd (Ⅱ) ions. One reason for repair inhibition appears to be the displacement of Zn (Ⅱ) and Mg (Ⅱ)[12]. Magnesium and Zn, that are cofactors for DNA polymerase, are effective protectors against carcinogenesis *in vivo*. Although Zn and Cu concentrations in serum and tissues of cancer patients have been studied extensively, the precise role of these metals in carcinogenesis is not clearly understood. While a great depth of literature is available regarding the alterations in the levels of trace elements in serum, relatively few studies are available on trace element levels in cancerous and non-cancerous human stomach tissue. Reddy and coworkers reported that the concentrations of essential metals including Fe, Zn and Cu are significantly lower in cancerous stomach tissue than in normal tissues[13]. Similarly, the lower Fe and Zn levels in cancerous stomach tissues than in normal tissues are also supported by von Czarnowski and coworkers[14]. Few studies have simultaneously determined both toxic and essential trace elements in cancerous and non-cancerous stomach tissues. On the other hand, most studies have been performed on dried and occasionally homogenized samples, that disturb the tissue from its natural physiological state. Ng and coworkers[15] reported that the wet-to-dry ratio of tumor (malignant) breast tissues is higher (more 2-times) than that of the normal tissues. Therefore the elevation of elemental contents in tumors is significantly different from that in normal tissues when concentrations are adjusted by using the wet-to-dry ratio of the samples. The same study noted that the wet-to-dry ratio varies significantly amongst specimens, not only of different types but also between samples of the same group[15]. Therefore, evaluation of trace element levels in dried samples should be regarded as incomplete in the absence of wet-to-dry ratios for individual specimens. It would appear that study of fresh and unprocessed specimens is preferable. On the other hand, the ratio of Cu to Zn (Cu/Zn) intake is widely utilized to assist diagnosis of various cancers or tumors[16]. The usefulness of the tissue-metal determination in cancer prevention, detection, monitoring, treatment and prognosis requires further investigation.

In our laboratory, atomic absorption spectrometry (AAS) being the most common analytical technique has been successfully used for trace metal analysis in biological samples[17-20]. To improve the sensitivity of flame atomic absorption spectrometry (FAAS), a slotted tube atom trap (STAT) has been used for some metals such as Cd, Pb and Cu in biological matrices[19-22]. In the current study, the concentrations of various minor and trace metals, including Cd, Ni, Cu, Zn, Fe, Mg and Ca in cancerous and non-cancerous stomach tissues, were determined by atomic absorption spectrophotometry. For digestion of the tissues, a microwave oven was used.

MATERIALS AND METHODS

Apparatus and reagents

An ATI UNICAM 929 flame atomic absorption spectrophotometer (FAAS) equipped with ATI UNICAM and KOTTO hollow cathode lamps was used for metal determinations. The optimum conditions for FAAS are given in Table 1. A STAT was used to improve the Cd and Cu sensitivities by FAAS. A domestic microwave oven (Kenwood) was used for digestion of the tissues. Unless stated otherwise, all chemicals used were of analytical grade. Throughout the analysis, doubly distilled water was used. All glass apparatus (Pyrex) were kept permanently full of 1 mol/L nitric acid when they were not used. In the digestion procedures, concentrated nitric acid (65%, Merck) and hydrogen peroxide (35%, Merck) were used. Stock solutions of metals (1000 mg/L) were prepared by dissolving their salts (Merck) in 1.0 mol/L nitric acid.

Preparation of samples

Fresh stomach tissue samples were taken since fresh and formalin-fixed tissues have been demonstrated to yield virtually the same results for essential and toxic metals including Ca, Mg, Fe, Cu, Zn, As, Cd, Hg and Pb[23]. In the current study, the samples were obtained in the formaldehyde solution from private Pathology Laboratories and the pathology laboratories of Firat University in Elazig, Turkey, after surgery and histopathologic examination. A total of eighteen samples were taken, of which four cancerous (malign) stomach tissue samples were taken from patients of different sex, age and living conditions, described as independent samples in this study, the other fourteen samples were taken from both cancerous (malign) and non-cancerous (normal) stomach tissues, described as paired samples in this study. All the patients were diagnosed as grade Ⅱ-Ⅲ or Ⅲ-Ⅳ adenocarcinoma except that one patient at the age of 80 years had grade I and poorly differentiated adenocarcinoma. Furthermore, most patients had metastatic and differentiated adenocarcinoma. The tissue samples were cut into small pieces with a stainless steel knife and transferred to a beaker.

Digestion of tissue samples

Exactly 2.0 mL of the mixture of HNO_3/H_2O_2 (2:1) was added to 0.7 g of the tissue samples. The mixture was placed into the water bath at 70℃ for 30 min and stirred occasionally. Then, 1.0 mL of the same acid mixture was added, and the mixture was transferred into a Teflon vessel bomb for the microwave oven. The bomb was closed, and the solution was placed inside the microwave

Table 1 Operation parameters for flame atomic absorption spectrophotometer

Parameter	Cd	Ni	Cu	Zn	Fe	Mg	Ca
Wavelength (nm)	228.8	232	324.8	213.9	248.3	285.2	422.7
HCL current (mA)	4	7.5	3	9.5	15	15	6
Acetylene flow rate (L/min)	0.5	0.5	0.5	0.5	0.5	0.5	4.2
N_2O flow rate (L/min)	-	-	-	-	-	-	4.7
Air flow rate (L/min)	4	4	4	4	4	4	-
Slit (nm)	0.5	0.2	0.5	0.5	0.2	0.5	0.5

Table 2 Equations of the curves

Equation		
Y = 2.4972x + 1.12	$R^2 = 0.99$	For Cd (4-100 ng/mL by STAT-AAS)
Y = 0.3278x - 0.2083	$R^2 = 1$	For Cu (25-400 ng/mL by STAT-AAS)
Y = 85x + 0.5	$R^2 = 0.99$	For Ni (0.2-2.0 mg/L)
Y = 302x + 0.75	$R^2 = 0.99$	For Zn (0.1-1.0 mg/L)
Y = 64x + 0.43	$R^2 = 1$	For Fe (0.20-3.0 mg/L)
Y = 515x + 7.0	$R^2 = 0.99$	For Mg (0.25-2.0 mg/L)
Y = 305x + 39	$R^2 = 1$	For Ca (0.25-2.0 mg/L)

oven. Radiation was applied for 3 min at 450 W. After addition of 0.5 mL of the same acid mixture, radiation was repeated for 3 min. After cooling for 5 min, 2.0 mL of 0.1 mol/L HNO_3 was added, and the solution was transferred into a Pyrex tube. After centrifugation, the clear solution was measured by FAAS. Three different portions of each sample were digested and the average value was calculated for the same tissue. Blank digests were carried out in the same way.

RESULTS

Calibration curves were obtained by using solutions of the studied elements at different concentrations. The graphs obtained were linear in the concentration range and the equations of the curves are described in Table 2.

Analytical performance

The accuracy of the method was studied by examining the recovery of metals from stomach tissue samples fortified with various amounts of the studied metals. The following metal amounts were added: 30 ng/g of Cd, 200 ng/g of Ni, 0.3 mg/kg of Cu, 10 mg/kg of Zn, 10 mg/kg of Fe, 100 mg/kg of Mg and 300 mg/kg of Ca. After digestion in microwave oven, the recoveries were found to be at least 90% for all studied metals. Furthermore, the standard addition method was used to remove possible interferences caused by the matrix. The slopes of the calibration curves for all studied elements were compared with those obtained by the standard addition method. The slopes of the calibration curves were found to be the same as those obtained with the standard addition method. In other words, all of the standard addition curves were parallel to the calibration curves. These results indicated the absence of chemical interference.

Levels of the metals including Cd, Ni, Cu, Zn, Fe, Mg and Ca in the reagent blanks in the analytical steps were found to be 0.5, 25, 10, 50, 50, 105 and 190 ng/mL with the standard deviations being 0.1, 4.0, 1.5, 9.0, 8.0, 20 and 35, respectively. Therefore, the detection limits for these elements defined as three times the s values of blanks were calculated as 0.3, 12, 4.5, 27, 24, 60 and 105 ng/mL. The precision of the standard deviations for 10 samples of the same tissue was found to be less than 10% for all studied elements.

Comparison of metal levels in cancerous and non-cancerous tissues

Metals are considered to act not only as carcinogens but also as co-carcinogens that activate carcinogenic chemicals. In evaluating the differences in cancerous and non-cancerous tissue samples, two comparisons were conducted: one between paired cancerous and non-cancerous tissue samples, the other between total cancerous and non-cancerous samples. *P* values (obtained by using *t*-test) less than 0.05 were considered significantly different between the two groups. The samples with exceptionally high values were disregarded in calculating the average and range.

Data related with carcinogenic effects of cadmium are available from the literature[24]. Multiple studies have linked occupational exposure to Cd with pulmonary cancer in humans, whereas a few studies showed that Cd exposure is associated with cancers of stomach and other sites in humans[24]. As it can be seen from Table 3, there was no significant difference in Cd concentrations between cancerous and non-cancerous stomach tissue samples, in the present study.

Nickel: The International Agency for Research on Cancer (IARC) has classified the carcinogenic substances in two groups. Group 1 includes substances "known to be human carcinogens or sufficient evidence of carcinogenicity from studies in humans", group 2 includes substances "reasonably anticipated to be a human carcinogen". The chemicals in group 2 are classified in two subgroups by IARC. Group 2A includes substances "probably carcinogenic to humans", and group 2B includes substances "possibly carcinogenic to humans" [8]. Kasprazak and coworkers[25] have made an overall evaluation of the carcinogenicity of nickel and found that Ni compounds are carcinogenic to humans (group 1), and metallic nickel is possibly carcinogenic to humans (group 2B).

vonCzarnowski *et al*[14] showed that Ni levels are lower in cancerous (malign) stomach tissues than in normal stomach tissues, whereas Reddy *et al*[13] reported that nickel concentrations are 6-time higher in cancerous stomach tissues than in normal stomach tissues. In this study, the Ni levels in cancerous stomach tissue samples were significantly higher ($P < 0.05$ for the paired samples) than those in the non-cancerous stomach tissue samples (Tables

Table 3 Trace metal concentrations in cancerous and non-cancerous stomach tissues. Every single cancerous tissue belongs to a different patient (mean ± SD, *n* = 3)

Tissue	Cd (ng/g)		Ni (ng/g)		Cu (mg/kg)		Zn (mg/kg)		Fe (mg/kg)		Mg (mg/kg)		Ca (mg/kg)	
(Age)	Cancerous	non-can-cerous	Cancerous	non-cancerous	Cancer-ous	non-cancerous	Cancer-ous	non-cancerous	Cancerous	non-cancerous	Cancer-ous	non-cancerous	Cancer-ous	non-cancerous
Stomach (65)	30 ± 9		230 ± 55		0.6 ± 0.1		11 ± 1		24 ± 3		508 ± 75[2]		526 ± 85	
Stomach (62)	156 ± 16		260 ± 30		0.9 ± 0.1		26 ± 2		8 ± 1		203 ± 18		545 ± 90	
Stomach (80)	50 ± 9		740 ± 84		0.9 ± 0.2		23 ± 24		65 ± 30		44 ± 17[2]		1010 ± 930	
Stomach (66)	29 ± 16		820 ± 113		0.7 ± 0.1		7 ± 1		60 ± 17		112 ± 30		480 ± 130	
Stomach (58)[1]	10 ± 3	33 ± 6	905 ± 690	328 ± 55	1.2 ± 0.1	1.0 ± 0.3	11 ± 1	23 ± 0.1	25 ± 4	19 ± 8	80 ± 10	200 ± 14	390 ± 76	834 ± 155
Stomach (60)[1]	33 ± 10	42 ± 7	1120 ± 265	840 ± 93	1.8 ± 0.1	1.5 ± 0.2	12 ± 2	17 ± 1	16 ± 2	21 ± 2	150 ± 12	84 ± 10	335 ± 41	450 ± 86
Stomach (57)[1]	68 ± 26	130 ± 21	510 ± 80	439 ± 57	1.5 ± 0.2	1.2 ± 0.1	16 ± 2	21 ± 4	37 ± 9	18 ± 5	210 ± 23	190 ± 15	432 ± 96	1047 ± 120
Stomach (59)[1]	90 ± 15	63 ± 8	2010 ± 356	1240 ± 112	1.7 ± 0.2	0.9 ± 0.1	25 ± 3	19 ± 1	40 ± 5	23 ± 3	106 ± 11	45 ± 5[2]	235 ± 34	492 ± 75
Stomach (52)[1]	107 ± 19	55 ± 5	335 ± 164	321 ± 237	1.5 ± 0.6	1.1 ± 0.3	22 ± 2	19 ± 1	34 ± 14	36 ± 21	210 ± 15	300 ± 128	911 ± 215	1199 ± 53
Stomach (51)[1]	22 ± 9	58 ± 8	740 ± 145	720 ± 95	1.1 ± 0.2	0.6 ± 0.1	18 ± 2	16 ± 1	35 ± 4	34 ± 3	24 ± 3[2]	30 ± 5[2]	403 ± 55	523 ± 41
Stomach (66)[1]	31 ± 3	33 ± 6	527 ± 116	241 ± 32	2.8 ± 0.1	0.9 ± 0.5	41 ± 4	30 ± 15	20 ± 2	23 ± 7	270 ± 15	241 ± 30	530 ± 10	624 ± 78
Stomach (60)[1]	30 ± 6	55 ± 6	360 ± 92	270 ± 32	0.5 ± 0.07	0.7 ± 0.1	25 ± 2	30 ± 3	9 ± 2	10 ± 3	200 ± 28	215 ± 25	600 ± 110	615 ± 65
Stomach (49)[1]	10 ± 1	33 ± 3	182 ± 72	190 ± 5	4.3 ± 0.5	2.1 ± 1.6	19 ± 4	23 ± 2	17 ± 3	20 ± 4	130 ± 21	110 ± 17	460 ± 62	484 ± 56
Stomach (55)[1]	30 ± 5	32 ± 4	500 ± 98	240 ± 28	2.8 ± 0.1	1.2 ± 0.2	33 ± 3	35 ± 4	12 ± 3	18 ± 4	190 ± 20	135 ± 15	533 ± 67	624 ± 60
Stomach (50)[1]	65 ± 17	85 ± 10	700 ± 85	750 ± 103	1.7 ± 0.1	0.8 ± 0.1	207 ± 19[2]	826 ± 55[2]	25 ± 2	13 ± 2	314 ± 30	184 ± 22	746 ± 86	713 ± 66
Stomach (53)[1]	60 ± 12	80 ± 11	300 ± 45	130 ± 12	2.4 ± 0.3	0.8 ± 0.1	410 ± 28[2]	380 ± 36[2]	26 ± 3	15 ± 2	207 ± 31	275 ± 10	685 ± 74	715 ± 61
Stomach (40)[1]	70 ± 12	105 ± 10	730 ± 98	850 ± 120	2.2 ± 0.2	1.9 ± 0.1	15 ± 2	16 ± 2	42 ± 4	25 ± 3	215 ± 10	200 ± 40	375 ± 42	417 ± 36
Stomach (51)[1]	32 ± 5	100 ± 12	410 ± 65	800 ± 112	2.2 ± 0.2	1.3 ± 0.1	17 ± 1	16 ± 2	42 ± 5	24 ± 2	327 ± 30	210 ± 25	476 ± 26	508 ± 52
Average	51 ± 37	65 ± 31	632 ± 430	526 ± 335	1.7 ± 1	1.1 ± 0.4	20 ± 9	22 ± 6	30 ± 16	21 ± 7	195 ± 72	195 ± 63	537 ± 196	660 ± 230
Range	10-156	32-130	230-2010	130-1240	0.5-4.3	0.6-2.1	7-41	16-30	8-65	10-36	24-508	30-300	235-1010	417-1199

[1]Cancerous and non-cancerous tissues in lines belong to the same persons; [2]These values are not included in calculation of average and in range data.

Table 4 Significant and tendentious elements in cancerous human stomach tissue except for the last one

Status	Stomach	*P* (for paired samples)	*P* (for total samples)
Significant	Ca (-)	0.009	0.122
	Cu (+)	0.001	0.036
	Fe (+)	0.046	0.065
Tendentious	Ni (+)	0.092	0.409
		0.025	

3, 4 and Figure 1).

Copper: Although copper is an essential element for humans and animals, high concentrations of Cu (above normal) could induce growth proliferation and cancer by damaging DNA with toxic free hydroxyl radicals[26]. Conflicting results regarding Cu concentrations have been observed in cancerous and normal stomach tissues[13,14]. VonCzarnowski and coworkers[14] reported that there are no differences in Cu concentrations between cancerous (malign) and normal stomach tissue samples, whereas Reddy and coworkers[13] described that Cu levels in cancerous stomach tissue samples are 3-time lower than those in normal stomach tissue samples. In the present study, the Cu levels in cancerous stomach tissues were significantly higher ($P = 8.10^{-4}$ for the paired samples and $P < 0.05$ for total samples) than those in non-cancerous stomach samples (Tables 3, 4 and Figure 2). The mechanism of copper elevation in cancerous tissues may be explained by modifications in the relationships among trace elements with reduced catabolism or by increased neoplastic synthesis of ceruoplasmin. Metal carcinogenesis is mediated either by the increased generation of highly ROS (Fenton reaction) and/or by interference with DNA repair processes[11,26]. Since almost all metals are able to generate ROS, further studies on the determination of trace element levels together with ROS production

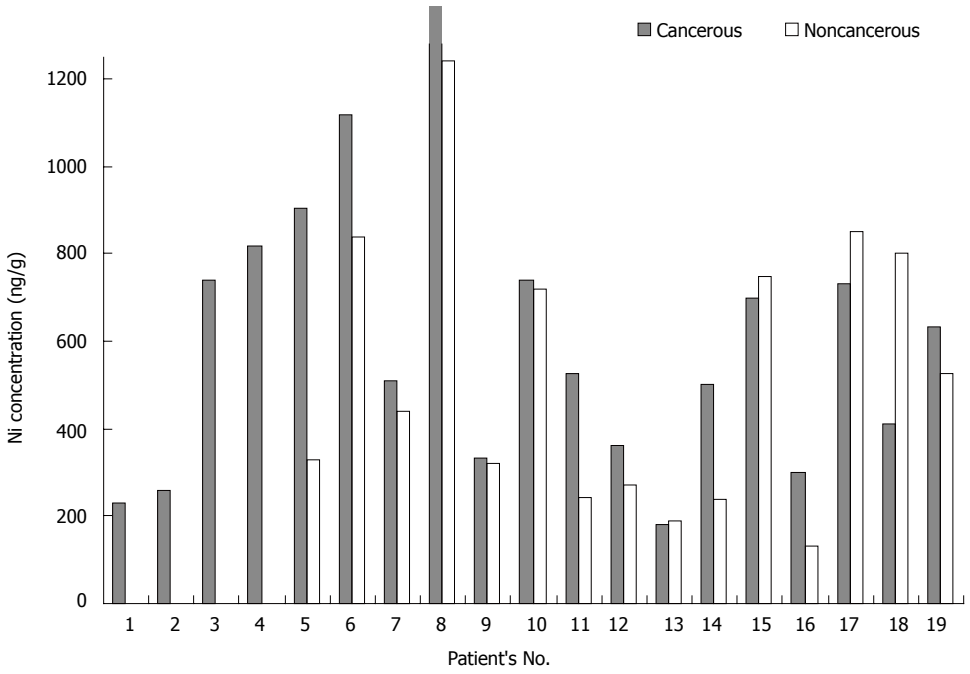

Figure 1 Comparison of Ni levels between cancerous and non-cancerous stomach tissue samples. The concentration of number 19 is average value.

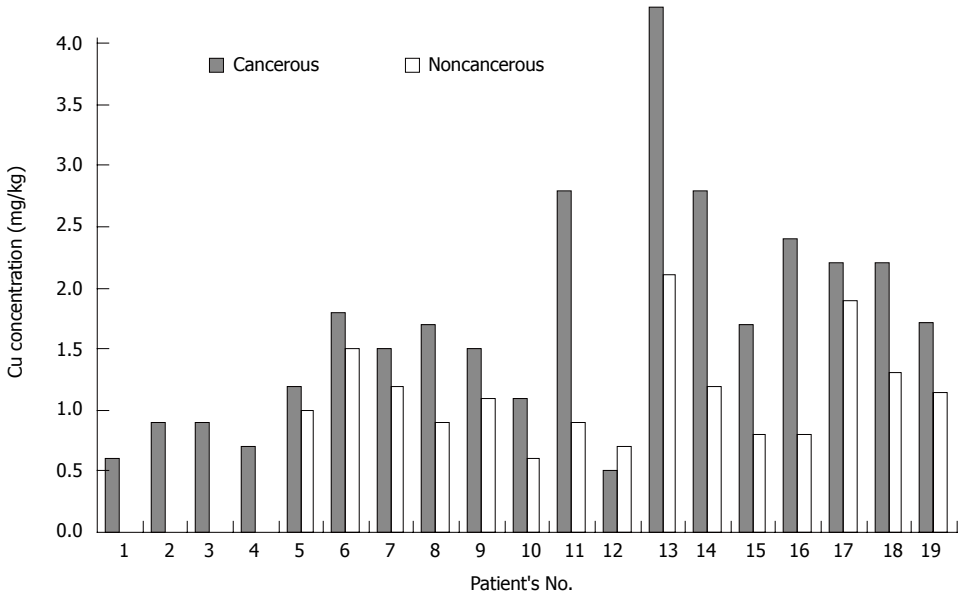

Figure 2 Comparison of Cu levels between cancerous and non-cancerous stomach tissue samples. The concentration of number 19 is average value.

are needed. Consequently, the physiological processes underlying tumor development can lead to uptake of trace elements by neoplastic cells because of the increased cellular and enzymatic activity.

Zinc: It was reported that Zn concentrations in cancerous stomach tissue are lower than those in normal tissues[13-14]. It can be seen from Table 3, there was no significant difference in Zn concentrations between cancerous and non-cancerous stomach tissue the paired samples. Excessive zinc concentrations were found in both cancerous and non-cancerous stomach tissues from two patients. Unfortunately, we could not explain these excessive Zn levels.

Iron: Although Fe is an essential nutritional element for all life forms, it is known that excess iron and iron deficiency also lead to oxidative DNA damage[27]. It was reported that iron levels are significantly decreased in cancerous stomach tissue in comparison with those in normal stomach tissue[13-14]. On the other hand, Hercberg and coworkers[28] reported that serum ferritin concentration >160 ng/mL is an increased risk of developing cancer in women but not in men. In this study, Fe levels in the cancerous stomach tissue samples were significantly higher ($P < 0.05$ for the paired samples and $P = 0.065$ for all samples) than those in the non-cancerous tissue samples (Tables 3, 4 and Figure 3). These findings can also be explained by the Fenton reaction described above.

Calcium: It was reported that calcium concentrations in cancerous stomach tissues are lower than those in the normal tissues[13], whereas steady Ca concentrations are observed in cancerous and normal stomach tissues[14]. In this study, Ca levels in the cancerous stomach tissue samples were significantly lower ($P < 0.01$ for the paired samples) than those in the non-cancerous tissue samples,

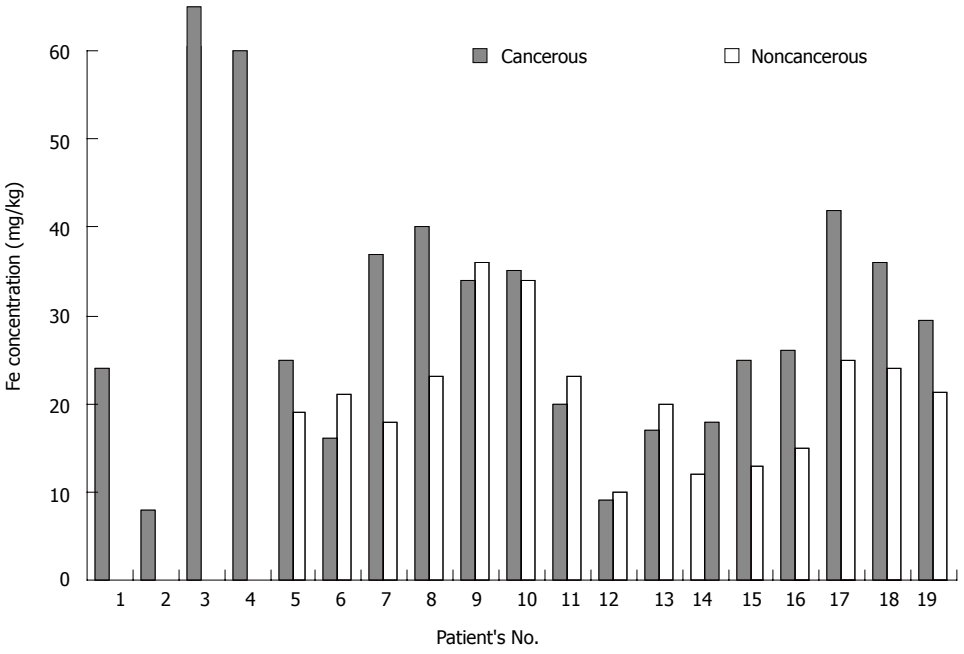

Figure 3 Comparison of Fe levels between cancerous and non-cancerous stomach tissue samples. The concentration of number 19 is average value.

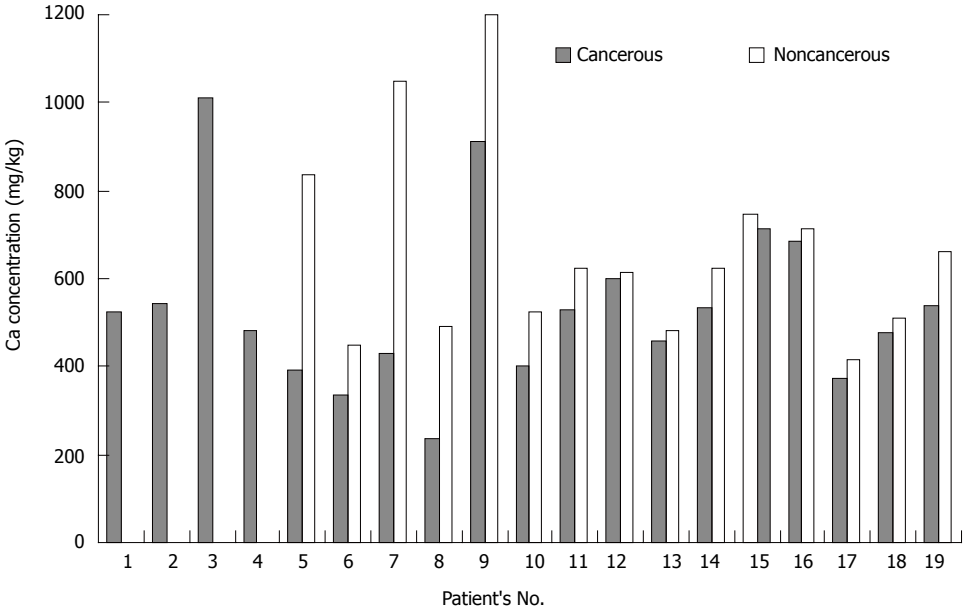

Figure 4 Comparison of Ca levels between cancerous and non-cancerous stomach tissue samples. The concentration of number 19 is average value.

similar to the results of Reddy coworkers (Tables 3, 4 and Figure 4). It was described that Ni^{+2} can block Ca^{+2} channels and hence, nickel releases the stored intracellular Ca^{+2} via a mechanism underlying the interaction between Ni^{+2} ions and the cell surface Ca^{+2} receptor[25]. The results of this study, involving the lower Ca concentrations in cancerous stomach tissue samples than in the non-cancerous tissue samples, agree with the observed higher Ni levels in cancerous tissues than in non-cancerous tissues.

Magnesium: There were no significant differences in the Mg concentrations between cancerous and non-cancerous stomach tissues (Table 3).

The significant and tendentious elements are listed in Table 4. The positive sign was used to illustrate accumulation of the elements in cancerous tissue, and the minus sign was used to indicate the depletion of elements in the cancerous stomach tissue samples in comparison to the non-cancerous tissue samples.

DISCUSSION

Reddy and coworkers[13] found that the concentrations of trace metals in normal/cancerous tissues (mg/kg) on dry weight basis are as follows: Fe = 2408/684, Cu = 63.5/21.2, Zn = 818/229, Ni = 10.5/60, Pb = 8.8/8.1 and Ca = 647/433. They described that the low iron level observed in carcinoma tissue of stomach might not initiate carcinoma in stomach, but the low absorption of iron may be due to the lack of HCl which in turn may be due to the carcinogenic nature of stomach. The lower iron levels observed in cancer tissue of stomach is supported by vonCzarnowski and coworkers[14]. In the present study, copper, iron and nickel concentrations in cancerous stomach tissue samples were higher than those in non-

cancerous stomach tissue samples. Although ROS were not measured in this study, these results are in agreement with the reported data[29]. Furthermore, we found that Ca levels in the cancerous stomach tissue samples were lower than those in the non-cancerous stomach tissue samples. It was reported that calcium and magnesium concentrations, similar to iron, nickel and zinc in cancerous prostate tissue are higher than those in non-cancerous prostate tissue[18]. The increase in calcium concentration and its heterogeneous distribution in malign prostate tissue in contrast to the data obtained in stomach tissue may be attributed to calcium functions and behaviors depending on the organ type. The organ-dependency on the changes in trace metal concentrations in cancerous and endometrial tissues[30] also supports these explanations. We think that the decreased Ca levels and the increased Ni concentrations in cancerous stomach tissues as well as its heterogeneous distribution in comparison to non-cancerous samples are very important for the investigation of cancer mechanism in this organ due to the displacement of Ni with Ca. The results in disagreement with the explanations above may be attributed to their subgroups of cancerous properties because the different mechanisms may be effective in such conditions.

In conclusion, STAT can be used to improve the sensitivity of copper and cadmium. In addition, the tissue digested in a microwave oven has very low blank values and can reduce the risk of metal loss or contaminations. The closed microwave digestion offers an easy and reliable method for the complete dissolution of tissues prior to the determination of trace metals.

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RAPID COMMUNICATION

Evaluation of the effect of partial splenic embolization on platelet values for liver cirrhosis patients with thrombocytopenia

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Abstract

AIM: To investigate the effect of partial splenic embolization (PSE) on platelet values in liver cirrhosis patients with thrombocytopenia and to determine the effective embolization area for platelet values improvement.

METHODS: Blood parameters and liver function indicators were measured on 10 liver cirrhosis patients (6 in Child-Pugh grade A and 4 in grade B) with thrombocytopenia (platelet values < 80 × $10^3/\mu L$) before embolization. Computed tomography scan was also needed in advance to acquire the splenic baseline. After 2 to 3 d, angiography and splenic embolization were performed. A second computed tomography scan was made to confirm the embolization area after 2 to 3 wk of embolization. The blood parameters of patients were also examined biweekly during the 1 year follow-up period.

RESULTS: According to the computed tomography images after partial splenic embolization, we divided all patients into two groups: low (< 30%), and high (≥ 30%) embolization area groups. The platelet values were increased by 3 times compared to baseline levels after 2 wk of embolization in high embolization area group. In addition, there were significant differences in platelet values between low and high embolization area groups. GPT values decreased significantly in all patients after 2 wk of embolization. The improvement in platelet and GPT values still persisted until 1 year after PSE. In addition, 3 of 4 (75%) Child-Pugh grade B patients progressed to grade A after 2 mo of PSE. The complication rate in < 30% and ≥ 30% embolization area groups was 50% and 100%, respectively.

CONCLUSION: Partial splenic embolization is an effective method to improve platelet values and GPT values in liver cirrhosis patients with thrombocytopenia and the ≥ 30% embolization area is meaningful for platelet values improvement. The relationship between the complication rate and embolization area needs further studies.

Key words: Partial splenic embolization; Liver cirrhosis; Thrombocytopenia

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INTRODUCTION

The spleen is known to be involved in thrombocytopenia associated with liver cirrhosis[1]. Thrombocytopenia in patients with liver cirrhosis has been reported to be caused by an increased platelet pool in the enlarged spleen[2], impaired platelet production in the bone marrow[3], decreased platelet function[4], and abnormalities in the platelet membranes[5]. Recently, an article[6] reported that decreased production of thrombopoietin (TPO) might also promote the development of thrombocytopenia in liver cirrhosis. Partial embolization of the splenic vessels has been used to treat hypersplenism of thrombocytopenia in liver cirrhosis patients. Mozes *et al*[7] described the benefit of partial splenic embolization (PSE) in reducing the prevalence of complications from splenic artery embolization. Miyazaki *et al*[8] reported the effectiveness of PSE in thrombocytopenia. Their reports suggested that PSE might be a safe and effective alternative to splenectomy in the treatment of thrombocytopenia. The aim of the current study was to investigate the effect of partial splenic embolization on platelet values in liver cirrhosis patients with thrombocytopenia and to determine

the effective embolization area for platelet values improvement.

MATERIALS AND METHODS

Ten liver cirrhosis patients with thrombocytopenia (platelet value < $80 \times 10^3/\mu L$) were transferred to our department to receive partial splenic embolization at Taipei Medical University Hospital from January 2004 to September 2005. All patients gave informed consent before their participation, and the Ethics Committee of Taipei Medical University Hospital approved the study. The Child-Pugh classifications of these 10 patients were grade A in 6 and grade B in 4 patients. They had no other chronic diseases. Before partial splenic embolization, measurements were taken of platelet, white blood counts, hemoglobin, bilirubin, alkaline phosphatase, and liver function indicators [aspartate aminotransferase, ASAT (GOT), and alanine aminotransferase, ALAT(GPT)].

Before the PSE, they underwent computed tomography (CT, HiSpeed CT/I; GE Medical Systems, Milwaukee, WI, USA) to observe the basal status of the spleen and calculate the volume of the spleen using the software of CT. After 2 to 3 d, angiography and PSE were performed, respectively. A femoral artery approach was used for superselective catheterization of the splenic artery. The catheter tip was placed as distally as possible in either the hilus of the spleen or the intrasplenic artery. Embolization was done by injections of a gelatin sponge (Gelfoam cube, Upjohn, Kalamazoo, USA) cut into 1 to 2 mm cube and suspended in 1 g cefazolin-containing solution. When the blood flow in this artery stopped, we finished the embolization. After 2 to 3 wk of PSE, we did CT scan again to confirm the embolization area via calculating the volume of the spleen. In addition, these patients took antibiotics orally to prevent sepsis. During the one year follow up period, we measured the blood parameters mentioned above biweekly, and recorded the blood parameters at every time point.

Statistical analysis

Blood parameters were compared using paired *t* test. $P < 0.05$ means significant differences.

RESULTS

The mean age of our patients was 56 years (from 19 to 71). According to the embolization areas presented on CT, we divided all patients into two groups: low embolization group (< 30%): 2 patients, and high embolization group (≥ 30%): 8 patients. The average embolization areas in these two groups were 20% and 40%, respectively. The effect of PSE on platelet values is listed in Table 1. In high embolization area group, the platelet values after 2 wk and 1 year of embolization were 2 to 3 times the basal values. In addition, we also evaluated the effect of different embolization areas on platelet values. We observed a marked variation of platelet values between low and high embolization groups. We found that an embolization area equal to or higher than 30% is an effective embolization area for improvement of platelet values.

Table 1 Effects of partial splenic embolization on platelet values

Basal values ($\times 10^3/\mu L$)	Embolization area (%)	After 2 wk ($\times 10^3/\mu L$)	After 1 year ($\times 10^3/\mu L$)
Low embolization			
56	15	69	66
54	25	66	51
Average platelet value		67.5	58.5
High embolization			
60	35	175[1]	136[1]
40	45	203[1]	158[1]
67	35	258[1]	140[1]
61	30	212[1]	185[1]
58	30	207[1]	149[1]
64	30	71	59
48	50	250[1]	195[1]
51	65	159[1]	141[1]
Average platelet value		192[2]	145[2]

[1]Means significant differences in platelet values between basal values and post-embolization values; [2]Means significant differences in platelet values between low and high embolization areas groups.

Table 2 Effect of partial splenic embolization on GPT values

Basal values (IU/L)	Embolization area (%)	After 2 wk (IU/L)	After 1 year (IU/L)
Low embolization			
145	15	46[1]	45[1]
95	25	57[1]	51[1]
Average GPT value		51.5	48
High embolization			
87	35	47[1]	52[1]
76	45	50[1]	51[1]
102	35	54[1]	40[1]
98	30	44[1]	51[1]
87	30	56[1]	44[1]
116	30	58[1]	54[1]
79	50	59[1]	55[1]
66	65	45[1]	35[1]
Average GPT value		52	48

[1]Means significant differences in GPT values between basal values and post-embolization values.

PSE cannot improve the GOT values markedly; however, the GPT values of these 10 patients after 2 wk of PSE decreased baldly and the range of degradation was from 32% to 68% (Table 2). We also found that the improvement of platelet and GPT values still persisted until 1 year after PSE. In addition, 3 of 4 (75%) Child-Pugh grade B patients progressed to grade A after 2 mo of PSE. It suggested that the PSE may improve the liver function with a long-term efficiency. The PSE procedure did not affect other blood parameters.

Regarding complications, there were fever in 10 (100%), pain in 8 (80%) and ascites in 1 (10%) of patients. Ascites occurred in high embolization group. The complication rate in < 30% and in ≥ 30% groups were 50% and 100%, respectively.

DISCUSSION

In 1973 Maddison[9] reported the first case of splenic

artery embolization. In 1979, Spigos[10] cured 14 hypersplenism cases using PSE. Since then, PSE has become a major therapy clinically for hypersplenism. The spleen represents one fourth of the total lymphatic mass, serves as a biological filter for the clearance of bacteria and also is essential for rapid antibody production after challenge with blood-borne particulate antigens in the absence of preexisting antibodies[11]. In addition, the spleen appears to be the site of production of a nonspecific leukophilic immunoglobin, tuftsin, that increases the phagocytic activity of polymorphonuclear leucocytes. Thus, it is apparent that the spleen has important and critical functions and its removal is not to be taken lightly[12]. Partial splenic embolization represents a potential alternative method to splenectomy when ablation of the splenic parenchyma is desired, particularly in compromised patients, where splenectomy carries significant morbidity and mortality rates. Partial splenic embolization has been successfully used experimentally in the treatment of thrombocytopenia or splenic trauma. Hematologic changes after embolization have been observed, especially in platelet values. There have also been sporadic reports[13,14] of successful splenic embolization in humans with thrombocytopenia due to hypersplenism of portal hypertension. Several studies[15,16] demonstrated that PSE could not only improve the symptoms of hypersplenism but also could reserve the spleen for immune function maintenance.

A major finding in this study is the effectiveness of PSE in improving platelet values and the extent of embolization seems to be critical in the efficacy of PSE. According to Bruno's study[17], embolization of 50% or less of the splenic mass was almost invariably associated with an elevation of platelet values. In another study[18], the authors demonstrated that 65%-70% embolization area is effectiveness in platelet values improvement in liver cirrhosis patients with thrombocytopenia. In Kimuro's study[19], the platelet counts were improved from $5.6 \times 10^3/\mu L$ to $36 \times 10^3/\mu L$ in 80% embolization area group and from $6.2 \times 10^3/\mu L$ to $25 \times 10^3/\mu L$ in 70% embolization area group, which is significantly higher than the former. According to the studies mentioned above, it is suggested that the improved efficacy of PSE on platelet values relates closely to embolization area. In our study, we observed a marked variation of platelet value between low and high embolization groups. We assume that an embolization area equal to or higher than 30% is an effective embolization area for platelet values improvement. Our result is not similar to previous studies. We presume that the limited patient number results in the fact that we cannot differentiate the age, basal platelet values and Child-Pugh classification of patients. Compared to the Bruno and Miyazaki's large-scale studies, there are more variabilities in our study. The various results probably have multiple causes, but patient selection, with different degrees of hepatic insufficiency, is probably of greatest importance.

The complications after PSE include pneumonia, ascites, bleeding, peritonitis, etc. In our study, there were fever in 10 patients, pain in 8 (7 in high embolization and 1 in low embolization) and ascites in 1 (in high embolization). The complication rate in $< 30\%$ and in $\geqslant 30\%$ groups was 50% and 100%, respectively. In Mukaiya's study[20], they divided all patients into three groups: $< 50\%$, 50%-70% and $\geqslant 70\%$ according to the embolization area. The complication rate was 28%, 56% and 95%, respectively. In another study[18], the authors also demonstrated that the complication rate associated with the embolization area, which is similar to our study. However, in Hong's study[21], they conducted linear regression analysis between complication rate and embolization area and the correlation coefficient was 0.587. Hong considered that embolization area will affect the complication rate, however, it is not an absolute factor. The relationship between these two parameters needs further study.

Our results showed that the GPT values of these 10 patients after 2 wk of PSE decreased badly and the range of degradation was from 32% to 68%. We also found that the improvement in GPT values persisted until 1 year after PSE. As compared with GPT, GOT is not a specific indicator for liver function. Generally speaking, GOT and GPT values will increase when hepatitis occurs, however, GOT also exists in erythrocytes, cardiac muscles and skeletal muscles. Some extrahepatic diseases, such as myocardial infarction, myocardial necrosis, hemolysis and several muscle related diseases could result in GOT increase. On the contrary, almost all GPT exists in the liver, so that GPT is more specific than GOT for liver function. This is the reason why PSE improved GPT values markedly while there was no effect on GOT values. In addition, 3 of 4 (75%) Child-Pugh grade B patients progressed to grade A after 2 mo of PSE. We suppose that the PSE may be able to improve the liver function, with a long term efficiency. In Noguchi's study[22], 1 liver cirrhosis patient with Child-Pugh grade C progressed to grade B after 6 mo of PSE; 3 of 5 Child-Pugh grade B patients progressed to grade A. In Vujic's study[23], they found liver function improvement in 56 of 128 patients (43.8%) who underwent PSE and their clinical expression included albumin increase and prothrombin formation time abridgement. According to Wang[24], the mechanism by which PSE improves liver function may involve immunologic mechanisms and hemodynamic changes. Noguchi *et al*[22] reported that after PSE the changes in the platelet count as compared with the preoperative value negatively correlate with the change in the platelet-associated immunoglobulin G levels. That study suggested that PSE improves the thrombocytopenia induced by immunologic mechanisms in cirrhotic patients. As for hemodynamic mechanisms, Barcena *et al*[25] reported that PSE decreased blood flow in the splenic artery and increased blood flow in the hepatic artery and superior mesenteric artery. In addition, Kato *et al*[26] found that the decrease in total portal blood flow and the relative increase in the mesenteric blood flow after PSE may decrease liver congestion, enhance the blood supply, and increase the supply of cytokines derived from the digestive tract.

In conclusion, partial splenic embolization is an effective method to improve platelet values in liver cirrhosis patients with thrombocytopenia. We also find that the $\geqslant 30\%$ embolization area is meaningful for

platelet values improvement. The relationship between complication rate and embolization area needs further studies.

COMMENTS

Background

The spleen is known to be involved in thrombocytopenia associated with liver cirrhosis. Thrombocytopenia in patients with liver cirrhosis has been reported to be caused by an increased platelet pool in the enlarged spleen, impaired platelet production in the bone marrow, a decreased platelet function, and abnormalities in the platelets membranes. Partial embolization of the spleen vessels has been used to treat hypersplenism of thrombocytopenia in liver cirrhosis patients. Studies suggested that PSE might be a safe and effective alternative to splenectomy in the treatment of thrombocytopenia.

Innovations and breakthroughs

The focus of most previous studies is to evaluate the effectiveness of partial splenic embolization on blood parameters (especially platelets values). The breakthrough of our article is that we found out the efficient embolization area for platelet value improvement, and this provides a meaningful reference for clinic physicians in their therapy.

Applications

Clinical physicians should adopt the minimal partial splenic embolization area, which could improve the platelet value for treatment of the liver cirrhosis patients with thrombocytopenia. The major advantage is fewer complications after embolization. The $\geqslant$ 30% efficient embolization area shown in our article is multivariate depending on the techniques and patient status; however, we provide another meaningful cerebration for clinical treatment.

Terminology

PSE: Partial splenic embolization.

Peer review

This article evaluated the effect of the degree of partial splenic embolisation on platelet values in cirrhotic patients with hypersplenism. The data suggest that at least 30% of the spleen area must be embolisation area in order to increase platelet counts sufficiently.

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RAPID COMMUNICATION

Therapeutic effects of Caspase-1 inhibitors on acute lung injury in experimental severe acute pancreatitis

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Abstract

AIM: To assess the therapeutic effect of Caspase-1 inhibitors (ICE-I) on acute lung injury (ALI) in experimental severe acute pancreatitis (SAP).

METHODS: Forty-two SD rats were randomly divided into 3 groups: healthy controls (HC, n = 6); SAP-S group (n = 18); SAP-ICE-I group (n = 18). SAP was induced by retrograde infusion of 5% sodium taurocholate into the bile-pancreatic duct. HC rats underwent the same surgical procedures and duct cannulation without sodium taurocholate infusion. In SAP-S group, rats received the first intraperitoneal injection of isotonic saline 2 h after induction of acute pancreatitis and a repeated injection after 12 h. In SAP-ICE-I group, the rats were firstly given ICE inhibitors intraperitoneally 2 h after induction of pancreatitis. As in SAP-S group, the injection was repeated at 12 h. Serum IL-1β was measured by ELISA. Intrapulmonary expression of Caspase-1, IL-1β and IL-18 mRNA were detected by semi-quantitative RT-PCR. The wet/dry weight ratios and histopathological changes of the lungs were also evaluated.

RESULTS: Serum IL-1β levels in SAP-S group were 276.77 ± 44.92 pg/mL at 6 h, 308.99 ± 34.95 pg/mL at 12 h, and 311.60 ± 46.51 pg/mL at 18 h, which were increased significantly (P < 0.01, *vs* HC). In SAP-ICE-I group, those values were decreased significantly (P < 0.01, *vs* SAP-S). Intrapulmonary expression of Caspase-1, IL-1β and IL-18 mRNA were observed in the HC group, while they were increased significantly in the SAP-S group (P < 0.01, *vs* HC). The expression of IL-1β and IL-18 mRNA were decreased significantly in the SAP-ICE-I group (P < 0.01, *vs* SAP-S), whereas Caspase-1 mRNA expression had no significant difference (P > 0.05). The wet/dry weight ratios of the lungs in the SAP-S group were increased significantly (P < 0.05 at 6 h, P < 0.01 at 12 h and 18 h, *vs* HC) and they were decreased significantly in the SAP-ICE-I group (P < 0.05, *vs* SAP-S). Caspase-1 inhibitors ameliorated the severity of ALI in SAP.

CONCLUSION: Caspase-1 activation, and overproduction of IL-1β and IL-18 play an important role in the course of ALI, and Caspase-1 inhibition is effective for the treatment of ALI in experimental SAP.

Key words: Severe acute pancreatitis; Caspase-1; Interleukin-1β; Interleukin-18; Acute lung injury

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INTRODUCTION

Patients with severe acute pancreatitis (SAP) is often complicated with acute lung injury (ALI), which is difficult to deal with clinically. IL-1β and TNF-α are currently believed to play an important role in promoting local tissue destruction and remote organ failure in the course of SAP[1,2]. IL-18 is a novel proinflammatory cytokine, sharing striking structural and functional similarities to IL-1β. Caspase-1, also termed IL-1β-converting-enzyme (ICE), is the first member of the family of cysteine proteases called Caspases, with the functions of proteolytic cleavage of IL-1β and IL-18 precursors into their active forms. Suppression of IL-1β and IL-18 by inhibiting the function of ICE, subsequently alleviating cascade reactions, may have a therapeutic significance for SAP and systemic inflammatory response syndrome (SIRS).

In this study, an experimental model of SAP was induced in SD rats. Serum IL-1β levels, intrapulmonary expression of Caspase-1, IL-1β and IL-18 mRNA were measured respectively, and the wet/dry weight ratios and histopathological alterations in the lungs were observed to assess the therapeutic effect of Caspase-1 inhibitors on ALI in SAP.

MATERIALS AND METHODS

Experimental animal models and grouping

Healthy adult male Sprague-Dawley rats weighing 230-250

g were provided by the Experimental Animal Center of Jingling Hospital in Nanjing. All forty-two rats were randomly divided into 3 groups: healthy controls (HC, n = 6); SAP-S group (n = 18); SAP-ICE-I group (n = 18). The latter two groups were further divided into 6, 12, and 18 h time points, and each contained 6 rats. SAP was induced by retrograde infusion of 5% sodium taurocholate into the bile-pancreatic duct in SD rats[3-7]. HC rats underwent the same surgical procedures and duct cannulation without sodium taurocholate infusion. In the SAP-S group, the rats received the first intraperitoneal injection of isotonic saline 2 h after induction of acute pancreatitis and a second injection after 12 h. In the SAP-ICE-I group, the rats were firstly given 0.25 mg of an ICE inhibitor (Ac-Tyr-Val-Ala-Asp-2,6-dimethylbenzoyloxymethylketone) dissolved in 1 mL sterile phosphate-buffered saline intraperitoneally 2 h after induction of pancreatitis. As in the SAP-S group, this was repeated at 12 h. Surviving rats were killed at certain time points, and all samples were obtained for subsequent analysis.

Measurement of serum IL-1β levels

Serum IL-1β levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (B&C Co.). All samples were tested in duplicate and expressed as the means.

RT-PCR examination of intrapulmonary Caspase-1, IL-1β and IL-18 mRNA

Reagents and primers: TRIZOL Reagent was purchased from Gibco BRL Life Technologies. One Step RNA PCR kit (AMV) was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. The sequences of IL-1β, IL-18 and β-actin primers (designed by Primer 3 software, synthesized by Sangon Biotechnology Co. Shanghai) were as follows: upstream and downstream primers, respectively: 5'-AAG GTC CTG AGG GCA AAG AG-3' and 5'-GTG TTG CAG ATA ATG AGG GC-3' for Caspase-1 (500 bp of amplification products); 5'-AGA AGC TGT GGC AGC TAC CT-3' and 5'-TTG GGA TCC ACA CTC TCC AG-3' for IL-1β (400 bp of amplification products); 5' -GCT GCA ATA CCA GAA GAA GG-3' and 5'-AGA TAG GGT CAC AGC CAG TC-3' for IL-18 (300 bp of amplification products); 5'-AGG GTG TGA TGG TGG GTA TG-3' and 5'-CAT AGC TCT TCT CCA GGG AG-3' for β-actin (600 bp of amplification products).

Total lung RNA extraction: Total RNA was extracted from the lung tissue by TRIZOL Reagent according to the manufacturer's protocol. One hundred mg of lung tissue was homogenized in 1 mL of TRIZOL Reagent. Following homogenization, insoluble material was removed from the homogenate by centrifugation at 12000 r/min for 10 min at 4℃ and the homogenized tissue was incubated for 5 min at a room temperature. Then 0.2 mL of chloroform was then added. The tube was shaken vigorously for 15 s and incubated at room temperature for 3 min. The sample was centrifuged at 12000 r/min for 15 min at 4℃ and the upper aqueous phase was transferred to another tube. After that 0.5 mL of isopropyl alcohol was added. The sample was incubated at room temperature for 10 min and centrifuged at 12000 r/min for 10 min at 4℃. The supernatant was discarded and the RNA pellets were washed with 1 mL of 75% ethanol. The samples were mixed by vortexing and centrifuged at 7000 r/min for 5 min at 4℃. At the end of the procedure, the RNA pellet was air-dried for 10 min, dissolved in 50 μL of DEPC water, and stored at -80℃. The A260/280 ratio was measured with an ultraviolet spectrophotometer and the RNA content was calculated (1A260 = 40 μg/mL).

RT-PCR was carried out using the One Step method. The total RT-PCR volume of each Eppendorf tube was 50 μL, including 5 μL of 10 × One Step RNA PCR buffer, 10 μL of $MgCl_2$ (25 mol/L), 5 μL of dNTPs (10 mol/L), 1 μL of RNase inhibitor (40 U/μL), 1 μL of AMV RTase XL (5 U/μL), 1 μL of AMV-Optimized Taq (5 U/μL), 1 μL of upstream specific primer, 1 μL of downstream specific primer, 1 μL of experimental sample(≤ 1 μg total RNA), 24 μL of RNase Free dH_2O. Each RT-PCR conditions were as follows: 30 min at 50℃ for RT reactions, 2 min at 94℃ for RTase inactivation, 30 s at 94℃, 30 s at 51℃, 90 s at 72℃ for 30 cycles (Caspase-1); 30 s at 94℃, 30 s at 53ºC, and 90 s at 72℃ for 30 cycles (IL-1β); 30 s at 94℃, 30 s at 55ºC, and 90 s at 72℃ for 30 cycles(IL-18); 30 s at 94℃, 30 s at 55℃, and 90 s at 72℃ for 35 cycles (β-actin). RT-PCR was terminated with an elongation step at 72℃ for 5 min, and PCR products were stored at 4℃. Five μL of the reaction products was visualized by electrophoresis in 2% agarose gels containing ethidium bromide. Ultraviolet illumination was used to visualize the DNA bands, and the gels were photographed digitally. Band intensity was determined by optical density with individual PCR product/β-actin cDNA ratios.

W/D ratio of the lung tissue

To assess tissue edema, the right lung was removed after the experiment, then weighed and dried in a 80℃ oven for 72 h until the weight was constant, and the ratio of wet weight to dry weight (W/D ratio) was then obtained.

Histologic examination

According to routine procedures, paraffin sections of the lung tissue samples were prepared by HE staining, and histologic alterations of lung tissue were observed by light microscopy.

Statistical analysis

All values were presented as mean ± SD. Statistical analysis was performed using SPSS 11.0 statistical software applying One-Way ANOVA. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

Serum IL-1β levels

In the SAP-S group and the SAP-ICE-I group, the serum IL-1β levels at all time points were significantly higher than those of HC ($P < 0.01$), whereas serum IL-1β levels were significantly decreased in the SAP-ICE-I group ($P < 0.01$) in comparison with the SAP-S group (Table 1).

Table 1 Serum IL-1β levels and W/D ratios of the lungs in rats with SAP (mean ± SD)

Group	*n*	IL-1β (pg/mL)	W/D ratio of lung (g/g)
HC	6	90.13 ± 21.13	4.32 ± 0.33
SAP-S			
6 h	6	276.77 ± 44.92[b]	4.85 ± 0.38[a]
12 h	6	308.99 ± 34.95[b]	4.97 ± 0.47[b]
18 h	5	311.60 ± 46.51[b]	5.03 ± 0.46[b]
SAP-ICE-I			
6 h	6	151.42 ± 27.26[b,d]	4.38 ± 0.36[c]
12 h	6	152.47 ± 29.60[b,d]	4.48 ± 0.37[c]
18 h	6	175.45 ± 29.72[b,d]	4.51 ± 0.36[c]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* HC group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* SAP-S group.

Table 2 Intrapulmonary expression of Caspase-1, IL-1β and IL-18 mRNA in rats with SAP (mean ± SD)

Group	*n*	Caspase-1 mRNA	IL-1β mRNA	IL-18 mRNA
HC	6	0.57 ± 0.06	0.42 ± 0.03	0.55 ± 0.05
SAP-S				
6 h	6	0.71 ± 0.04[b]	0.75 ± 0.05[b]	0.82 ± 0.05[b]
12 h	6	0.71 ± 0.05[b]	0.81 ± 0.06[b]	0.83 ± 0.06[b]
18 h	5	0.72 ± 0.04[b]	0.79 ± 0.07[b]	0.82 ± 0.07[b]
SAP-ICE-I				
6 h	6	0.72 ± 0.05[b]	0.52 ± 0.05[b,d]	0.58 ± 0.06[d]
12 h	6	0.72 ± 0.06[b]	0.50 ± 0.04[b,d]	0.55 ± 0.04[d]
18 h	6	0.69 ± 0.08[b]	0.49 ± 0.04[a,d]	0.57 ± 0.04[d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* HC group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* SAP-S group.

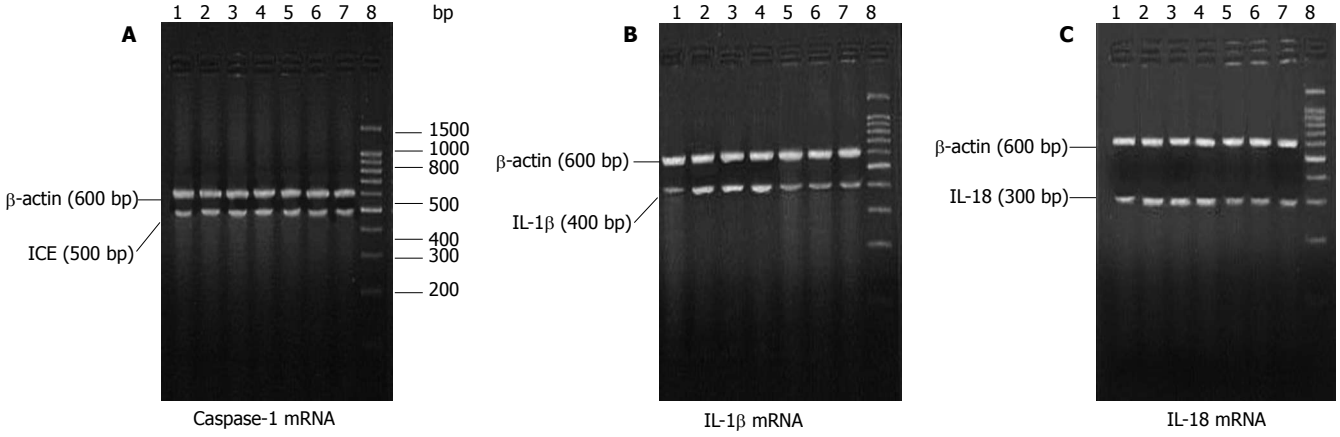

Figure 1 Intrapulmonary expression of Caspase-1, IL-1β and IL-18 mRNA in rats with SAP. Lane 1: HC; lanes 2-4: 6-18 h in SAP-S; lanes 5-7: 6-18 h in SAP-ICE-I; lane 8: Marker.

Intrapulmonary expression of Caspase-1, IL-1β and IL-18 mRNA

Intrapulmonary expression of Caspase-1, IL-1β and IL-18 mRNA were observed in the HC group, which were increased significantly in the SAP-S group ($P < 0.01$ *vs* HC). The expression of IL-1β and IL-18 mRNA were significantly decreased with ICE inhibition ($P < 0.01$), whereas Caspase-1 mRNA expression had no significant difference ($P > 0.05$) (Table 2, Figure 1 A-C).

W/D ratio of the lung tissue

In the SAP-S group, W/D ratios were increased significantly ($P < 0.05$ at 6 h, $P < 0.01$ at 12 h and 18 h, *vs* HC), and those were significantly attenuated with ICE inhibition ($P < 0.01$) (Table 1).

Pathologic alterations in lung tissue

Observed under a light microscope, the pulmonary structures of the HC group were basically normal. However, characteristics of typical lung injury could be seen in the SAP-S group, represented by marked congestion, edema and masses of inflammatory cell infiltration in pulmonary interstitium and aveoli, thickened alveolar septum, which became progressively severe after SAP induction. Compared with the SAP-S group, such pathologic changes were much less in the SAP-ICE-I group.

DISCUSSION

SAP is often complicated with multiple systemic organ failure (MSOF), which is the major cause of death in SAP. In extra-pancreatic organs, ALI is most prominent. About 20% of cases with SAP may develop adult respiratory distress syndrome (ARDS). One third of the patients die during the early stages of SAP, a half of which die as a result of ARDS[8,9]. At present, the role of cytokines in the pathogenesis of SAP has become a hot issue in the research field. Of the numerous cytokines, IL-1β and TNF-α have been confirmed to play an important role in the development of systemic complications of SAP. However, their roles in mediating ALI during SAP are not completely understood.

Caspase-1/ICE is one member of the family of cysteine proteases called Caspases. One of the major functions of ICE is the proteolytic cleavage of the 31 000 molecular weight IL-1β precursor into its biologically active 17 000 form. IL-1β is an inflammatory cytokine produced by activated lymphocytes and monocytes. IL-1β and TNF-α are primary inducers of IL-6 and IL-8 production, and are known to cause fever, hypoperfusion, circulatory collapse, shock, metabolic acidosis, cardiac dysfunction, and the occurrence of ARDS. Norman *et al*[10] showed that no IL-1β mRNA expression was found in the lungs of healthy mice, and that intrapulmonary IL-1β mRNA and protein expression were increased after SAP

induction. Paszkowski *et al*[11] demonstrated that IL-1β mRNA expression could be found in the lungs of healthy rats, and that intrapulmonary IL-1β mRNA expression was upregulated in SAP, in proportion to the severity of ALI, suggesting the existence of a significant correlation between overproduction of IL-1β in the lungs and ALI in SAP.

Ac-Tyr-Val-Ala-Asp-2,6-dimethylbenzoyloxymethylketone used in this study is a highly competitive and irreversible inhibitor of ICE. It inactivates the enzyme and is relatively inert toward other bionucleophiles such as glutathione. Previous studies showed changes in serum amylase and pancreas in SAP[12,13]. In this study, the ICE inhibitor was injected into rats intraperitoneally 2 h after SAP was induced by retrograde infusion of 5% sodium taurocholate into the bili-pancreatic duct. A remarkable elevation of serum IL-1β levels, an upregulation of intrapulmonary Caspase-1 and IL-1β mRNA expression, an increased W/D ratio of the lung and obvious pathological changes after induction of SAP were observed; serum and intrapulmonary IL-1β expression were decreased significantly, with an improvement of pathological changes in rats treated with ICE inhibitors. These suggest that the therapeutic effects of ICE inhibitors on ALI in SAP may be associated with a reduced IL-1β-mediated injury. Besides generating large numbers of active IL-1β, ICE is also known to process the inactive precursor of IL-18 into its bioactive forms[14-17]. IL-18, formerly called IFN-γ-inducing factor, is a novel proinflammatory cytokine with an 18000 molecular weight, sharing striking structural and functional similarities to IL-1β. In addition, the biological activity of IL-18 is closely related to that of IL-1β: IL-18 induces the gene expression and synthesis of IL-1, TNF, and several chemokines by means of a putative IL-18 receptor complex. Also, IL-18 plays an important role in the Th-1 response to the stimulation of virul antigens, primarily because of its ability to induce IFN-γ production in T cells and NK cells. Rau *et al*[18] reported that local and systemic IL-18 concentrations are significantly elevated in patients with AP, and that serum IL-18 concentrations closely correlate with the development of pancreatic necrosis and remote organ failure. In the current study, intrapulmonary IL-18 mRNA expression was measured by RT-PCR. Marked upregulation of IL-18 mRNA was observed in the lungs after induction of SAP, and intrapulmonary IL-18 expression was significantly decreased in rats treated with ICE inhibitors. In addition, it is well established that IL-1β, IL-18, and TNF-α share a close interrelationship by inducing the synthesis of each other[14,19,20]. Paszkowski *et al*[11] indicated that intrapulmonary TNF-α mRNA expression was uniformly downregulated in rats receiving the treatment of ICE inhibitors, suggesting that the therapeutic effects of ICE inhibitors on ALI in SAP may correlate with a decrease in TNF-α-mediated injury. Considering that the synthesis of IL-1β, IL-18, and TNF-α could be induced by each other, plus the alterations in W/D ratios of the lungs and histopathologic changes in SAP, we speculate that as with TNF-α and IL-1β, overproduction of IL-18 in the lungs plays an important role in the course of SAP complicated with ALI, and that therapeutic effects of ICE inhibitors on ALI in SAP may be associated with the inhibition of IL-18.

In summary, activation of Caspase-1/ICE, and overproduction of IL-1β and IL-18 in the lungs play an important role during the course of ALI and ARDS in SAP, and ICE inhibitors are effective against ALI in SAP. The mechanisms for ICE inhibition may be associated with decreased cytokine-mediated injury, such as IL-1β, IL-18 and TNF-α. Therefore, studies of the mechanism for ALI in SAP may shed new light on understanding of SIRS and MSOF, as well as the prevention and treatment for SAP.

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RAPID COMMUNICATION

Evaluation of prognostic markers in severe drug-induced liver disease

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Abstract

AIM: To analyze the outcome of patients with severe drug-induced liver disease (DILD) associated with jaundice classified as hepatocellular, cholestatic or mixed liver injury and to evaluate the validity of Hy's rule and the most important predictors for outcome.

METHODS: The Adverse Drug Reaction Advisory Committee was set up in 1997 in our hospital to identify all suspicions of DILD following a structured prospective report form. Liver damage was divided into hepatocellular, cholestatic, and mixed types according to laboratory and histologic criteria when available. Further evaluation of causality assessment was performed.

RESULTS: From January 1997 to December 2004, 265 patients were diagnosed with DILD, and 140 (52.8%) of them were female. hepatocellular damage was the most common (72.1%), the incidence of death was 9.9% in patients with hepatocellular damage and 9.5% in patients with cholestatic/mixed damage ($P < 0.05$). There was no difference in age of dead and recovered patients. The proportion of females and males was similar in recovered and dead patients, no difference was observed in duration of treatment between the two groups. The serum total bilirubin ($P < 0.001$), direct bilirubin ($P < 0.001$) and aspartate transaminase (AST) ($P = 0.013$) values were higher in dead patients than in recovered patients. Chinese herbal medicine was the most frequently prescribed, accounting for 24.2% of the whole series. However, antitubercular drugs (3.4%) were found to be the primary etiological factor for fetal DILD. Factors associated with the development of fulminant hepatic failure were hepatic encephalopathy (OR = 43.66, 95% CI = 8.47-224.95, $P < 0.0001$), ascite (OR = 28.48, 95% CI = 9.26-87.58, $P < 0.0001$), jaundice (OR = 11.43, 95% CI = 1.52-85.96, $P = 0.003$), alcohol abuse (OR = 3.83, 95% CI = 1.26-11.67, $P = 0.035$) and direct bilirubin (OR = 1.93, 95% CI = 1.25-2.58, $P = 0.012$).

CONCLUSION: Death occurs in 9.8% of patients with DILD. Chinese herbal medicine stands out as the most common drug for DILD. While antitubercular drugs are found to be the primary etiological factor for fetal DILD, hepatic encephalopathy, ascites, jaundice, alcohol abuse and direct bilirubin levels are associated with the death of DILD patients.

Key words: Drug-induced liver disease; Prognosis; Prognostic marker; Mortality

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INTRODUCTION

Drug-induced liver disease (DILD) is an adverse drug reaction-induced disease. Almost all drugs can elevate liver enzyme level and cause DILD. However, the majority of drugs exhibit low incidences of hepatic adverse reactions. Therefore, DILD is mostly identified only after broad clinical drug application (phase Ⅳ). Well-established causes of DILD include non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, antiepileptics, statins, tuberculostatics and herbal medicines[1,2].

High serum aminotransferase (hepatocellular injury) and jaundice levels induced by different drugs have been reported to result in a mortality of 10%-50%[3-5]. These observations have been named "Hy's rule" after Hyman Zimmerman, who first described them. The rule states that if both drug-induced hepatocellular injury and jaundice occur simultaneously without biliary obstruction, a mortality of at least 10% can be expected[3,5,6]. Hy's rule defined as DILD with serum alanine aminotransferase (ALT) levels 3 or more times the upper limit of normal

(ULN) + serum bilirubin levels 2 or more times the ULN level, has been advocated by the US Food and Drug Administration for use in the assessment of hepatotoxicity of newly developed drugs[5,7]. However, this rule has never been scientifically validated. The sensitivity and specificity of clinical jaundice for the outcome in patients with drug-induced hepatocellular (HC) injury are unknown. The most important predictors of outcome in DILD with HC injury have not been analyzed in a large number of patients. Furthermore, information about the prognosis in other forms of DILD (e.g., DILD with cholestatic or mixed damage) is limited.

In our hospital, a systematic monitoring system for DILD has been in use since 1997, with regular causality assessment offering the opportunity to evaluate a large number of patients with DILD. The aim of this study was to analyze the outcome of patients with severe DILD associated with jaundice classified as HC, cholestatic (CS), or mixed liver injury and to evaluate the validity of Hy's rule and the most important predictors for outcome.

MATERIALS AND METHODS

All reports of suspected drug-induced liver injury received by the Adverse Drug Reaction Advisory Committee (ADRAC) in our hospital between 1997 and 2004 have been computerized and are available for legally acceptable users with a password online. Our analysis was restricted to patients with serum bilirubin levels two-fold higher than the ULN level. Furthermore, in patients with HC injury, our analysis was restricted to those patients with ALT levels 3 or more times the ULN level as well as serum bilirubin levels 2 or more times the ULN level.

A total of 301 reports fulfilling these criteria were evaluated using international consensus criteria [RousselUclaf causality assessment method (RUCAM)][8,9] to assess the probability of a causal relationship between drug exposure and liver disease. Causality assessment was performed based on information about the onset time of reaction of the drug, the development of liver tests after cessation of the drug, the presence of risk factors, and known hepatotoxicity of the suspected drug and concomitant drug or drugs[8]. Furthermore, investigations were performed to exclude non-drug causes for the reaction. Thus, abnormal liver tests shortly after the use of a new drug, rapid decline of abnormal liver test values after stopping the drug, and exclusion of other causes gave high scores compatible with the drug as a possible, probable, or highly probable cause of the reaction[8]. If the report did not receive a high enough score to consider a causal relationship with the suspected drug, the reaction did not likely occur in accordance with the criteria or the relationship was excluded. Each author scored approximately one fifth of the cases. We all performed assessment of 100 cases independently and found very low intraobserver variability with no disagreement in the assessment of cases.

Because many patients had been exposed to several drugs at the time when liver injury occurred, it is not always possible to deduce which drug is most likely responsible for it. In such cases, the reaction was judged to bepotentially caused by more than one drug. On the other hand, if there was a close temporal relationship between the liver injury and treatment of patients with only one of many drugs, which was then considered to be the suspected drug.

The computerized reports include all relevant facts from medical records and the results of laboratory investigations. The following information was collected from the reports: duration of exposure, drug(s) suspected to be responsible, age and sex of the patients, duration of treatment, type of liver injury, results of AST and ALT as well as alkaline phosphatase (ALP) and bilirubin tests, nondrug causes, and outcome of the patients (recovery, death or liver transplantation).

The type of liver damage was classified according to the International Consensus Meeting criteria[8,9], using ALT and alkaline phosphatase activity, expressed as a multiple of the upper limit of normality, to determine the ratio (R) of ALT/AP. The type of liver damage was hepatocellular when $R > 5$, cholestatic when $R < 2$, and mixed when $R > 2$ but < 5. The liver tests used for the classification of liver damage were the first blood test available after liver injury.

Statistical analysis

For descriptive purposes, Fisher's exact test was used to test differences in dichotomous variables between groups. Mann-Whitney test was used for continuous variables. Stepwise logistic regression was performed for multivariate purposes to predict death. All tests were two-tailed and conducted at a 5% significance level.

RESULTS

During 1997-2004, ADRAC received 301 reports of suspected DILD. Of which 265 reports of DILD fulfilled the RUCAM criteria for at least a possible relationship. According to the RUCAM criteria, 22 reports (8.3%) had a possible relationship, 183 (69.1%) a probable relationship, and 60 (22.6%) a highly probable relationship. These 265 reports with a possible/probable/highly probable relationship to drug(s) included 191 with HC injury, 51 with CS injury, and 23 with mixed liver injury (Table 1).Table 1 shows the age and sex, duration of treatment, and peak liver test values in patients with different types of DILD.

There were no differences in the age of patients with HC or CS or mixed injury ($P = 0.127$). A higher proportion of females were observed in all different subgroups, in which females accounted for 53%. The total protein was different both in patients with HC and CS injury ($P = 0.003$) and in those with CS and mixed injury ($P = 0.041$).The difference in albumin between patients with HC and CS injury was significant ($P < 0.001$). Total and direct bilirubin levels were higher in patients with CS injury than in those with HC injury ($P = 0.043$ and $P < 0.001$ respectively) and mixed injury ($P < 0.001$ and $P = 0.01$ respectively). Obviously, the ALT, AST and ALP values were different in patients with mixed injury or with HC or CS injury.

Twenty-six (9.8%) of the 265 patients died of liver

Table 1 A age and sex, duration of treatment, and peak liver test values in patients with different types of DILD

	Total	Hepatocelluar	Cholestatic	Mixed
Number	265	191	51	23
Age ($\bar{x} \pm s$)	48.6 ± 13.7	47.8 ± 13.8	52.1 ± 13.0	47.3 ± 14.0
Sex (F/M)	140/125	100/91	27/24	13/10
Duration of	25.9	24.6	31.4	21.7
treatment (d)	(1-121)	(1-120)	(8-121)	(1-47)
Total bilirubin	95.7	67.5	236	105.5
(μmol/L)	(7-701)	(7-701)	(11-615)	(14-630)
Direct bilirubin	49.4	31.8	126.8	47.5
(μmol/L)	(2-461)	(2-338)	(3-461)	(3.3-281)
Total protein (g/L)	66.9 ± 9.4	67.7 ± 9.6	63.3 ± 8.8	68.1 ± 8.0
Albumin (g/L)	38.1 ± 7.3	39.1 ± 6.9	34.9 ± 8.3	36.4 ± 6.2
ALT (U/L)	351.5	313.8	123.4	557
	(13-2652)	(20-2652)	(13-1079)	(92-1600)
AST (U/L)	230.1	282.9	111.8	393
	(18-1925)	(19-1925)	(18-1165)	(48-1553)
ALP (U/L)	141.8	69.6	117.8	318.8
	(51-1165)	(51-370)	(70-1165)	(147-801)
GGT (U/L)	200.7	112.9	254	241
	(16-3102)	(17-788)	(16-3102)	(87-969)

The laboratory parameters are all peak values before treatment.

Table 2 Comparison between died and recovered patients with DILD

	Died	Recoverd	*P*
Age	52 ± 13.8	48 ± 13.7	0.187
Sex (F/M)	13/13	127/112	NS
Duration of treanment (d)	29 (1-79)	25.7 (1-121)	NS
Total bilirubin	333.3 (38-695)	82.7 (7-701)	< 0.001
Direct bilirubin	218.2 (11.5-330)	44.0 (2-461)	< 0.001
ALT	351 (78-1906)	341.8 (13-2652)	0.293
AST	339 (52-1700)	213.8 (18-1925)	0.013
ALP	139.1 (51-561)	144.4 (51-1165)	0.805
GGT	124.8 (44-1483)	203.8 (16-3102)	0.193

The laboratory parameters are all peak values. Results are expressed as mean ± SD or medians.

failure (Table 2).The following drugs were associated with death: antitubercular drugs (n = 9), medicinal herbs (n = 5), immunodepressants (n = 2), antimycotic drugs (n = 2), antiinfection drugs (n = 1), nonsteroidal anti-inflammatory drugs (n = 1), antidepressant drugs (n = 1), antithyroid drugs (n = 1), antigout preparation (n = 1), and other drugs (n = 3). There were no differences in age of the dead and recovered patients. The proportion of females and males was similar in recovered and dead patients, and no difference was observed in duration of treatment between them.

The levels of serum total bilirubin, direct bilirubin and AST were higher in dead patients than in recovered patients, whereas the levels of ALT, ALP and GGT were similar in the two groups.

A comparison between the dead and recovered patients in the HC group revealed no differences in age, sex, duration of treatment, ALT or ALP (Table 3). Total bilirubin and AST levels were higher in deceased patients with HC injury than in those with CS/mixed injury, while total bilirubin levels were significantly higher only in deceased patients (Table 3).

Table 3 Comparison between died and recovered patients with hepatocellular injury or with cholestatic/mixed liver injury

	Hepatocellular injury		Cholestatic/mixed injury	
	Died	Recovered	Died	Recovered
Number	19	172	7	67
Age	51.3 ± 13.4	47.5 ± 13.9	54.0 ± 15.7	50.3 ± 13.2
Sex, F/M (%)	11/8	89/83	2/5	38/29
Duration of	30.8	23.8	22	30
treatment	(1-79)	(1-120)	(13-40)	(1-121)
Total	330.5	67.6	169.6	83.8
bilirubin	(38-695)	(7-701)[b]	(50-630)	(11-615)[b]
ALT	488.5	414.5	230.5	192.5
	(106-2652)	(20-1906)	(78-692)	(13-1600)
AST	392.3	270.8	265.5	143.0
	(99-170)	(19-1925)[a]	(52-1272)	(18-1553)
ALP	133.7	130.1	268	310.1
	(51-370)	(51-266)	(70-561)	(72-1165)

The laboratory parameters are all peak values. Results are expressed as mean ± SD or medians. [a]$P < 0.05$, [b]$P < 0.001$ *vs* the control group.

Table 4 Factors associated with death of the patients with DILD

Independent variables	Coefficient	OR (95% CI)	*P*
HE	2.232	43.66 (8.47-224.95)	< 0.001
Ascite	2.883	28.48 (9.26-87.58)	< 0.001
Jaundice	1.124	11.43 (1.52-85.96)	0.003
Alcohol abuse	1.511	3.83 (1.26-11.67)	0.035
Direct bilirubin	-0.007	1.93 (1.25-2.58)	0.012

CI = confidence interval; OR = odds ratio, HE = hepatic encephalopathy, Constant = -15.37.

Logistic regression analysis showed that hepatic encephalopathy ($P < 0.001$), ascite ($P < 0.001$), jaundice ($P = 0.003$), alcohol abuse ($P = 0.035$) and direct bilirubin ($P = 0.012$) could independently predict death (Table 4).

The drugs associated with DILD are listed in Table 5. The largest number of reports on drug-induced fatal HC injury was related to antitubercular drugs (because only 2 DILDs were associated with antigout drug, we did not calculate the mortality induced by this drug). In this group, 7 out of the 20 patients died (35%).The second most commonly reported drug type associated with mortality was antifungal agents (33.3%). The mortality ranging from 35% of antitubercular drugs to 0% in reports is related to many other drugs. As in CS/mixed injury, the highest number of reports of death is related to immunosuppressive agent (28.6%). The mortality ranges from 0% with most of the drugs to 28.6%. Overall, antitubercular drugs (32.1%) are the primary etiological factor for fetal DILD.

DISCUSSION

Drug-induced hepatotoxicity remains a challenge to modern hepatology. Hepatotoxicity is typically detected when several thousands of patients are exposed to drugs, and regulatory authorities are often compelled to make dicisions based on scanty, fragmentary, and incomplete

Table 5 Patients with hepatocellular, cholestatic or mixed liver injury and their death due to different drugs

	Hepatocellular	Death	Cholestatic/mixed	Death	Total study group	Death
Antituberculous drugs	20	7	8	2	28	9
Immunodepressant	26	0	7	2	33	2
Antineoplastic agent	8	0	6	0	14	0
Antibiotics	17	1	8	0	25	4
Chinese herbal medicine	51	5	13	0	64	5
Antipyretic analgesic	8	1	2	0	10	1
Antidepressant drug	5	0	5	1	10	1
Cardiovascular drugs	10	0	1	0	11	0
Sedative hypnotics	1	0	0	0	1	0
Drugs for peptic ulcer	1	0	0	0	1	0
Antithyroid drugs	12	0	11	1	23	1
Antifungal agent	6	2	2	0	8	2
Hypoglycemic agent	5	0	2	0	7	0
Drugs for prostate	1	0	0	0	1	0
Antigout drug	2	1	0	0	2	1
Others	18	2	9	1	27	3

epidemiologic data. In addition, a major challenge is the ability to identify predisposed subjects before they receive drugs. The susceptibility of individuals to genetic and environmental factors is still poorly understood. In this study, we analyzed cases of toxic liver injury prospectively collected from our hospital during the past 8 years.

Hyman Zimmerman, the pioneer in the field of DILD, observed that combined HC injury (high aminotransferase) and jaundice induced by a drug is associated with the poor prognosis of patients, with a fatality rate of 10%-50% for different drugs involved (Hy's rule)[3-6]. It was reported that a new drug should be stopped in patients if their AST and ALT levels are 3-fold higher than ULN level, and bilirubin levels are 2-fold higher than ULN level[7]. Concomitant jaundice and hepatocellular injury observed in clinical trials of new drugs are considered to cause serious troubles concerning safety in the postmarketing phase, when a much larger number of patients are exposed to drugs[5].

Our analysis is unique because it was performed in a large cohort of patients with severe DILD, giving the opportunity to elucidate the most important predictors for outcome. Adverse drug reactions are significantly underreported. The true incidence of hepatic adverse drug reactions has been recently observed. However, a recent prospective survey of drug-induced liver injury in the general population in France suggests that at most, only 1 out of 16 cases of DILD in France is actually reported[10].

Heptatotoxicity was found in a higher proportion of females (53%) in our study, which is consistent with the reported epidemiologic data[10,11]. No difference was found in age of the deceased and recovered patients in our study. A recent study from Japan reported that there is also no difference in age of decreased and recovered patients with DILD[12]. The levels of serum total bilirubin and AST were higher in deceased patients than in recovered patients, whereas ALT, ALP and GGT levels were similar in the two groups in the current study, suggesting that hepatic encephalopathy, ascites, jaundice, alcohol abuse and direct bilirubin increase the risk of death in patients with DILD.

We found that the main causative drugs were Chinese herbal medicine (24.2%), followed by immunosuppressive agents (12.5%), antituberculous drugs (10.6%), antibiotics (9.4%), antithyroid drugs (8.7%). But antituberculous drugs (32.1%) were the leading cause of fatal DILD.

In summary, Chinese herbal medicine is the most common drug associated with liver injury, and the mortality rate is 9.8% in patients with DILD. Hepatic encephalopathy, ascites, jaundice, alcohol abuse and direct bilirubin are associated with the death of patients with DILD. Our ADRAC has proved to be an effective instrument in detecting cases of idiosyncratic liver disease and delineating a profile of risk factors for severity. Further efforts must be made to prevent hepatic adverse reactions to drugs.

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RAPID COMMUNICATION

Diagnosis and management of colonic injuries following blunt trauma

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Abstract

AIM: To retrospectively evaluate the preoperative diagnostic approaches and management of colonic injuries following blunt abdominal trauma.

METHODS: A total of 82 patients with colonic injuries caused by blunt trauma between January 1992 and December 2005 were enrolled. Data were collected on clinical presentation, investigations, diagnostic methods, associated injuries, and operative management. Colonic injury-related mortality and abdominal complications were analyzed.

RESULTS: Colonic injuries were caused mainly by motor vehicle accidents. Of the 82 patients, 58 (70.3%) had other associated injuries. Laparotomy was performed within 6 h after injury in 69 cases (84.1%), laparoscopy in 3 because of haemodynamic instability. The most commonly injured site was located in the transverse colon. The mean colon injury scale score was 2.8. The degree of faecal contamination was classified as mild in 18 (22.0%), moderate in 42 (51.2%), severe in 14 (17.1%), and unknown in 8 (9.8%) cases. Sixty-seven patients (81.7%) were treated with primary repair or resection and anastomosis. Faecal stream diversion was performed in 15 cases (18.3%). The overall mortality rate was 6.1%. The incidence of colonic injury-related abdominal complications was 20.7%. The only independent predictor of complications was the degree of peritoneal faecal contamination (P = 0.02).

CONCLUSION: Colonic injuries following blunt trauma are especially important because of the severity and complexity of associated injuries. A thorough physical examination and a combination of tests can be used to evaluate the indications for laparotomy. One stage management at the time of initial exploration is most often used for colonic injuries.

Key words: Colonic injuries; Blunt trauma; Operation; Faecal stream diversion; Rretrospective study

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INTRODUCTION

Although the colon is often injured in case of penetrating abdominal trauma, a significant proportion of colonic injuries caused by road accidents is a grossly destructive blunt type associated with damage to multiple organs[1-3]. The diagnosis and management of blunt colon injuries are still debatable. The aim of this retrospective study was to evaluate the preoperative diagnostic methods and management of colonic injuries following blunt abdominal trauma.

MATERIALS AND METHODS

Subjects

All patients with colonic injuries caused by blunt trauma presenting to the Emergency Center of the Second Affiliated Hospital of School of Medicine of Zhejiang University between January 1992 and December 2005 were enrolled. The criterion for inclusion in the study was full thickness perforation of colon injuries requiring surgical repair. Data were collected on clinical presentation, investigations, diagnostic methods, associated injuries, operative management, morbidity and mortality.

Haemodynamic status was determined based on their heart rate and systolic blood pressure (BP) on admission. A systolic BP equal to or < 90 mmHg on admission was interpreted as haemodynamic instability or presence of shock. The time from injury to operation was recorded. The site of colon injury (right colon defined as the right of the middle colic vessels, left colon the left of the vessels) and major associated injuries of the head, thorax, pelvis, axial skeleton, major blood vessels and long bones were recorded.

The severity of colon injury was graded according to the colon injury scale (CIS) score[4]. CIS score was definited as follows: grade 1: contusion and serosal tear without devascularization, grade 2: laceration of less than 50% of the wall, grade 3: laceration of 50% or greater of the wall, grade 4: 100% transection of the wall, and grade 5: complete transection with tissue loss and devascularization, an advanced grade for multiple injuries to the colon. The degree of faecal spillage (the gross extent of intra-abdominal faecal contamination) was categorized as mild: stool contamination on local or one quadrant, moderate: stool contamination on 2 to 3 quadrants, and severe: stool contamination on all four quadrants[5].

Methods

All patients were resuscitated and received intravenous antibiotics in the emergency room. The discretion of operative options was based on Stone's exclusion factors for primary repair[6] and surgeons' experience. The outcome variables of the study included colonic injury-related mortality and abdominal complications (anastomotic leak, intra-abdominal abscess or peritonitis, and colon obstruction or necrosis, if it was judged to be directly related to the colonic trauma).

Statistical analysis

All analyses were carried out by SPSS 12.0 statistical software. Independent predictors for colostomy and post-operative complications were determined by entering potential confounders into a multivariate stepwise (backward elimination) logistic regression. Variables considered in the model for colostomy included age, mechanism of injury, shock on admission, CIS, degree of peritoneal faecal contamination, location of colon injury, and associated intra-abdominal injury. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic data

A total of 82 patients were included in this study. There were 77 males (93.9%) and 5 females (6.1%). Their age ranged 15-67 years with a mean of 37.6 years. Colonic injury was found in 57 patients (69.5%) due to motor vehicle accidents, in 18 (22.0%) due to building accidents, in 6 (7.3%) due to criminal assault, and in 1 (1.2%) due to burst injury.

Clinical presentation

Abdominal signs could not be detected in 8 cases (9.8%) because of head injuries, intoxication or sedation. Seventy patients (94.6%) had moderate to severe abdominal tenderness, 18 (24.3%) had diffuse peritonism, 23 (28.0%) had shock on admission. In addition, hematuria was found in 12 patients (14.6%), paraplegia in 2 (2.4%), aerocele of scroticles in 2 (2.4%) patients. Plain abdominal radiograph was performed to find pneumoperitoneum and intestinal obstruction in 54 patients. Diagnostic peritoneal lavage (DPL) or paracentesis was performed in 65 cases, which was positive in 43 cases (noncongested blood in 20 cases, pus in 23 cases). Abdominal ultrasonography (US) and computed tomography (CT) were performed in 58 and 10 cases respectively. Among them, 12 were diagnosed as gastrointestinal injury with intraperitoneal free fluid.

Table 1 Associated injuries in 82 patients with blunt colonic injuries

	n
Intra-abdominal	
Small bowel	42
Spleen	11
Liver	9
Kidney	4
Urinary bladder	3
Pancreas	2
Ureter	2
Stomach	2
Duodenum	1
Diaphragm	1
Extra-abdominal	
Head	12
Chest	6
Vascular peripheral	5
Fracture vertebral lumbar	5
Fracture pelvis	2

Associated injuries

Fifty-eight patients (70.3%) were found to have one or more associated injuries (Table 1). The most commonly associated intra-abdominal injury occurred in the small bowel (51.2%), followed by in the speen, liver, and kidney. Multiple colonic wounds were observed in 4 cases (4.9%), Isolated colon injury in 20 cases (24.4%). The range of intra-abdominal organs injured was 1-4, with a mean of 2.3.

Timing and indications for laparotomy

Seven patients (8.5%) underwent immediate laparotomy (< 2 h after injury), 4 for severe peritonitis and 3 due to haemodynamic instability. Laparotomy was performed between 2 h and 6 h after injury in 62 cases (75.6%). Of them, 33 had a laparotomy because of abdominal signs with evidence of peritonitis at admission or during observation, 35 because of positive DPL or paracentesis. Eighteen (51.4%) of these patients had more than one significant intra-abdominal injury. An abdominal CT scan or US imaging with diagnostic or suspicious findings was the main reason for laparotomy in 15 cases (18.3%). Colonic injuries were found in 2 patients at diagnostic laparoscopy (Figure 1).

Site and nature of injuries

A total of 87 colonic injuries were found in 82 patients. The most often wounded site was located in the transverse colon (32 cases, 36.8%). The right colon injury was found in 21 cases, the descending colon injury in 16, the sigmoid colon injury in 13, and the intraperitoneal rectum injury in 5. The mean CIS score was 2.8 ± 1.2. The degree of faecal contamination was classified by the operating surgeon as mild in 18 cases (22.0%), moderate in 42 (51.2%), severe in 14 (17.1%), and unknown in 8 (9.8%).

Management and prognosis

Therapeutic options were considered: two-stage management for those with any type of faecal stream diversion, while one stage management for those undergoing primary repair of the injured colon with or without anastomosis. The successful rate for colonic wounds without diversion was 81.7% (67 cases). Primary repair was undertaken in 37 cases with resection and primary anastomosis in a further 30 cases. Two-stage operation was performed in 15 cases (18.3%): repair and protective ostomy in 11 cases, exteriorisation of the repaired bowel in 3 cases, Hartmann's operation in 1 case. The overall mortality rate was 6.1% (5/82). The overall incidence of colonic injury-related abdominal complications was 20.7% (17/82). The most common complications were anastomotic leak (12 cases), intra-abdominal abscess (10 cases), wound infection (12 cases) and colon obstruction or necrosis (4 cases). The only independent predictor of complications was the degree of peritoneal faecal contamination (P = 0.02). There was no significant correlation between age, mechanism of injury, shock on admission, location of colon injury, therapeutic options and outcome in terms of morbidity and mortality.

DISCUSSION

Injuries of the hollow viscera are far less common in blunt abdominal trauma than in penetrating abdominal trauma. Blunt abdominal trauma accounts for approximately 5% to 15% of all operative abdominal injuries[3,7]. The majority of colonic injuries caused by penetrating trauma are dominant[1-3,5]. Nevertheless, in our experience about 6.5% of patients with blunt trauma at admission had injuries to the colon and rectum, which is slightly higher than the reported 5%[8]. Despite their infrequence, traumatic blunt injuries to the colon are extremely destructive and generally associated with damage to multiple organ systems, making diagnosis and treatment difficult. It was reported that delayed management of colonic injuries results in a high incidence of morbidity[9]. Therefore, further researches on guidelines for the diagnosis and surgical management of colonic injuries following blunt trauma are especially important.

No clinical investigations are available to compare with gastrointestinal tract injuries. Moreover, clinical assessment can be unreliable in patients following blunt trauma due to distracting injuries, head and spinal cord injuries, and shock. Less than 50% of gastrointestinal tract injuries resulting from blunt trauma are reported to have sufficient clinical findings to indicate the need for laparotomy[10]. In this study, 3 patients with unstable haemodynamics undergoing immediate laparotomy (< 2 h) showed marked evidence for abdominal injury. The other 4 patients with gross abdominal distension and marked tenderness were also immediately operated. In 6 patients presented within two hours, abdominal signs were vague at initial evaluation but became marked over a few hours at a repeated examination. The finding of abdominal signs in the other 27 cases presented between two and six hours after trauma resulted in laparotomy. Tenderness or other abdominal findings were usually apparent within 24 h.

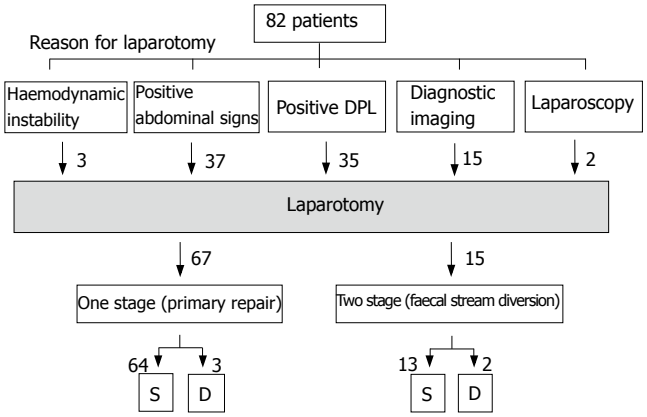

Figure 1 Outcome of 82 patients with colonic injuries. S = survived, D = died.

Physical examination and diagnostic tests can be used to evaluate patients with blunt abdominal trauma, including DPL, US, CT, and diagnostic laparoscopy. Speed and efficiency are important factors in the performing such tests[3]. It is reported that peritoneal lavage cell count may also be useful in early detection of hollow viscus injury[11,12]. Although DPL is sensitive in identifying haemoperitoneum and associated hollow viscus injury, it has been criticised for its higher rate of non-therapeutic laparotomy (NTL) and inconvenience in practice[12]. In this study, the presence of positive DPL or paracentesis was an important clinical finding. The routine use of diagnostic celiocentesis to detect possible intra-abdominal injuries in cardiovascularly stable patients has been used to differentiate between injuries that require a therapeutic laparotomy and those that do not[13]. Suspicious diagnosis of gastrointestinal tract injuries was indicated in 35 cases in this study. However, the diagnostic rate of colonic injuries by DPL or celiocentesis was decreased over the study period, which may be due to the increased use of imaging techniques to assess haemodynamically stable trauma patients.

US is convenient, cheap and noninvasive. A positive study is defined as evidence of free fluid or solid-organ parenchymal injury. Abdominal CT is also useful in the diagnosis of abdominal injuries as it accurately delineates solid organ injuries and retroperitoneal lesions. While some advocate limiting imaging tests to evaluation of patients with DPL-positive results and haemodynamic stability, US and CT remain the preferred tool in the evaluation of blunt abdominal trauma[3,14]. The accuracy of abdominal US for evaluating blunt abdominal trauma is comparable to the reported accuracy[15]. However, only 10 out of the 58 scans in our study could diagnose intra-abdominal gastrointestinal tract injuries with 5 being suspicious of a significant intra-abdominal injury. Some patients with free fluid but no evidence of a solid viscus injury might presumably be overlooked.

Although the role of laparoscopy in abdominal trauma is controversial[16], diagnostic laparoscopy has been introduced in our emergency center. Its indications have expanded from identifying the causative pathology of acute abdominal pain to avoidance of unnecessary laparotomies, treatment of intra-abdominal lesions, and

can be used as a resource for evaluating blunt abdominal trauma. Diagnostic laparoscopy was performed in 2 cases in our study and some direct indications for colonic injuries (such as faecal spillage, colon rupture) were found in both cases. Take together, the indications for laparotomy were determined according one of the following findings: haemodynamic instability with reasonable clinical suspicion of an intra-abdominal cause, positive abdominal signs, positive DPL, positive diagnostic imaging and abdominal finding by laparoscopy.

The management of colonic injuries has changed significantly from "faecal diversion dogma" to primary repair[2,3]. Although several studies showed that diversion is not mandatory, additional considerations in management should be taken into account regarding grossly destructive colon injuries. In our study, mild, moderate and severe faecal contamination was found in 22.0%, 51.2%, and 17.1% of patients, respectively at laparotomy. In 15 patients (18.3%), primary laparotomy was terminated before the completion of definitive surgery (abbreviated laparotomy or damage control).

It was reported that the mortality of colonic injuries have declined to 2%-12%[1,3,17]. Primary closure or resection and anastomosis can be used in patients with colonic injury. The results are generally favorable, due to the advances in intensive care techniques and antibiotic therapy. Primary repair reduces operation and postoperative complications, avoids a second operation, stoma complications, and the financial burden related to colostomy care. A number of factors have been traditionally accepted to be associated with higher mortality and morbidity of primary colonic repair. It was reported that patients should be excluded from primary repair in the presence of shock, major blood loss, > two organs injured, faecal contamination higher than 'mild', delay of repair > 8 h and destructive wounds of the colon or abdominal wall requiring resection[6]. The grade of colonic injuries trends to be independently associated with intra-abdominal complications. In our study, the overall mortality rate was 6.1%. Although neither grade of injury nor ostomy formation demonstrated a significant impact on morbidity, peritoneal faecal contamination has shown its significant predictive value for complications. We advocate that peritoneal faecal contamination should be thoroughly removed during operation to reduce postoperative abdominal septic morbidities. There was no difference between patients with primary repair and faecal stream diversion. However, other organ injuries must be kept in mind. Colostomy may be indicated due to unusual conditions, such as intramural hematomas causing compression ischemia and delayed perforation, mesenteric hematomas causing vascular compression with subsequent infarction, and perforations in omentum or other surrounding organs[3]. All together, the decision for a primary anastomosis, especially after segmental resection in the descending colon, should be individualized according to the injuries in different patients.

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CASE REPORT

A case of acute infectious mononucleosis presenting with very high ferritin

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Abstract

Hepatitis is an important but uncommon manifestation of acute Epstein Barr infection. Infectious mononucleosis is usually a disease of young adults. We report a case of infectious mononucleosis in a 72-year old jaundiced gentleman with ferritin level of 2438 that normalised on clinical improvement.

Key words: Epstein Barr virus; Infectious mononucleosis; Ferritin; Jaundice; Liver function tests

Thoufeeq MH, Ali Khan SL, Jain SK, Al-Shakerchi H, Hussain M. A case of acute infectious mononucleosis presenting with very high ferritin. *World J Gastroenterol* 2007; 13(4): 637-638

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INTRODUCTION

Hepatitis is an important but uncommon manifestation of acute Epstein Barr infection. Infectious mononucleosis is usually a disease of young adults. We report a case of infectious mononucleosis in a 72-year old jaundiced gentleman with ferritin level of 2438 that normalised on clinical improvement.

CASE REPORT

A 72-year old retired farmer was admitted with a three-week history of feeling generally unwell. He gave a history of loss of appetite with stable weight. He denied any history of fever. Having a long history of prostatism, he was found to be in urinary retention at the same time for which he was catheterised. He was not started on any new medications and denied taking any over the counter medications. He said his urine was dark and sometimes had dysuria. He denied any gastro-intestinal symptoms.

His past history included osteoarthritis, previous myocardial infarction in 1993, gastro-oesophageal reflux disease and laparoscopic herniorrhaphy,

His medications were 75 mg aspirin, 10 mg atorvastatin, 300 mg quinine sulphate, 20 mg omeprazole and 5 mg amlodipine once daily. He said he was allergic to codeine phosphate. He was living on his own and his wife had recently passed away. His alcohol intake was negligible. There was no family history of any clinical significance.

On clinical examination he was noticed to be jaundiced, afebrile, haemodynamically stable. There were no stigmata of chronic liver disease. His abdomen was soft with mild tenderness elicited in the right hypochondrium. No organomegaly or masses were noted. Examinations of cardiovascular and respiratory systems were normal.

His blood tests were as follows: Hb = 14.7 g/dL (13.5-16.9), WBC = 6.1 (4.5-13.0), platelets = 161 (150-400), MCV = 92 fL (84-99.0), neutrophils = 1.65 (2.0-7.5), lymphocytes = 3.97 (1.5-4.0). A blood film showed a moderate number of atypical lymphocytes.

Serum ferritin was high at 2438 μg/L (25-400). Haemochromatosis screen was negative. PT, APTT and fibrinogen were normal. Sodium was 135 mmol/L (135-145), potassium 4.2 mmol/L (3.5-5.3), urea 6.1 mmol/L (3-7) and creatinine 102 μmol/L (53-115) (Table 1).

Bilirubin was 50 μmol/L (0-20), alanine aminotransferase (ALT) 254 U/L (0-37), alkaline phosphatase (ALP) 677 U/L (39-128), gamma glutamic transpeptidase(GT) 817, albumin 34 g/dL (35-52), calcium 2.18 mmol/L (2.1-2.6), C reactive protein (CRP) 21 mg/L (0-8), alfa- fetoprotein 3.5 (0-5.8), C3 and C4 (complements) were normal; IgM was 3.73 g/L (0.5-2).

Blood and urine cultures were negative. Hepatitis A, B, C and CMV serology were negative. Anti Epstein Barr virus (EBV) capsid antigens IgG and IgM were detected, Anti EBV nuclear antigen IgG was also detected.

Anti nuclear antibody (ANA) was negative. Anti neutrophil cytoplasmic antibody (ANCA), antimitochondrial, smooth muscle, liver kidney microsomal antibodies were all negative.

Chest and abdominal X-rays showed no radiological abnormalities. He had an abdominal CT showing moderate

Table 1 Abnormal blood tests in patient

1	Ferritin 2438 (25-400)
2	Bilirubin 50 (0-30)
3	ALT 254 (0-30)
4	ALP 677
5	Gamma GT 817
6	CRP 21 (0-8)
7	IgM 3.73 (0.5-2)
8	Anti EBV nuclear IgG detected
9	Anti EBV capsid antigen IgM and IgG detected
10	Atypical lymphocytes

splenomegaly and liver changes that were suggested as possibly secondary to hepatitis.

He was managed conservatively, his LFTs improved. He remained very well and was discharged after 2 wk of hospitalisation.

He was seen in Outpatient Follow-up Clinic after 4 mo, he was well and his weight was stable. His ferritin and LFT at that time became normal.

DISCUSSION

Infectious mononucleosis (IM) was first described in 1920. It was not until 1968 that EBV infection was described as a causative agent of IM. Incubation period is 25-50 d. It is usually a self-limiting disease.

There is a mortality rate of 0.1% associated with IM. Ninety percent of patients have asymptomatic deranged liver function tests, but jaundice is rare. EBV hepatitis is an important cause of viral hepatitis.

Fulminant hepatitis is rare but has been reported[1]. Severe hepatitis is rare, although it has been reported. Hepatomegaly is frequently present. Jaundice is rare. Occasionally, jaundice may be due to autoimmune haemolysis. Cholestasis can be noticed[2]. Splenomegaly is common and 50%-75% of patients develop it. Dommerby *et al*[3] reported that all patients have ultrasonic splenomegaly but that is palpable in only a few. Splenic rupture should be ruled out in patients who present with severe abdomen pain. Rupture can be precipitated by sports activity or can occur spontaneously and 0.1%- 0.5% of cases can result in rupture[4]. Spleen is most vulnerable to rupture in the 2nd and 3rd week of infection. Preparation with amoxicillin can result in rash frequently. Fever can be noted in 90 % of cases, usually low grade. Maculo- popular rash can be noticed in 5%-10% of cases, but is more common in children.

Malaise and fatigue may persist for weeks or months after IM. Persistent EBV infection is not a cause of chronic fatigue syndrome (CFS). High titres of antibodies to EBV may be noticed in patients with CFS that are identical to healthy EBV- sero positive adults.

Table 2 Gastrointestinal manifestations of EBV infection

1	Asymptomatic deranged LFTs
2	Viral hepatitis, usually self limiting
3	Fulminant hepatitis
4	Cholestasis
5	Auto-immune haemolysis causing jaundice
6	Splenomegaly
7	Splenic rupture (rare)

Duncan syndrome, a rare X linked recessive disorder, was reported by Purtilo *et al* in 1975 which is characterised by a defect in the immune response against EBV resulting failure to prevent EBV replication and hence leading to fatal complications[5]. Patients with combined immunodeficiency, Wiskott- Aldrich syndrome and Ataxia telangiectasia develop severe IM[6]. Likewise, patients with acquired immunodeficiency states like patients with AIDS or those receiving immunosuppressive agents develop severe IM and malignant lymphoma[7].

Treatment for IM is supportive with analgesic as required and rest. Due to the risk of splenic rupture physical activity in excess is to be avoided. There is no place for oral glucocorticoids in uncomplicated IM. In patients with severe tonsillar enlargement that might predispose to airway obstruction, severe thrombocytopenia or haemolytic anaemia, Prednisolone 40 to 60 mg once daily for 2-3 d with tapering over 3 wk is adviced.

In conclusion, acute EBV hepatitis should be ruled out in acutely jaundiced patients with high ferritin at any age group (Table 2).

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CASE REPORT

An autopsy case showing massive fibrinoid necrosis of the portal tracts of the liver with cholangiographic findings similar to those of primary sclerosing cholangitis

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Abstract

An 81-year-old Japanese man with jaundice was strongly suspected clinically of having primary sclerosing cholangitis based on clinical examinations and later died of hepatic failure. The entire course of the disease lasted about 10 mo. The autopsy revealed extensive fibrinoid necrosis in the liver, kidney, spleen, pancreas, lung, lymph nodes, and pleura. Particularly extensive fibrinoid necrosis in the portal tracts of the liver induced severe stenoses of the intrahepatic bile ducts, resulting in cholestasis in association with prominent liver injury. There were no findings indicating primary sclerosing cholangitis. The hepatic lesions in this case did not coincide with any known disease including collagen diseases. To clarify the cause of irregular stenoses of the intrahepatic biliary trees on cholangiographic findings, we postulate that some form of immunological derangement might be involved in pathogenesis of fibrinoid necrosis. However, the true etiology remains unknown.

Key words: Jaundice; Fibrinoid necrosis; Cholangiography; Primary sclerosing cholangitis; Liver; Autopsy

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INTRODUCTION

We herein present an unusual autopsy case showing systemic fibrinoid necroses in various organs. The case was strongly suspected clinically of primary sclerosing cholangitis (PSC) based on the cholangiographic findings; however, the autopsy disclosed systemic fibrinoid necroses instead of histological features of PSC. Particularly noteworthy was the presence of fibrinoid necrosis in the portal tracts of the liver caused stenoses of the biliary trees in association with cholestasis. We could not find any similar case reported in the literature. Presently, the etiology remains unknown.

CASE REPORT

About 10 mo before admission to our hospital, an 81-year-old Japanese man was admitted to a local hospital because of jaundice. Laboratory examinations revealed elevated levels of serum total bilirubin and other serum enzymes such as alkaline phosphatase, γ-glutamyltranspeptidase, leucine aminopeptidase. Serum autoantibodies such as anti nuclear antibody, anti mitochondrial antibody and anti smooth muscle antibody were negative. Endoscopical retrograde cholangiogram showed characteristic irregularity and beading of the intrahepatic biliary tree (Figure 1). No extrahepatic involvement was found. The patient was strongly suspected clinically of PSC. The pathologic diagnosis of the liver biopsy specimen was liver damage with cholestasis. Concentric fibrosis around the bile ducts suggesting PSC was not found. The patient was transferred to our hospital 18 d before death because of aggravation of jaundice, anorexia and easy fatigability. Chronological laboratory data is shown in Table 1. Serum fibrinogen was within normal lower limit. Plasminogen and D-dimer in serum were not examined. Serologic markers for hepatits B and C viral hepatitis were negative. These findings indicated that the liver cell damage rapidly worsened with aggravation of cholestasis, especially in end stage. Despite symptomatic and supportive treatment, the jaundice became progressively worse, and ascites and hepatic encephalopathy developed. The patient eventually died of hepatic failure.

An autopsy was performed about two hours after his death. The diagnoses at autopsy except for systemic fibrinoid necrosis were cholestatic liver damage, acute

Table 1 Laboratory data

			d 1	d 38	d 48	d 54				
Peripheral blood							*Serological tests*			
WBC	/μL	(4500-8500)	8400	8800	9200	16900	IgG	mg/dL	(800-1800)	1629
Hemoglobin	g/dL	(13.5-16.5)	12.1	12.9	11.5	11.4	IgA	mg/dL	(130-290)	182
Platelet	$\times 10^4$/μL	(15.0-35.0)	26.8	26.6	20.8	18.6	IgM	mg/dL	(100-180)	68
							C_3	mg/dL	(70-100)	99
Coagulation tests							C_4	mg/dL	(11-44)	46.5
Prothrombin time	%	(> 70%)	55	37	52	26	CH_{50}	U/mL	(28-45)	44.6
Hepaplastin test	%	(> 70%)	90	44	57	38	Serum-Cu	μg/dL	(78-131)	247
Fibrinogen	mg/dL	(150-400)		158	176	160				
							Viral markers			
Blood chemistry							HBsAg			(-)
AST	IU/L	(10-30)	185	134	98	422	HCVAb			(-)
ALT	IU/L	(6-40)	126	91	59	174				
LDH	IU/L	(160-325)	450	646	439	812	*Auto antibodies*			
ALP	IU/L	(120-400)	1359	834	692	556	Anti nucleic antibody			(-)
LAP	IU/L	(105-235)		528	472	425	LE test			(-)
γ-GTP	IU/L	(4-70)	296	151	119	73	Anti DNA antibody			(-)
T-Bil	mg/dL	(0.1-0.8)	10.7	15.8	16.2	18.5	Anti SMA antibody			(-)
D-Bil	mg/dL	(0-0.3)	5		9.1	10.9	Anti mitochondrion antibody			(-)
TP	g/dL	(6.7-8.3)	6.4	6.3	5.2	4.9				
Alb	g/dL	(3.5-5.2)	3.3	2.8	2.8	2.6	*Tumor markers*			
TC	mg/dL	(120-220)	260	319	193	158	AFP	ng/mL	(1-15)	2
BUN	mg/dL	(8-20)		32	68	150	CEA	ng/mL	(5.8↓)	6.1
Cr	mg/dL	(0.5-1.1)	1.2	1.3	3.1	7.7	CA19-9	U/mL	(37↓)	2470
Serological test	mg/dL									
CRP		(0-0.5)	1.8	1.3	1.5	8.8				

D: hospital day; Underline: abnormal value.

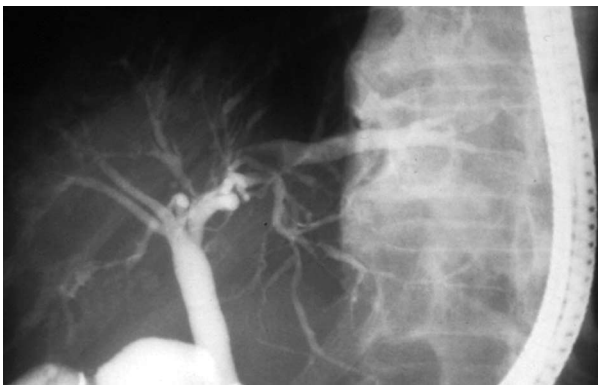

Figure 1 Endoscopic retrograde cholangiogram. Intrahepatic biliary branches showed irregularity and extensive beading (arrows). The long arrow indicates the dilatation of the bile duct due to stenosis caused by the pressure of cysts.

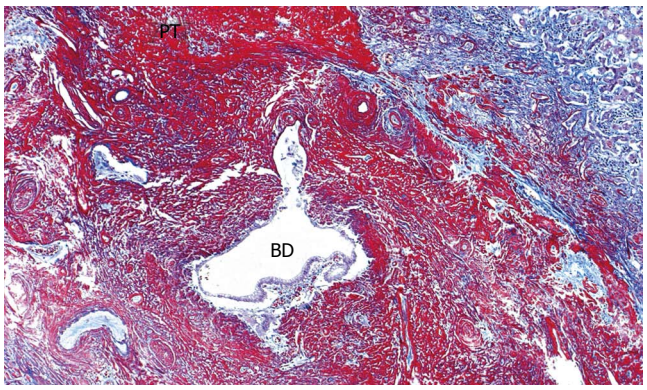

Figure 2 Extensive fibrinoid necrosis in large portal tract. Septal bile duct is buried in the fibrinoid material (Masson's trichrome x 1). PT: portal tract, BD: bile duct.

renal swelling, acute pancreatitis, mild cardiac hypertrophy (350 g) and latent adenocarcinoma of the prostate. Pathologic findings concerning fibrinoid necrosis are described as follows.

The liver (1380 g) showed a deeply yellow-brown cut-surface due to cholestasis with scattered simple small cysts. Gross examination could not detect remarkable changes of the common bile duct and bilateral hepatic ducts. Microscopically the most striking feature was a massive deposition of homogeneous material stained scarlet with Masson's trichrome in the portal tracts (Figure 2). Careful examinations disclosed that the material was deposited in the walls of arteries and portal veins as well as in the connective tissue (Figure 3). In the bile ducts the deposits circumscribed the lumen leaving the epithelial lining intact and caused marked stenosis. The area of the involved vessels and ducts extended from the distal interlobular portion to the proximal septal portion (Figures 2 and 3). The material was stained violet with PTAH and immunohistochemically was positive for fibrinogen and negative for immunoglobulins such as IgG, IgM, and IgA, C3 and C1q (Figure 4A). Electron microscopic findings of the liver were electron dense materials deposited in the involved tissue (Figure 4B). The results of these stainings and electronmicrogram suggested that the lesion with homogeneous material deposition was fibrinoid necrosis. In addition, the portal tracts were enlarged and

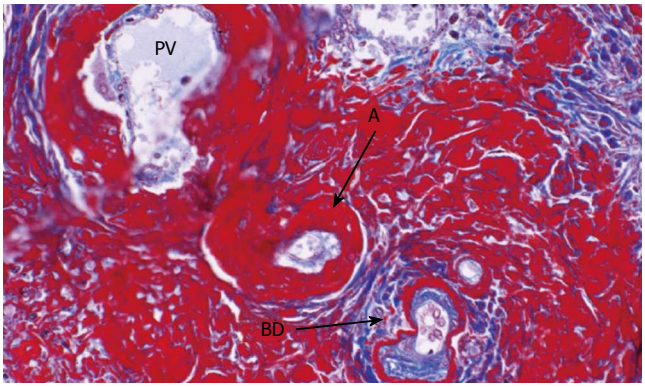

Figure 3 A smaller portal tract. Fibrinoid material is deposited in the connective tissue, walls of the arteries and periductular connective tissue (Masson's trichrome x 20). PV: portal vein, A: artery, BD: bile duct.

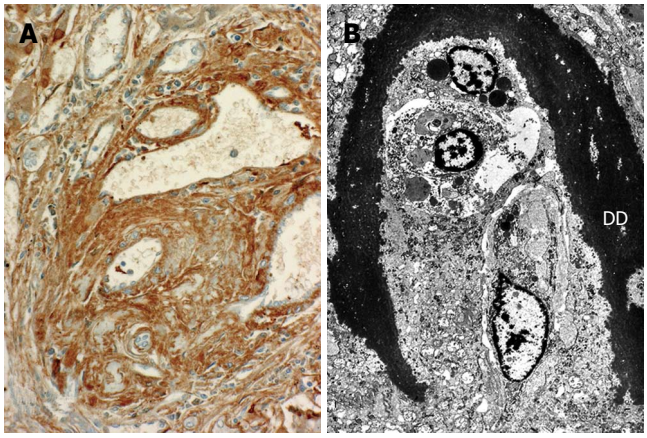

Figure 4 **A**: Immunohistochemical staining demonstrates the positivity of the fibrinoid material for fibrinogen; **B**: An electron micrograph shows dense deposit materials suggestive of fibrinoid necrosis around the small bile ducts in the portal tract of the liver (original magnification x 2000).

infiltrated with lymphocytes and plasma cells with an occasional intermingling of polymorphonuclear cells, ductular proliferation and fibrosis. The histologic features were biliary interface activity, resulting from cholestasis. However, it was noted that the lesion with fibrinoid necrosis was scarcely accompanied by inflammatory reactions. Cholestasis also caused conspicuous parenchymal damage including feathery degeneration or necrosis. Although the liver was extensively examined histologically, there were no detectable features of PSC.

The kidney (250 g, respectively) showed marked swelling. Histologically fresh fibrinoid necrosis was found in various degrees mainly in the wall of the interlobular arteries (Figure 5). However, it was not accompanied by any inflammatory reaction. Elastica-Van Gieson stain also demonstrated the destructive changes of the arterial walls suggestive of old vascular lesions. Occasionally, focal fibrinoid necrosis involved tubules and connective tissue.

Intensive fibrinoid necrosis was noticed in lymph nodes and the spleen. Old vascular lesions were also found frequently in the spleen. The pancreas, lung and pleura showed scattered foci of fresh fibrinoid change.

On re-examination of the liver biopsy specimen, the same fibrinoid necrosis as observed on autopsy materials was seen in the portal tracts.

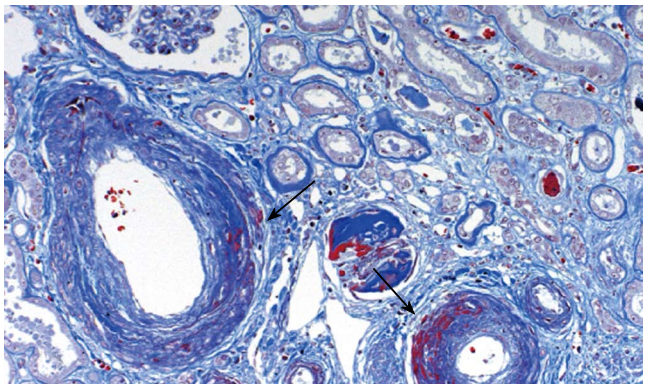

Figure 5 Kidney. Fibrinoid necroses were seen in the walls of the interlobular arteries (arrows) (Masson's trichrome x 10).

DISCUSSION

The case reported here is characterized with unusual systemic fibrinoid necroses in the walls of blood vessels and connective tissue in various organs, especially in the liver. It is clear that massive fibrinoid material around the bile ducts compressed the lumen and caused various degrees of stenoses. It was considered that such stenosis of the bile ducts resulted in the findings similar to those of PSC on the cholangiogram[1,2]. Obstructive jaundice over time aggravated and severely damaged the liver parenchyma. This was considered to reflect the progressive deposition of fibrinoid material in the portal tracts.

Klemperer proposed the term, collagen diseases, to describe systemic connective tissue disorders that are characterized histologically by fibrinoid necrosis of the connective tissue[3,4]. Thereafter many studies clarified that fibrinoid material is composed mainly of fibrins, while other minor components are immunoglobulins. Although the pathogenesis of fibrinoid necrosis has not yet been fully elucidated, it is assumed to result from the insudation of immunoglobulins into blood vessel walls, leading to activation of the coagulation cascade and deposition of fibrins[5]. Furthermore, fibrinoid necrosis of blood vessels is also known to develop in non-autoimmune diseases like malignant hypertension[6,7]. In our case laboratory findings or symptoms were not suggestive of any autoimmune disease or malignant hypertension. The liver pathology was also quite different from that of reported cases with collagen diseases[8-10]. On a search of the literature using key words such as fibrinoid necrosis, vasculitis, we could not find any case similar to ours.

It is well known that the main cholangiographic features of PSC are a beaded appearance, very short strictures, and diverticulum-like outpourchings[11]. Cholangiographic differential diagnosis of PSC involves cholangiocarcinoma, cirrhosis, acute cholangitis, and advanced primary biliary cirrhosis in general[12]. In addition, it might be necessary, in diagnosing PSC, to keep in mind that the lesions severely involving the portal tracts like our case also cause severe damage, besides stenosis of the biliary tracts.

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CASE REPORT

Pouchitis and pre-pouch ileitis developed after restorative proctocolectomy for ulcerative colitis: A case report

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Abstract

We report a case of pouchitis and pre-pouch ileitis, and inflammation in the neo-terminal ileum proximal to the pouch, developed after restorative proctocolectomy for ulcerative colitis. A 35-year old female presented with fever and abdominal pain five weeks after ileostomy closure following proctocolectomy. Computed tomography showed collection of feces in the pouch and proximal ileum. A drainage tube was placed in the pouch perianally, and purulent feces were discharged. With antibiotic treatment, her symptoms disappeared, but two weeks later, she repeatedly developed fever and abdominal pain along with anal bleeding. Pouchscopy showed mucosal inflammation in both the pouch and the pre-pouch ileum. The mucosal cytokine production was elevated in the pouch and pre-pouch ileum. With antibiotic and corticosteroid therapy, her symptoms were improved along with improvement of endoscopic inflammation and decrease of mucosal cytokine production. The fecal stasis with bacterial overgrowth is the major pathogenesis of pouchitis and pre-pouch ileitis in our case.

Key words: Mucosal cytokines; Pouchitis; Pre-pouch ileitis; Restorative proctocolectomy; Ulcerative colitis

Iwata T, Yamamoto T, Umegae S, Matsumoto K. Pouchitis and pre-pouch ileitis developed after restorative proctocolectomy for ulcerative colitis: A case report. *World J Gastroenterol* 2007; 13(4): 643-646

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INTRODUCTION

Restorative proctocolectomy with ileal pouch-anal anastomosis has become the surgical procedure of choice for patients with ulcerative colitis (UC)[1-3]. Pouchitis is the most common complication following restorative proctocolectomy for UC[1-5]. Patients with UC may develop inflammation in the neo-terminal ileum (pre-pouch ileum) proximal to the pouch, so called pre-pouch ileitis. So far, there has been only one study reporting the details of pre-pouch ileitis. Bell and colleagues[6] retrospectively investigated the clinicopathological characteristics of pre-pouch ileitis using their pouch database, however the pathogenesis of pre-pouch ileitis is unknown. We have recently experienced one patient in whom both pouchitis and pre-pouch ileitis developed after restorative proctocolectomy for UC. The purpose of this article is to report the case of our patient, and to discuss the pathogenesis of pre-pouch ileitis based on the clinicopathological and immunological findings.

CASE REPORT

Clinical presentations

In July 2005, we received a 35-year old female with a six-year history of UC requiring surgical treatment. Small bowel follow-through, barium enema and ileocolonoscopy with histological examinations confirmed UC but not Crohn's disease. She had a history of allergy to sulfasalazine and mesalazine. She was treated with long-term corticosteroid therapy, and the cumulative dose of prednisolone administered before operation was more than 20 g. Selective granulocyte and monocyte adsorption apheresis therapy was not effective. She had no history of extraintestinal manifestations. Endoscopically, the extent of disease was pancolitis without backwash ileitis. The indication for surgery was steroid-dependency and chronic continuous disease. A laparoscopic-assisted proctocolectomy with ileal pouch-anal canal anastomosis was performed. The J-shaped pouch (15 cm in length) was constructed using a linear stapler. A stapled ileo-anal canal anastomosis was performed at approximately 2 cm from the dentate line. There was no difficulty in mobilizing the ileal pouch sufficiently to achieve a tension-free anastomosis. The covering ileostomy was constructed to protect the anastomosis. After operation, she developed no serious complications, and was doing well with her ileostomy. Histological diagnosis of the colectomy specimen was UC, and there were no findings of inflammation in the terminal ileum.

Before ileostomy closure, a pouchgram, pouchscopy with biopsy, and manometric study were performed. The pouchgram showed no findings of anastomotic leak, fistulae and strictures, decreased pouch compliance, a long efferent loop, or decreased pouch emptying. Both

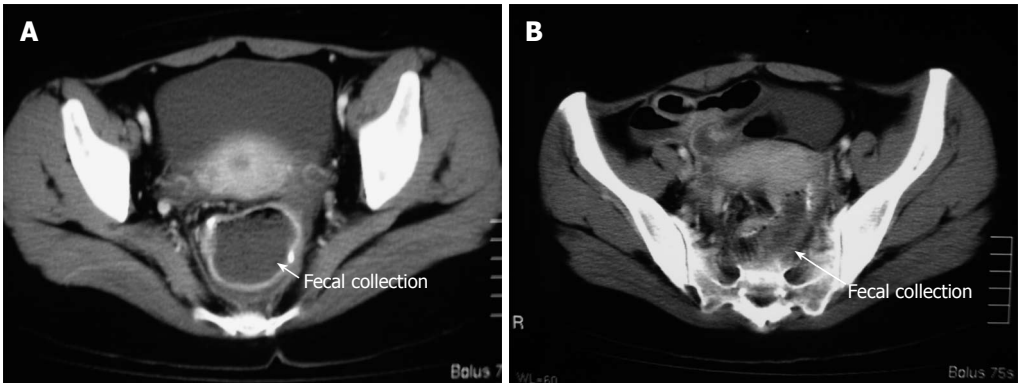

Figure 1 Abdominal computed tomography showing a massive collection of feces in the pouch (**A**) and proximal ileum (**B**).

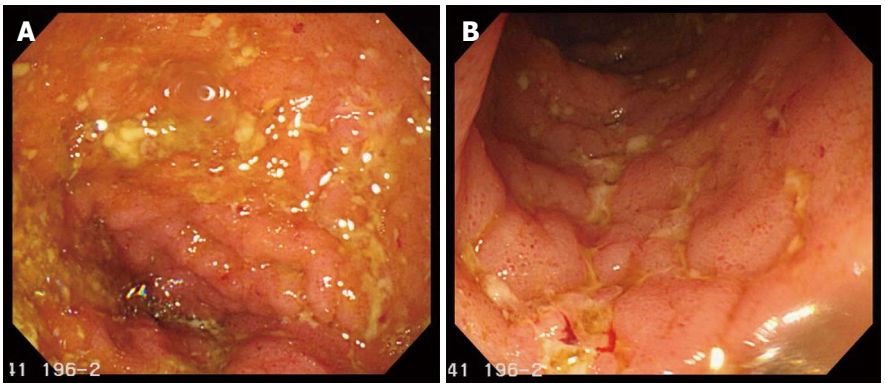

Figure 2 Pouchscopy showing inflammation conditions such as edema, granularity, friability, loss of vascular pattern, and erosions in both the pouch (**A**) and the pre-pouch ileum (continuous 30 cm proximal to the pouch) (**B**).

endoscopically and histologically, there were no findings of inflammation in the pouch and proximal ileum. In the manometric study, the resting anal pressure was 42 mmHg compared with 65 mmHg before pouch construction. The squeezing pressure was 110 mmHg compared with 128 mmHg before pouch construction. In March 2006, she had her ileostomy closed. Postoperatively, she was doing well, and on a normal diet. However, five weeks after operation, she developed high fever (40℃) and severe abdominal pain. Retrospectively, the number of defecations did not change (7-10 times/d), but the fecal volume on each defecation remarkably decreased for several days before her symptoms occurred. Abdominal computed tomography (CT) showed a massive collection of feces in the pouch and proximal ileum (Figure 1). A drainage tube was placed in the pouch perianally, and a large amount of purulent feces were discharged. In fecal culture, *Salmonella, Shigella, Campylobacter, Escherichia coli* O157, *Yersinia, Vibrio* and *Clostridium difficile* were negative, whereas *Escherichia coli* and *Staphylococcus aureu*s were positive. She was managed with total parenteral nutrition in combination with intravenous antibiotic administration (imipenem, 1.0 g/d for one week). Her symptoms disappeared after the five-day drainage of the feces, and she was back on a normal diet. However, two weeks later, she repeatedly presented with fever (40℃) and severe abdominal pain along with mild anal bleeding. Pouchscopy showed inflammatory conditions such as edema, granularity, friability, loss of vascular pattern, and erosions in both the pouch and the pre-pouch ileum (continuous 30 cm proximal to the pouch, Figure 2). The inflammation was milder more proximally. Biopsies were taken for histological examination and mucosal cytokine measurement. Histologically, moderate leukocyte infiltration without crypt abscess formation was observed in both the pouch and the pre-pouch ileum. According to the pouchitis disease activity index by Sandborn[5], her score was 12, and she was diagnosed as having pouchitis (≥ 7 points). Antibiotics (cefmetazone sodium, 2.0 g/d for one week), and prednisolone (30 mg/d for one week) were administered intravenously. Her symptoms were rapidly improved, and pouchscopy after the one-week treatment revealed improvement in mucosal inflammation. Thereafter, predonisolone was orally given (15 mg/d for two weeks, and then 10 mg/d for two weeks). Four months after ileostomy closure following pouch operation, she was doing well with normal bowel function (defecation, 5-7 times/d; soiling, 1 time/2 wk).

Laboratory data

Using the biopsy specimens obtained during pouchscopy, the mucosal cytokine (interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) levels were measured by the enzyme-linked immunosorbent assay as previously reported (Table 1)[7]. In this patient, the mucosal cytokine levels were examined before ileostomy closure, at the time of diagnosis of pouchitis and pre-pouch ileitis, and one week after the treatment for inflammation, and compared with those in 28 patients without pouchitis or pre-pouch ileitis three months after ileostomy closure following ileal pouch construction for UC in our previous study[7]. At the diagnosis of pouchitis and pre-pouch ileitis, the mucosal cytokine levels were remarkably elevated in both the pouch and the pre-pouch ileum compared with those before ileostomy closure. White blood cell (WBC) count

Table 1 Change of mucosal cytokine production in the pouch and pre-pouch ileum

	Before ileostomy closure	At the diagnosis ofpouchitis and pre-pouch ileitis	After treatment	Patients without pouchitis or pre-pouch ileitis[1]
Pouch (pg/mg of tissue)				
IL-1β	25	730	98	34.5 (26-39)
IL-6	590	14000	2700	900 (580-1480)
IL-8	31	840	290	58 (34-76)
TNF-α	10	350	67	38.5 (27-60)
Pre-pouch ileum (pg/mg of tissue)				
IL-1β	17	560	91	10.5 (5-22)
IL-6	600	17000	3600	570 (420-720)
IL-8	22	710	190	19.5 (10-41)
TNF-α	13	270	82	12 (5-18)

IL: Interleukin; TNF: tumor necrosis factor. [1]Median values (interquartile range) for 24 patients without pouchitis or pre-pouch ileitis three months after ileostomy closure following restorative proctocolectomy for ulcerative colitis[7].

was 12300/mm^3, platelet cont was 443000/mm^3, and C-reactive protein (CRP) level was 19.34 mg/dL. After the one-week administration of antibiotics and prednisolone, the mucosal cytokine levels markedly decreased along with endoscopic improvement of mucosal inflammation. At that time, WBC count was 10800/mm^3, platelet count was 506000/mm^3, and CRP level was 0.09 mg/dL.

DISCUSSION

The etiology of pouchitis is still unknown. A variety of factors such as fecal stasis, bacterial overgrowth, immune alternation, bile acid toxicity, short-chain fatty acid deficiency, and ischemia may affect the development of pouchitis[4,8,9]. In our research[7], to examine the impact of the fecal stream and stasis on immunological reactions in the pouch and proximal ileum, mucosal cytokine production was measured before and after ileostomy closure following proctocolectomy for UC. No patients developed pouchitis or pre-pouch ileitis during the study period. We found that immunological reactions in the pouch occurred soon after ileostomy closure, and continued thereafter[7]. In contrast, cytokine production was not elevated in the proximal ileum, where the fecal stasis does not often occur. Thus, the fecal stasis may play an important part in the pathogenesis of immunological reactions in the pouch[7].

Bell and colleagues[6] reported that 15 (2.6%) of 571 inflammatory bowel disease patients undergoing restorative proctocolectomy developed pre-pouch ileitis. However, this incidence may be underestimated because their study was retrospective, and the pre-pouch ileum was not routinely examined. The median age of the 15 patients (eight females) was 36 years, and the median duration from pouch construction to diagnosis of pre-pouch ileitis was three years. The most common clinical presentations were frequency of defecation (40%) and bowel obstruction (40%). Preoperatively, backwash ileitis was observed in 18% of the patients, and 27% had a history of extraintestinal manifestations. All patients had continuous disease from the neo-terminal ileum-pouch junction for a distance of 1 cm to more than 50 cm proximally becoming milder more proximally. Concomitant pouchitis was observed in 47% of the patients. Nine patients had narrowing of the lumen in the pre-pouch ileum, and in two of these there was a severe stricture. One patient had a fistula from the pre-pouch ileum to the vagina. Eleven patients with symptoms were treated, of whom eight required surgery (resection in six, strictureplasty in one, defunctioning ileostomy in one).

In the study by Bell and colleagues[6], there were no statements about the pathogenesis of pre-pouch ileitis. Only half of their patients with pre-pouch ileitis had concomitant pouchitis. Several patients presented with tight stricture, deep ulcerations or fistula. We suspect that these patients may have Crohn's disease, although the histological diagnosis based on the colectomy specimen was confirmed as UC[6]. Wolf *et al*[10] reported that afferent limb ulcers predict Crohn's disease in patients with ileal pouch-anal anastomosis. In our case, findings suggesting Crohn's disease were not observed.

The pathogenesis of pre-pouch ileitis is also unknown. Our patient developed a massive collection of feces in the pouch and pre-pouch ileum in the early postoperative period after ileostomy closure, which was detected by CT. Before her symptoms (fever and abdominal pain) occurred, the fecal volume remarkably decreased, although the number of defecations did not change. The cause of fecal collection is unknown. Before ileostomy closure, pouchgram showed no evidence of anastomotic strictures, decreased pouch compliance, or decreased pouch emptying. The results in our manometric study were similar to those in patients with normal pouch function after restorative proctocolectomy in the previous study[11]. Although a longer follow-up was necessary, she had neither evacuation problems nor impaired pouch emptying at the time of our report (four months after ileostomy closure). If she had the similar symptoms such as fever and abdominal pain along with a decrease in volume of feces, further studies were needed to examine her pouch function.

In our case, endoscopic and histological features of mucosal inflammation were similar in the pouch and pre-pouch ileum, although the inflammation was milder more proximally. Bell and colleagues[6] also found that the histological findings of pouchitis and pre-pouch ileitis are similar. In our institution, mucosal cytokine production is routinely investigated before and after ileostomy closure following restorative proctocolectomy for UC[7]. In this report, at the diagnosis of pouchitis and pre-pouch ileitis, the mucosal IL-1β, IL-6, IL-8, and TNF-α levels were remarkably elevated in both the pouch and the pre-pouch ileum compared with those before ileostomy closure, which were much higher than those in patients without pouchitis or pre-pouch ileitis in our previous study (Table 1)[7], suggesting that both pouchitis and pre-pouch ileitis are associated with increased mucosal cytokine production. There seemed to be no significant difference in cytokine production between pouchitis and pre-pouch ileitis. These cytokine levels remarkably decreased after antibiotic and steroid medication. Purulent feces were discharged through a drainage tube placed in the pouch. In fecal culture, *E.*

coli and *Staphylococcus aureus* were detected, which may have caused severe clinical symptoms such as high fever and abdominal pain.

In conclusion, fecal stasis along with subsequent bacterial overgrowth is the major pathogenesis of both pouchitis and pre-pouch ileitis. The pathogenesis may be similar in pouchitis and pre-pouch ileitis due to the similar endoscopic, histological and immunological features. Further studies are needed to fully understand the pathogenesis of pouchitis and pre-pouch ileitis after restorative proctocolectomy for UC.

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CASE REPORT

Cytomegalovirus hepatitis and myopericarditis

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Abstract

Cytomegalovirus (CMV) infection in inmunocompetent hosts generally is asymptomatic or may present as a mononucleosis syndrome but rarely can lead to severe organ complications. We report a case of simultaneous hepatic and pericardic CMV infection in a 36-year old immunocompetent man. He was admitted to coronary unit with fever, chest pain radiated to shoulders, changes on electrocardiogram with diffuse ST elevation and modest laboratory elevations in the MB fraction of creatine kinase (CK-MB) of 33.77 μg/L (0.1-6.73), serum cardiac troponin T of 0.904 ng/mL (0-0.4), creatine kinase of 454 U/L (20-195) and myoglobin of 480.4 μg/L (28-72). Routine laboratory test detected an elevation of aminotransferase level: alanine aminotransferase 1445 U/L, aspartate aminotransferase 601 U/L. We ruled out other causes of hepatitis with normal results except IgM CMV. The patient was diagnosed with myopericarditis and hepatitis caused by cytomegalovirus and started symptomatic treatment with salicylic acid. In few days the laboratory findings became normal and the patient was discharged.

Key words: Cytomegalovirus; Hepatitis; Myopericarditis; Pericarditis

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INTRODUCTION

Cytomegalovirus (CMV) infection in an immunocompetent host, rarely can lead to severe organ specific complications[1,2]. A variety of presentations have been described, ranging from a common form of infectious mononucleosis to systemic disease (gastrointestinal, cardiovascular, hepatic, neurologic manifestations) with significant morbidity. We report a case of simultaneous hepatic and pericardic CMV infection in an immunocompetent host.

CASE REPORT

A 36-year old man was admitted to our hospital with fever and chest pain radiated to shoulders. He had no other relevant medical history and physical examination was normal. Electrocardiogram showed diffuse ST elevation and laboratory test displayed modest elevations in MB fraction of creatine kinase (CK-MB) of 33.77 μg/L (0.1-6.73), serum cardiac troponin T of 0.904 ng/mL (0-0.4), creatine kinase of 454 U/L (20-195) and myoglobin of 480.4 μg/L (28-72). The patient was diagnosed with myopericarditis and admitted to the coronary unit. Routine laboratory tests detected an elevation of aminotransferase level: alanine aminotransferase 1445 U/L (6-41), aspartate aminotransferase 601 U/L (6-38). Other laboratory parameters were normal including gamma glutamyl transpeptidase, alkaline phophatase, bilirubin, cholesterol, electrolytes, complete blood cell count and coagulation test. We ruled out other causes of hepatitis: medications, alcohol abuse, hepatitis B, hepatitis C, hereditary hemochromatosis, hepatic steatosis and steatohepatitis, thyroid disorders, celiac disease, autoimmune hepatitis, Wilson´s disease and alpha 1 antitrypsin deficiency. Finally we looked for a less common infection etiology (Epstein Barr virus, cytomegalovirus and atypical bacteria). The results were normal except for IgM CMV. The patient was diagnosed with myopericarditis and hepatitis caused by cytomegalovirus and started symptomatic treatment with salicylic acid. In few days the laboratory findings became normal and the patient was discharged.

DISCUSSION

CMV infection in immunocompetent patients is common with a substantial morbidity and mortality. It is generally asymptomatic in immunocompetent hosts or may present

as a mononucleosis syndrome. Occasionally primary CMV infection can lead to severe organ specific complications[1,2]. Although these cases are rare, gastrointestinal, cardiovascular, neurologic, hepatic disorders have been reported.

Liver function abnormalities are frequently encountered in patients with symptomatic CMV infection. Subclinical transaminitis is the most common finding in immunocompetent patients, and elevations of alkaline phosphatase and total bilirubin are less typical[1,3,4]. Pericarditis and myocarditis have been described in immunocompetent patients with acute CMV infection[5,6]. We have found only one case reporting simultaneous infection of liver and myopericardia with cytomegalovirus[7].

Since symptomatic CMV infection is generally self-limited with complete recovery over a period of days to weeks, it is difficult to prove whether antiviral therapy has a significant impact on the clinical outcome.

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Indian patients with nonalcoholic fatty liver disease presenting with raised transaminases are different at presentation

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TO THE EDITOR

We read with great interest the article, "Non-alcoholic fatty liver disease may not be a severe disease at presentation among Asian Indians" by Madan *et al*[1] in the recent issue of *WJG*. Twenty-eight (55%) out of 51 patients with non-alcoholic fatty liver disease (NAFLD) who presented with abnormal transaminases had histological evidence of nonalcoholic steatohepatitis (NASH). The majority of patients had grade 1 [32 (63%)] or grade 2 [16 (31%)] inflammation and either had no [23 (45%)] fibrosis or stage I [19 (37%)] fibrosis. None of the patients had cirrhosis[1]. We agree with Madan *et al*[1] that Asian Indians with NAFLD who present with unexplained increase in transaminases may have mild disease at presentation on the basis of similar observations made by us[2]. NAFLD has a spectrum which includes patients with only steatosis and NASH that can progress to cirrhotic and hepatocellular carcinoma[3]. Of the 127 NAFLD patients (July 2001-March 2006) who presented with raised transaminases for at least 6 mo with negative viral, autoimmune and metabolic workup analyzed in our study, 43 underwent liver biopsy (Table 1). Only half of them [22 (51%)] had histological evidence of NASH as defined by either class Ⅲ [8 (19%)] or class Ⅳ [14 (32%)] NAFLD according to Matteoni *et al*[4]. The other 21 (49%) patients either had class Ⅰ [2 (5%)] or class Ⅱ [19 (44%)] disease not amounting to histological NASH (Table 1). In the 22 patients with histological NASH evaluated as per Brunt *et al*[5], the majority had mild to moderate inflammation and either no fibrosis or stage Ⅰ to Ⅱ fibrosis. Only 18% patients had stage Ⅲ fibrosis and none of the patients had cirrhosis of the liver (Table 1).

The majority of patients studied by us were males with a mean age of 39.2 ± 10.7 years (Table 2), which is similar to data shown by Madan *et al*[1]. In addition to the mild histological disease at presentation, there are other differences in NAFLD patients from India and those from the West[2]. When we used the Asia Pacific criteria[6,7], even though most of our patients had central obesity [104 (82%)] and were either overweight [27 (21%)] or obese [86 (68%)] they did not have the kind of morbid obesity seen in patients from the West (Table 2). The mean body weight and body mass index (BMI) of our patients were 71 kg and 28.7 kg/m^2 respectively, much less than those reported from the West[8,9], but were similar to the data shown by Madan *et al*[1] who also found that the median BMI is 26.7 (range 21.3-32.5) kg/m^2 and the majority of them are obese (69%) according to the Asian Pacific criteria. When the ATP Ⅲ criteria with modified waist were used in 81 of our patients to define metabolic syndrome, around half of them [39 (48%)] had metabolic syndrome, also less than reported in patients from the West (Table 2)[10]. We attributed low prevalence of metabolic syndrome in our patients to the lower prevalence of diabetes mellitus [16 (13%)] and hypertension [13 (10%)] at presentation, which is similar to the data reported by Madan *et al*[1] (10% and 11.8% respectively) in their study. Furthermore, the low prevalence of metabolic syndrome (20.9%) in the study of Madan *et al*[1] could be due to their use of BMI as a surrogate marker for waist, which may not always be true. Indians may have a normal BMI with an abnormal waist which is related to more of central obesity rather than overall obesity. It is possible that diabetes mellitus occurs late in the course of this disease when the degree

Table 1 Liver histology in 43 patients with nonalcoholic fatty liver disease (NAFLD), *n* (%)

Class Ⅰ	2 (5)
Class Ⅱ	19 (44)
Class Ⅲ	8 (19)
Class Ⅳ	14 (32)
NASH (class Ⅲ + Ⅳ) on histology (*n* = 22)	
Grade 1	10 (45)
2	12 (55)
3	0
Stage 0	6 (27)
1	7 (32)
2	5 (23)
3	4 (18)
4	0
Perls' Prussian blue staining on liver biopsy (*n* = 30)	
0	20 (67)
1+	6 (20)
2+	4 (13)
3+	0
4+	0

Table 2 Clinical and laboratory parameters in 127 patients with nonalcoholic fatty liver disease (NAFLD)

mean age ± SD (yr)	39.2 ± 10.7
Males	84
Mean body weight (range) (kg)	71 (45-100)
Mean BMI (range) (kg/m^2)	28.7 (19-34)
Overweight	27 (21%)
Obesity	86 (68%)
Abnormal waist	104 (82%)
Insulin resistance	48/58 (83%)
Diabetes mellitus	16 (13%)
Hypertension	13 (10%)
Dyslipidemia	67 (53%)
Metabolic syndrome	39/81 (48%)

of insulin resistance increases and our patients could represent patients in the early spectrum of NAFLD with less severe disease and diabetes mellitus at presentation with raised transaminases. Diabetes mellitus is one of the risk factors for severe liver disease in NAFLD and absence of this risk factor in majority of our patients may explain the mild disease on liver biopsy.

Insulin resistance is very common in patients with NAFLD irrespective of the methodology used. Eighty percent of 51 patients in the study by Madan *et al*[1] had abnormal homeostasis model assessment for insulin resistance (HOMA-IR). We found insulin resistance in all of our 22 patients initially studied by insulin tolerance test (ITT) and later in 48 (83%) of 58 patients studied by HOMA-IR (Table 2)[2,11,12].

Though not studied by Madan *et al*[1], another difference in Indian patients and those from the West is the presence of serum and liver iron abnormalities and HFE gene mutations[2,13,14]. Only 4 (5%) of our 87 patients had abnormal serum ferritin or transferrin saturation and 4 (13%) of 30 patients studied were heterozygotes for H63D mutation. None of the patients had C282Y HFE gene mutation. The majority of our patients had negative Perls' staining for iron on liver biopsy (Table 1) and there was no correlation between the iron staining and degree of necro-inflammation and fibrosis, suggesting that serum and liver iron and HFE gene mutations play a very little role in Indian patients with NAFLD[2,13,14].

In conclusion, Indian patients with NAFLD who present with incidental detection of raised transaminases representing a part of spectrum of patients with NAFLD have a milder disease at presentation. Whether NAFLD in Indian patients is overall mild or overall different from other parts of the world requires analysis of full spectrum of NAFLD patients.

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Treatment regimen design in clinical radiotherapy for hepatoma

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TO THE EDITOR

Recently, the paper by Wang *et al*[1] published in *World Journal of Gastroenterology* has given rise to great interest of many researchers. It is well known that hepatoma is one of the lethal diseases with a high incidence in the world, especially in Asia. Radiotherapy is the main treatment modality of hepatoma in clinical practice. Unfortunately, intrinsic radiosensitivity of cancer cells is not fully understood, though a large number of papers on it are now available. Yang and colleagues[2] have developed the premature chromosome condensation technique for clinical radiotherapy of hepatoma. A precise and quick measurement of cell radiosensitivity can detect the high-risk results after exposure to a large dose.

Premature chromosome condensation technique can quickly and precisely detect radiation-induced chromosome damage[3-5]. Chromatid breaks are regarded as a good radiodosimetry, which highly correlates with cell survival and radiosensitivity[6]. However, they are not a negative value as described by Wang *et al*[1]. I recommend her to further measure them in order to perfect this promising approach.

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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