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EDITORIAL

Current and emerging pharmacological therapy for non-alcoholic fatty liver disease

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Abstract

The main treatment of patients with non-alcoholic fatty liver disease (NAFLD) is life style modification including weight reduction and dietary regimen. Majority of patients are safely treated with this management and pharmacologic interventions are not recommended. However, a subgroup of NAFLD patients with non-alcoholic steatohepatitis (NASH) who cannot achieve goals of life style modification may need pharmacological therapy. One major obstacle is measurement of histological outcome by liver biopsy which is an invasive method and is not recommended routinely in these patients. Several medications, mainly targeting baseline mechanism of NAFLD, have been investigated in clinical trials for treatment of NASH with promising results. At present, only pioglitazone acting as insulin sensitizing agent and vitamin E as an antioxidant have been recommended for treatment of NASH by international guidelines. Lipid lowering agents including statins and fibrates, pentoxifylline, angiotensin receptor blockers, ursodeoxycholic acid, probiotics and synbiotics are current agents with beneficial effects for treatment of NASH but have not been approved yet. Several emerging medications are in development for treatment of NASH. Obeticholic acid, liraglutide, elafibranor, cenicriviroc and aramchol have been tested in clinical trials or are completing trials. Here in, current and upcoming medications with promising results in clinical trial for treatment of NAFLD were reviewed.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Vitamin E; Pioglitazone; Pharmacological therapy; Obeticholic acid

Core tip: Non-alcoholic fatty liver disease (NAFLD) is an increasing liver disease worldwide. However, most of patients are treated with life style modification including weight loss and dietary regimen. Pharmacologic therapy may be indicated in a group of patients with non-alcoholic steatohepatitis. Here in, the current and emerging medications for treatment of NAFLD was reviewed briefly with regard of their beneficial effects

on histological outcomes.

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INTRODUCTION

Prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing and NAFLD is probably the most common cause of abnormal liver enzymes worldwide[1]. The spectrum of NAFLD is ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and liver cirrhosis[2]. While simple steatosis is generally considered a benign condition, it may gradually progress to NASH, liver cirrhosis and eventually hepatocellular carcinoma (HCC)[3]. This has been resulted in significant attention of physicians and health care providers toward detection and treatment of NAFLD in recent years.

NASH has been estimated to affect 2%-5% of western population and is associated with 10-fold increase in liver related mortality[4]. In addition to steatosis, NASH is defined by cellular injury and inflammation that may eventuate in liver fibrosis[5]. Hepatocellular injury and fibrosis in NASH is mainly a result of free fatty acid lipotoxicity mediated by Kupffer and hepatic stellate cells causing collagen deposition[6].

The main treatment of NAFLD is life style modification including exercise and weight loss. It has been suggested that more than 7% weight reduction sustained for 48 wk may cause significant improvement in histology of NASH[7]. However, some patients will not achieve goals of lifestyle modification or cannot maintain them for long term period. On the other hand, there are patients with advanced liver disease needing targeted therapy. Most experts suggest pharmacologic therapy for NASH only in individuals with advanced disease or those at high risk of liver cirrhosis[8]. No drug has been approved specifically for treatment of NAFLD yet, however, some drugs are now routinely prescribed or are under trial. Here in, current medications and future drug candidates for treatment of NAFLD are briefly reviewed (Table 1).

DISCUSSION

Pathogenesis of NAFLD

The pathogenesis of NAFLD is complex including multiple environmental and genetic factors. High calorie dietary regimens rich in carbohydrates and saturated fatty acids causing weight gain; are probably the most important environmental factor. The significant rising prevalence of NAFLD in recent years is attributed to change in life style especially dietary regimen[9]. Insulin resistance (IR) and metabolic syndrome are central in pathogenesis of NAFLD. Several other mechanistic pathways involved in pathogenesis of NAFLD finally result in IR. Metabolic derangements and IR in NAFLD are mediated by dysregulation of metabolic pathways which are naturally regulated by nuclear receptors such as peroxisome proliferator-activated receptors (PPARs), farnesoid X receptors (FXRs) and liver X receptors (LXRs)[10]. Nuclear receptors have been targeted for production of drugs with beneficial effects in NAFLD.

Lipotoxicity, defined as fat induced injury to hepatocytes, oxidative stress, mediated by hypoxia-inducible transcription factors-1α and 2α, and chronic inflammation, through several cytokines and chemokines, are also involved in initiation and progression of NAFLD[11]. These are other targets for medical therapy in NAFLD.

Genetic predisposition to NAFLD has been described in some studies. Patatin-like phospholipase domain containing-3 (PNPLA3) is a gene responsible for encoding a lipase acting for clearance of lipid droplets from the hepatocytes[12]. Loss of function mutation of PNPLA3 results in hepatocytes injury and steatohepatitis. The other important genetic factor is loss of function mutation in trans-membrane 6 superfamily member 2 (*TM6SF2*) gene that is a predisposing risk for NAFLD[13]. Alteration of adipocytokines and micronutrients[14], thyroid hormone abnormalities[15], and vitamin D deficiency[16] are other proposed contributing factors in pathogenesis of NAFLD. Understanding pathogenesis and epidemiology of NAFLD may help clinicians for estimation of more precise burden of disease in population. While surveillance strategies applied to all individuals is not cost-effective, screening may be suggested for high risk groups such as those with metabolic syndrome, diabetes mellitus and hypertension[17].

Insulin sensitizer

Since the main defined mechanism for NAFLD is IR, drugs targeting IR have been implemented as treatment of NAFLD. Peroxisome proliferator-activated receptor (PPAR)-γ is a nuclear receptor and a member of PPAR superfamily that is expressed exclusively in adipose tissue and involved in glucose metabolism and lipogenesis[18]. Thiazolidinediones, agonists of PPAR- γ, are primarily used for treatment of diabetes mellitus and act by improvement of insulin sensitivity[19]. Thiazolidinediones also have anti-inflammatory, antfibrotic properties and increase serum adiponectin level[20-22]. The largest clinical trial investigating the effect of thiazolidinedions in NASH patients, PIVENS trial, randomized 247 patients with biopsy proven NASH to receive pioglitazone (30 mg/d), vitamin E (800 IU/d) or placebo for 96 wk[23]. This study showed that

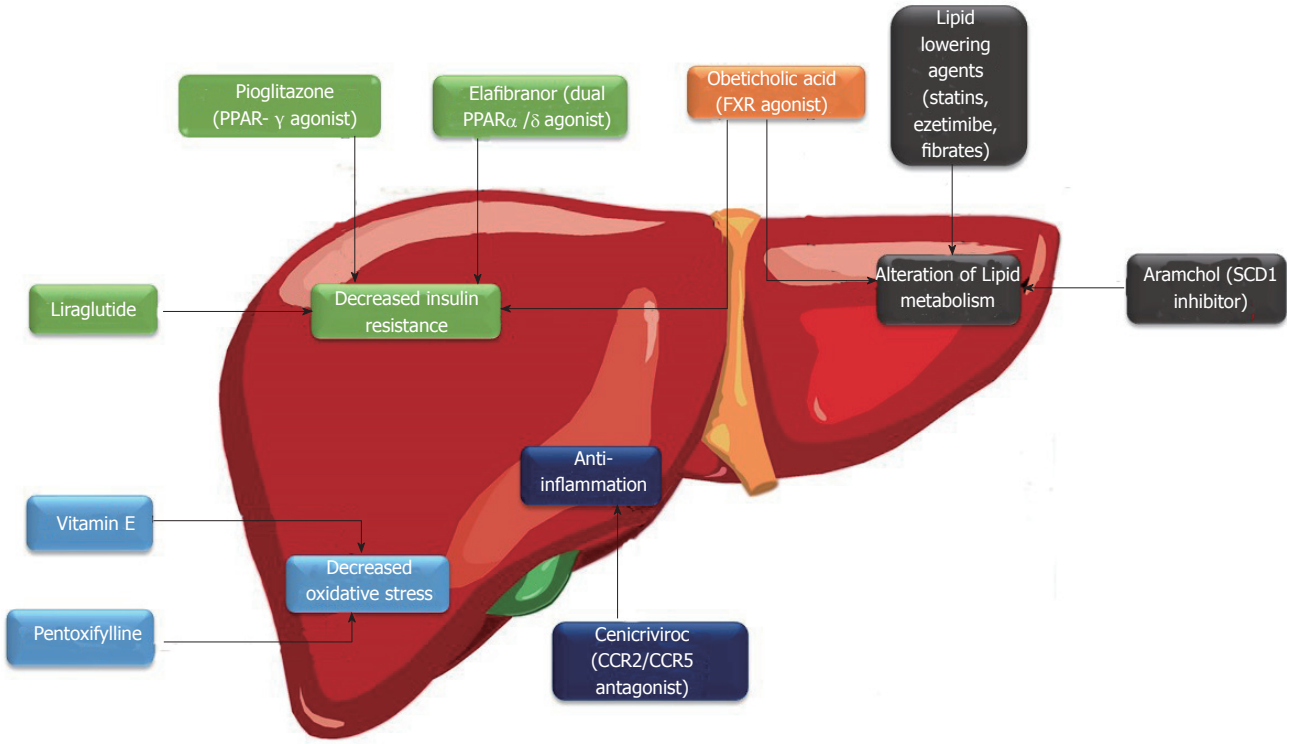

Figure 1 Current and emerging drugs for non- alcoholic fatty liver disease and their mechanism of action. PPAR: Peroxisome proliferator-activated receptor; SCD1: Stearoyl CoA desaturase-1; FXR: Farnesoid X receptor.

Table 1 Current medications that have been used for treatment of non- alcoholic fatty liver disease

Medication	Mechanism	Effect on histology	Recommended by AASLD/EASL
Pioglitazone	PPAR-γ	Improvement of steatosis, lobular inflammation and ballooning	Yes
Vitamin E	Anti-oxidant	Improvement of hepatocyte ballooning	Yes
Metformin	Amelioration of IR	No beneficial effect	No
Statins	HMG-CO A reductase inhibition	No beneficial effects	No
Ezetimibe	Inhibition of cholesterol absorption	Improvement of hepatocyte ballooning	No
Fibrates	PPAR-α	Improvement of hepatocyte ballooning	No
Pentoxifylline	Inhibition of TNF-α and anti-oxidants	Improvement of inflammation and ballooning	No
Losartan	ARB	Improvement of steatosis, lobular inflammation, ballooning and fibrosis	No
UDCA	Prevention of apoptosis/inflammation	Lacking data	No
Synbiotic and probiotics	Modulation of gut microbiota	Lacking data	No

PPAR-γ: Peroxisome proliferator-activated receptor–γ; HMG-CO A: Hydroxyl-methyl-glutaryl-coenzyme A reductase; ARB: Angiotensin receptor blockers; UDCA: Ursodeoxycholic acid; AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of Liver.

pioglitazone could improve steatosis and histological alterations in patients with definite NASH in their liver biopsies. FLIRT trial showed improvement of steatosis and serum aminotransferase level by rosiglitazone in patients with NASH[24]. However, use of rosiglitazone has been prohibited in Europe and highly restricted in United States based on FDA concerns regarding increase in cardiovascular side effects with this drug[25]. Some studies showed beneficial effects of thiazolidinedines in amelioration of hepatic fibrosis[17,26]. American Association for the Study of Liver Diseases (AASLD) and European Association for the Study of Liver (EASL) have suggested use of pioglitazone for treatment of patients with NASH[27,28].

It should be noted that beneficial effects of thiazolidinediones are abrogated after discontinuation and NASH is returned in liver biopsies[29]. Other limitation of thiazolidinedions, restricting their widespread application, is their side effects including weight gain, increased bone loss and fracture risk, deterioration of heart failure and increased risk of bladder cancer with pioglitazone[30-32].

Metformin

Metformin is used to treat type Ⅱ diabetes acting through amelioration of IR by decreasing hepatic gluconeogenesis and triglyceride production[33]. Metformin is no longer considered as a treatment for

Table 2 Emerging medications for treatment of non- alcoholic fatty liver disease

Medication	Mechanism	Histology benefit
Obeticholic acid	Farnesoid X receptor agonist	Improvement of steatosis, lobular inflammation, ballooning and fibrosis
Aramchol	Inhibition of SCD1	Lacking data
Elafibranor	PPAR α/δ agonist	Improvement of steatosis and fibrosis
Cenicriviroc	Inhibition of CCR2/CCR5	Lacking data
Liraglutide	Glucagon-like peptide-1 agonist	Improvements in steatosis and hepatocyte ballooning

SCD1: Stearoyl CoA desaturase-1; PPAR: Peroxisome proliferator-activated receptor.

NAFLD. While it had some promising results in animal studies, both pediatric and adult clinical trials failed to demonstrate histological improvement of NASH in human by metformin[34]. AASLD and EASL guidelines do not recommend metformin as a treatment for NAFLD at present[27,28].

Anti-oxidants

Oxidative stress has a major role in NASH pathogenesis especially activation of hepatic stellate cells (HSCs) and promoting liver fibrosis[35]. Vitamin E is a fat soluble anti-oxidant that is capable of repairing oxidizing radicals and prevent lipid peroxidation[36]. Vitamin E regulates PPAR and transforming growth factor-β1 (TGF-β1) pathways and is involved in inflammation, apoptosis and fibrosis process[37,38]. Vitamin E has been tested in clinical trials to evaluate its efficacy in treatment of patients with NASH. Vitamin E with a dose of 800 IU/d compared to placebo for treatment of patients with NASH in PIVENS trial. After 96 wk of therapy, amelioration of hepatic steatosis, lobular inflammation and hepatocyte ballooning were seen in vitamin E treated group[23]. However, no improvement of fibrosis was observed. The Treatment of NAFLD in Children (TONIC) trial is a randomized trial allocating 800 IU/d vitamin E and metformin (1 g/d) to children with NAFLD. Vitamin E resulted in improvement of hepatocyte ballooning but failed to improve hepatic inflammation, fibrosis and steatosis[39]. Based on promising results of these studies, AASLD and EASL have suggested use of vitamin E for treatment of non-diabetic, non-cirrhotic patients with NASH[27,28]. Safety profile of vitamin E is still controversial. Vitamin E treatment was associated with an increase in all cause related mortality in a meta-analysis[40]. Some studies also showed an increase in hemorrhagic stroke and prostate cancer with high doses and long term use of vitamin E respectively[41,42]. Therefore, it is better to individualize use of vitamin E for treatment of NASH based on these cautions. Medications against NAFLD and their mechanisms of action were outlined in Figure 1.

Lipid lowering agents

NAFLD is considered the hepatic manifestation of metabolic syndrome. Many patients with NAFLD have features of metabolic syndrome including diabetes mellitus, dyslipidemia and obesity. Accumulation of free cholesterol in hepatocytes have been also suggested in pathogenesis of NAFLD[43]. Statins that inhibit hydroxyl-methyl-glutaryl-coenzyme A reductase are widely used as cholesterol lowering agents and can be theoretically useful in patients with NAFLD[44]. Athyros *et al*[45] showed that daily atorvastatin use can improve liver enzymes and reduce cardiovascular morbidity in patients with mild to moderate abnormal liver tests. In an animal model of NASH, simvastatin was associated with amelioration of liver fibrosis by inhibition of hepatic stellate cells *via* nitric oxide synthase pathway[46]. However, simvastatin therapy was not associated with improvement of liver enzymes, hepatic steatosis and fibrosis in a group of patients with biopsy proven NASH[47]. In a retrospective cohort, statin use was associated with decreased risk of advanced fibrosis in patients at risk for NASH[48]. While there are concerns about risk of using statins in patients with chronic liver disease[49], most recent studies showed that statins are generally safe in patients with NAFLD[50]. Since most of patients with NAFLD have components of metabolic syndrome such as dyslipidemia and DM Ⅱ, statin use may be considered in this group of patients.

Ezetimibe is a cholesterol absorption inhibitor that is used for treatment of patients with elevated cholesterol level. In a mice model of hepatic steatosis induced by high fat diet, ezetimibe therapy prevented hepatic steatosis and decreased hepatic insulin resistance[51]. In MOZART trial, ezetimibe could not significantly reduce hepatic steatosis as assessed by magnetic resonance imaging-derived proton density fat fraction[52]. In a meta-analysis of 6 studies, Nakeda *et al*[53] reported that ezetimibe therapy resulted in improvement of liver enzymes and hepatocyte ballooning in patients with NAFLD. Colesvelam is a bile acid sequestrant that is used clinically to decrease low density lipoprotein (LDL). A placebo controlled clinical trial showed that colesvelam was associated with increased liver fat in patients with NASH as assessed by magnetic resonance imaging and magnetic resonance spectroscopy[54].

Fibrates are activator of peroxisome proliferator-activated receptor alpha (PPAR-α) that are used as anti-hyperlipidemic agents and mainly decrease serum triglyceride level. Fibrates also improve insulin resistance, stimulate oxidation of fatty acids and have

anti-inflammatory effects[55]. In an animal model of NAFLD, fibrates therapy was associated with resolution of hepatic steatosis, steatohepatitis and fibrosis[56]. A pilot trial showed improvement of metabolic syndrome and glucose metabolism by fenofibrate in patients with NAFLD but with only minimal effects on liver histology[57]. Co-treatment with pentoxifylline plus fenofibrate was effective in reduction of liver stiffness and markers of liver fibrosis in patients with NAFLD[58].

Pentoxifylline

Pentoxifylline is an inhibitor of tumor necrosis factor-alpha (TNF-α) with anti-oxidant properties that initially was known to be effective in treatment of alcoholic hepatitis[59]. Most animal models suggested beneficial effects of pentoxifylline in reducing liver enzymes and hepatic inflammation[60,61]. In a randomized placebo trial, pentoxifylline significantly improved liver fibrosis and decreased NAFLD activity score[62]. They also showed that beneficial effects of pentoxifylline in NASH are mediated by decreasing free-radical-mediated lipid oxidation[63]. Satapathy *et al*[64] showed that 12 mo pentoxifylline therapy was associated with biochemical and histological improvement in patients with NASH. Two meta-analysis reported beneficial effects of pentoxifylline in terms of reduction of liver enzymes and improvement of histology in NASH patients[65,66]. However, long-term safety and efficacy of pentoxifylline in patients with NASH needs to be investigated as there are some reports of aggravation of fatty liver in mice by pentoxifylline therapy[67].

Angiotensin receptor blockers

Angiotensin Ⅱ receptor blockers are a group of medications widely used for treatment of hypertension. Angiotensin Ⅱ probably promotes liver fibrosis via activation of transforming growth factor-β (TGF-β) and toll-like receptor-4 signaling[68,69]. In a series of 7 patients with NASH, 48 mo therapy with an angiotensin Ⅱ receptor blocker, losartan (50 mg/d), resulted in amelioration of necro-inflammatory response and improvement of hepatic fibrosis[70]. These beneficial effects was reported to be mediated *via* inhibition of HSC in this group of patients[71]. Fogari *et al*[72] reported that combination of simvastatin and losartan improved hepatic steatosis indices and decreased visceral adipose tissue diameter. In FANTASY trial, telmisartan therapy for 12 mo was associated with decreased serum free fatty acid levels without significant improvement in liver enzymes[73]. Addition of losartan to rosiglitazone had no extra histological benefit than rosiglitazone alone in patients with NASH[74]. In a rat model of type Ⅱ diabetes, valsartan could reduce hepatic fibrosis and steatosis correlated with reduction of tissue expression of TNF-α and monocyte chemoattractant protein-1 (MCP-1)[75]. Further studies are needed to confirm therapeutic role of angiotensin Ⅱ receptor blockers in treatment of NAFLD.

Ursodeoxycholic acid

It has been postulated that ursodeoxycholic acid (UDCA) may prevent progression of NAFLD because of anti-inflammatory and anti-apoptotic properties[76]. In a randomized placebo controlled trial, Ratziu *et al*[77] showed that high dose UDCA was effective in improvement of serum aminotransferase and markers of liver fibrosis in patients with biopsy proven NASH. A systematic review of 12 trials reported beneficial effects of UDCA for treatment of NASH[78]. However, UDCA is not currently recommended for treatment of NASH in international guidelines due to lacking of well-designed large randomized trials and lack of evidence about histological benefits.

Synbiotics and probiotics

Probiotics are live, human origin, non-pathogenic microorganisms with beneficial effects when consumed adequately. Prebiotics are not live microorganisms but chemicals causing growth of microorganisms. Nutritional supplements composed of probiotics and prebiotics are called synbiotics[79]. The role of gut microbiota has been confirmed in pathogenesis of insulin resistance and NAFLD[80]. Therefore, modulation of gut microbiota using probiotics and synbiotics has been suggested as a treatment option in NAFLD. Several studies with different preparations of probiotics have been conducted among NAFLD patients. A meta-analysis of 4 randomized trials reported that probiotics had beneficial effects on liver enzymes, lipid profile and improved insulin resistance in patients with NAFLD/NASH[81]. A double blind randomized clinical trial showed that Synbiotic + life style modification including physical activity and dietary regimen was superior to life style modification alone in treatment of patients with NAFLD[82]. Beneficial effects of synbiotics was also confirmed in lean NAFLD[83]. Despite promising results, data regarding histologic benefits of synbiotics and probiotics is lacking. Larger studies are needed to further elucidate the issue.

EMERGING PHARMACOLOGICAL OPTIONS

Obeticholic acid

Farnesoid X receptor (FXR) is a nuclear receptor which is expressed in the liver and is involved in bile acid synthesis. Binding of bile acids to FXR results in down-regulation of bile acid synthesis, hepatic lipogenesis, hepatic gluconeogenesis and improved peripheral insulin sensitivity[84]. FXR activation resulted in prevention of weight gain and decreased liver/muscle fat deposition and hepatic steatosis in obese rats[85]. Obeticholic acid (OCA) is a synthetic bile acid derivatives that acts as agonist of FXR and has been shown to be capable of reduction of hepatic steatosis in mice[86]. In a randomized double blind placebo controlled trial, 25 mg or 50 mg OCA was

given to patients with type Ⅱ diabetes and NAFLD for 6 wk. After completion of the study, OCA group had significant reduction in liver enzymes and markers of liver fibrosis compared to those in placebo group. However, the histologic features were not evaluated in this study[87]. FLINT trial is a multi-central randomized trial reporting improvement of histological features of NASH with 25 mg daily OCA for 72 h[88]. An unfavorable outcome was a rise in total cholesterol and LDL cholesterol accompanied by a fall in HDL cholesterol[88]. OCA seems to be a promising agent to be included in treatment of NASH in future.

Aramchol

Aramchol is a conjugate molecule composed of two components, cholic and arachidonic acid, which is primarily used for treatment of cholesterol gallstone[89]. Aramchol is an inhibitor of stearoyl CoA desaturase-1 (SCD1) an enzyme which is involved in lipid metabolism and hepatic insulin resistance[89,90]. Administration of 100 or 300 mg aramchol for 3 mo in patients with NAFLD resulted in decreased liver fat content without significant improvement of liver enzymes[91]. Larger clinical trials are needed to further elucidate the role of this agent in treatment of NAFLD.

Elafibranor

Peroxisome proliferator-activated receptor alpha (PPAR-α) is a member of PPAR superfamily that is expressed in adipose tissue, liver, skeletal muscle, heart and is involved in regulation of lipid and glucose metabolism. PPAR-α gene expression has been shown to have negative correlation with severity of NASH and visceral adiposity in patients with NAFLD[92]. Activation of another PPAR, PPAR-δ, is associated with improvement of insulin resistance, increase in oxidation of fatty acids and decrease in hepatic gluconeogenesis[93]. Elafibranor, a dual agonist of PPAR α/δ, has improved lipid profile and reduced hepatic fat in animal studies[94]. In a cross-over randomized trial, 80 mg daily administration of elafibranor in obese subjects was associated with improvement of hepatic and peripheral insulin resistance[95]. A recent randomized clinical trial showed that daily oral 120 mg elafibranor for 52 wk was associated with improvement of hepatic steatosis and fibrosis in patients with NASH in a dose dependent manner. Elafibranor therapy was also associated with improvement of systemic inflammation, lipid/glucose profiles and liver enzymes when compared to the placebo group[96]. Authors reported a reversible rise in serum creatinine in elafibranor group, otherwise, the drug was well-tolerated. Elafibranor may be considered as a candidate for treatment of patients with NASH after completion of ongoing phase Ⅲ trial.

Cenicriviroc

Overexpression of inflammatory chemokines CCL2 (MCP-1) and CCL5 (RANTES) have been established in patients with NASH leading to worsening of hepatic inflammation and fibrosis[97]. CCR2 and CCR5 are chemokine receptors for CCL2 and CCL5 that are inhibited by cenicriviroc. Anti-fibrotic properties of cenicriviroc have been approved in thioacetamide model of hepatic fibrosis in mice[98]. CENTAUR is an ongoing randomized clinical trial evaluating efficacy of cenicriviroc in patients with NASH and hepatic fibrosis[99].

Liraglutide

Liraglutide is an incretin mimetic that acts as an agonist of glucagon-like peptide-1 receptor and was primarily used for treatment of type Ⅱ diabetes. In animal model, liraglutide therapy was associated with amelioration of hepatic steatosis in mice fed with high fat/high fructose[100]. In Wistar rats, liraglutide therapy improved insulin resistance and hepatic steatosis by activation of AMP-activated protein kinase[101]. In a randomized trial, addition of liraglutide to insulin glargine was not superior to insulin glargine alone in improvement of glycemia and hepatic steatosis[102]. In a small randomized trial, 1.8 mg liraglutide administered subcutaneously was effective in improvement of histological features in patients with NASH[103]. Table 2 outlined new emerging medications for NAFLD.

CONCLUSION

Several medications including thiazolidindiones, metformin, vitamin E, statins, pentoxifylline, losartan, ursodeoxycholic acid, probiotics and synbiotics have been applied for treatment of NAFLD/NASH with promising but conflicting results. Future candidate medications for this purpose with ongoing or completing clinical trials are OCA, elafibranor, aramchol, cenicriviroc and liraglutide targeting different underlying mechanisms in NASH. Some of them may have beneficial effects on histological features of NAFLD/NASH.

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REVIEW

Intestinal epithelium, intraepithelial lymphocytes and the gut microbiota - Key players in the pathogenesis of celiac disease

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Abstract

Celiac disease (CD) is a chronic immune-mediated disorder triggered by the ingestion of gluten in genetically predisposed individuals. Before activating the immune system, gluten peptides are transferred by the epithelial barrier to the mucosal lamina propria, where they are deamidated by intestinal tissue transglutaminase 2. As a result, they strongly bind to human leucocyte antigens (HLAs), especially HLA-DQ2 and HLA-DQ8, expressed on antigen-presenting cells. This induces an inflammatory response, which results in small bowel enteropathy. Although gluten is the main external trigger activating both innate and adaptive (specific) immunity, its presence in the intestinal lumen does not fully explain CD pathogenesis. It has been hypothesized that an early disruption of the gut barrier in genetically susceptible individuals, which would result in an increased intestinal permeability, could precede the onset of gluten-induced immune events. The intestinal barrier is a complex functional structure, whose functioning is dependent on intestinal microbiota

homeostasis, epithelial layer integrity, and the gut-associated lymphoid tissue with its intraepithelial lymphocytes (IELs). The aim of this paper was to review the current literature and summarize the role of the gut microbiota, epithelial cells and their intercellular junctions, and IELs in CD development.

Key words: Celiac disease; Intestinal microbiota; Epithelium; Intraepithelial lymphocytes; Intestinal barrier

Core tip: There is evidence that the host-microbiota homeostasis is disrupted in celiac disease (CD) patients. Dysbiosis, meaning an imbalance in the gut microbiota and its metabolome, may activate innate immunity leading to pro-inflammatory changes, which induces intraepithelial lymphocyte infiltration and epithelial barrier damage, ultimately resulting in increased transfer of gluten peptides and inflammatory activation leading to CD development. The intestinal microbiota also has a direct effect on the breakdown of gluten and formation of immunogenic peptides. As colonization of the gut with microorganisms may be dependent on genetic factors, future prophylactic strategies may focus on gut microbiota modulation in genetically predisposed infants.

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INTRODUCTION

Celiac disease (CD) is a chronic immune-mediated disorder triggered by the ingestion of gluten in genetically predisposed individuals[1]. Gluten is a storage protein that consists of alcohol-insoluble glutenins and soluble prolamines, such as gliadin in wheat, secalin in rye, and hordein in barley. CD development requires the presence of gluten, the intestinal enzyme tissue transglutaminase 2 (TTG2), which modifies gluten peptides, and the genes encoding human leucocyte antigen (HLA)-DQ2 or HLA-DQ8[2]. Gluten from food products is degraded by gastrointestinal tract enzymes into peptides, which then are transferred through the epithelial barrier into the mucosal lamina propria.

In CD individuals, some of these peptides can bind to HLA-DQ2 or HLA-DQ8 heterodimers expressed on the surface of antigen-presenting cells (*e.g.,* macrophages, lymphocytes or dendritic cells) and, after triggering T-cell responses, lead to local tissue damage[3]. TTG2 converts glutamine residues present in gluten peptides into glutamic acid, and this conversion generates deamidated gluten peptides (DGP), which strongly bind to HLA-DQ2/-DQ8 molecules. Consequently, increased gluten antigenicity amplifies a gluten-specific T-cell response.

Gluten-activated T cells release pro-inflammatory cytokines [(mainly interferon-gamma (IFN-γ), interleukin (IL)-21 and IL-17)], which induce mucosal inflammation and have a direct cytotoxic effect on the epithelium, all of which finally leads to villous atrophy in the small intestine. Moreover, specific T cells induce B cells to produce antibodies directed against DGP and TTG2[4]. Thus, this adaptive (specific) T-cell response is a requirement for CD development. Nonetheless, innate immunity also plays an important role in CD development. The increased transfer of gluten peptides through the epithelial barrier could be a consequence of earlier activation of innate (non-specific) immunity, dependent on the function of both the epithelium and the lymphocytes located between epithelial cells, *i.e.* intraepithelial lymphocytes (IELs)[5].

Some of the gluten peptides can directly react with epithelial cells and activate production of pro-inflammatory cytokines, especially IL-15. IL-15 plays a key role in enhanced cytolytic activity of IELs *via* increasing the expression of both intestinal epithelial cell surface ligands (such as MICA and MICB, *i.e.* major histocompatibility complex class I chain-related molecules), which are targeted by cytotoxic, natural killer (NK)-like IELs, and NK receptors, such as NKG2D and CD94/NKG2C, on the surface of IELs. Finally, IL-15 activation leads to innate cytotoxic disruption of epithelial cells, resulting in increased intestinal permeability to different luminal macromolecules, including immunogenic gluten peptides[6].

Although gluten is the main external trigger of CD, gluten ingestion does not fully explain CD pathogenesis. Introduction of gluten into the diet starts in early childhood, but CD can develop at any point during a person's lifetime. The role of both breastfeeding and the time when gluten is first introduced into the diet in the risk of CD has long been debated. Retrospective data from Sweden indicated that introducing gluten in small amounts to breastfed infants at the age between 4 mo and 6 mo reduced the risk of CD compared with introducing gluten in larger amounts at older ages[7,8]. However, a recently published systematic review with meta-analysis of studies that assessed the effect of gluten consumption on CD development showed that for infants at high genetic risk of CD, gluten introduction at the age of 4 mo, 6 mo or 12 mo, resulted in similar rates of CD diagnosis in childhood, and neither breastfeeding as such (at any time during an infant's life) nor breastfeeding during gluten introduction were shown to reduce the risk of CD[9]. Also, the recently

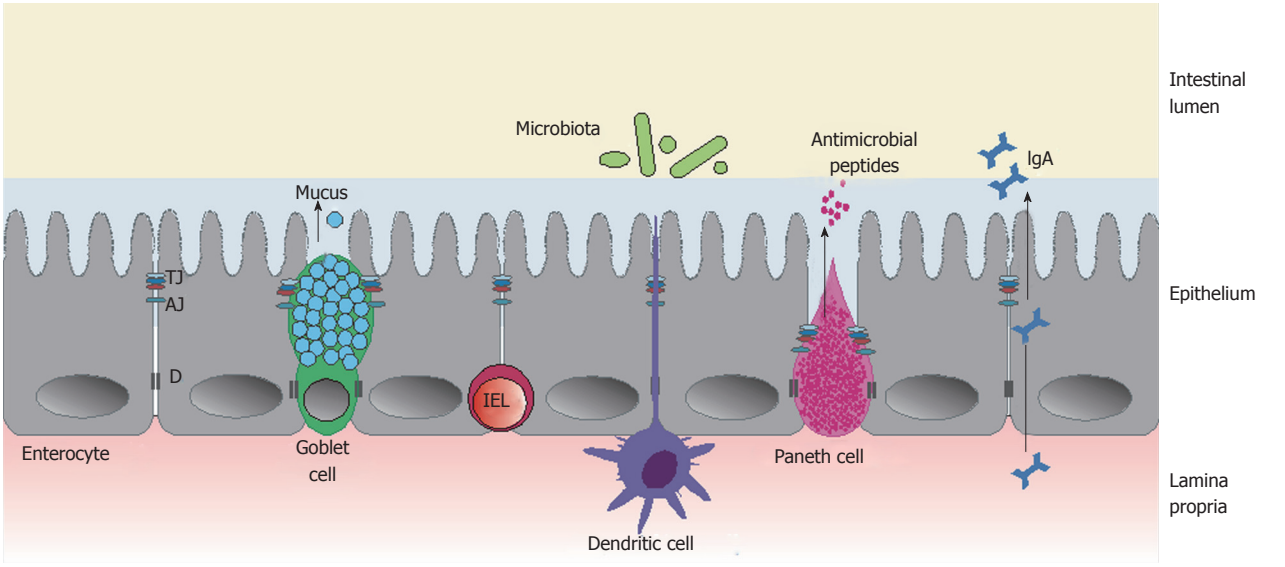

Figure 1 Schematic illustration of the intestinal barrier. The three main components of the intestinal barrier: the microbiota; epithelium, with its specialized cells (goblet cells, Paneth cells and enterocytes), together with a layer of mucus; and gut-associated lymphoid tissue cells, including IELs and dendritic cells. AJ: Adherens junction; D: Desmosome; IEL: Intraepithelial lymphocyte; TJ: Tight junction.

published prospective PreventCD cohort study showed that neither the gluten consumption pattern nor the amount of gluten consumed at the age of 11 mo to 36 mo influenced CD development in children with a genetic risk[10].

Thus, the time of gluten introduction into the diet seems not to play a key role in CD development. In addition, gluten-free diet (GFD) has been reported to improve mucosal lesions and decrease specific antibody levels, but not to correct the increased activation of pro-inflammatory mediators, which is characteristic for CD[11]. That is why it has been hypothesized that an early disruption of the gut barrier in genetically susceptible individuals, which is not associated with gluten peptides and results in an increased intestinal permeability, could precede the onset of gluten-induced immune events.

The intestinal barrier is a complex structure that separates the internal milieu from the luminal environment[12]. It consists of three main functional components: the microbiota that colonize the intestines; the epithelium, with its specialized mucus-producing cells and cells producing antimicrobial peptides; and gut-associated lymphoid tissue, composed of various immune cells (including IELs, which come in direct contact with gut luminal antigens, and lamina propria cells, producing secretory IgA) (Figure 1).

This review summarizes the role of epithelial cells and their intercellular junctions as well as IELs and the gut microbiota in the activation of early processes leading to the pathomechanisms associated with CD.

EPITHELIAL JUNCTIONS - STRUCTURES RESPONSIBLE FOR GUT PERMEABILITY

The small intestinal epithelium is organized into a monolayer of specialized cells: enterocytes (constituting approximately 80%), goblet cells (secreting mucus), Paneth cells (synthesizing defensins and other antimicrobial agents), endocrine cells (secreting hormones), and intestinal stem cells (responsible for epithelial cell homeostasis and regeneration)[13,14]. Epithelial cells form a continuous layer thanks to being sealed together by intercellular junctions, including tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions[15].

The ultrastructure of epithelial junctions is presented in Figure 2. TJs and AJs are supported by a dense perijunctional ring of actin and myosin, and they form the apical junctional complex and regulate epithelial paracellular permeability[16,17]. TJs are located near the apical surface of enterocytes and they act as a gate in the paracellular transport of ions, solutes, water, and cells. TJs are highly dynamic structures, whose degree of sealing varies in response to external stimuli as well as physiological and pathological conditions. TJs are composed of transmembrane proteins: occludin, claudins, junction adhesion molecules, tricellulin, and scaffold proteins - zonula occludens (ZO-1, ZO-2 and ZO-3)[17].

Occludin is an integral membrane protein with two extracellular loops, a short cytoplasmic N-terminal region, and a long cytoplasmic C-terminal region,

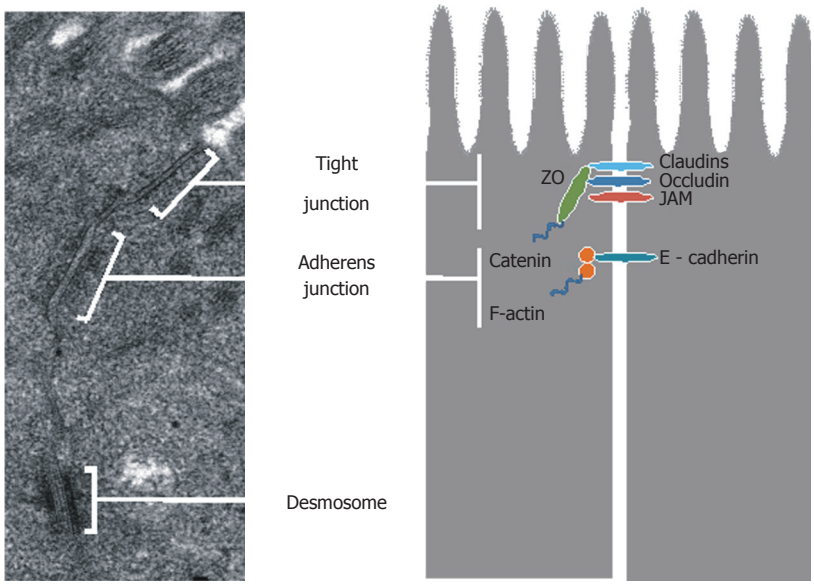

Figure 2 Ultrastructure and corresponding schematic representation of intercellular junctions. Transmission electron microscopy (JEOL JEM-1011, Japan; × 60000) was used to show the ultrastructure of intercellular junctions in the human small intestine. The transmission electron micrograph comes from our own research. JAM: Junction adhesion molecule.

which interacts with a ZO-1 protein that links occludin to the actin cytoskeleton[18,19]. Occludin plays a role in TJ maintenance and assembly, which are regulated by phosphorylation of serine (Ser), threonine (Thr), and tyrosine (Tyr) residues[20]. In an intact epithelium, occludin is highly phosphorylated on Ser and Thr residues[21,22] and poorly phosphorylated on Tyr residues[23]. Dephosphorylation of Ser/Thr residues and increased phosphorylation of Tyr residues reduces occludin's interaction with ZO-1, leading to its separation from the junctional complex and TJ disruption[24,25].

The claudin family can be divided into sealing proteins (claudins 1, 3, 4, 5 and 8), which reduce permeability, and pore-forming proteins (claudins 2, 7, 10 and 12), which increase permeability[26]. Thus, claudins 1, 3, 4, 5 and 8 strengthen the intestinal barrier, whereas claudins 2, 7, 10 and 12 weaken it. The extracellular loops of claudins are involved in the formation of ion-selective channels[27], while the intracellular C-terminal domain is connected to the cytoskeleton *via* a domain containing ZO-1, ZO-2 and ZO-3[28,29]. ZO-1, ZO-2 and ZO-3 are multidomain bridging proteins that function as cross-linkers, anchoring the TJ strand proteins to the actin cytoskeleton[30].

Recently, tricellulin has been identified as a co-mponent maintaining TJ structure and regulating the passage of macromolecules through the junctions[31]. TJ development may be dependent on AJ formation, since the ability of ZO-1 proteins to migrate apically to join occludin was observed only after AJ assembly[32]. The main component of AJ is E-cadherin, a transmembrane protein that forms homodimers with other cadherin molecules on adjacent cells. This protein is connected to the actin cytoskeleton by a complex of cytoplasmic proteins: α-, β- and γ-catenins[33].

Despite the major progress in knowledge on TJ structure and function, the mechanisms regulating TJs are still incompletely understood. The discovery of the *Vibrio cholerae*-derived Zonula occludens toxin, which reversibly regulates TJ permeability, helped identify its intestinal mammalian analogue - a human protein named zonulin[34,35]. Zonulin was identified as pre-haptoglobin 2. Structural analysis of this protein revealed similarities with several growth factors, such as hepatocyte growth factor or epidermal growth factor, which affect intercellular TJ integrity[36,37].

Zonulin was shown to induce TJ disassembly and a subsequent increase in intestinal permeability. Zonulin transactivates the epidermal growth factor receptor through proteinase-activated receptor 2, and then activates phospholipase C, which hydrolyzes phosphatidylinositol to release inositol 1, 4, 5-tris phosphate and diacylglycerol[38, 39]. Protein kinase Cα is then activated, either directly (*via* diacylglycerol) or through the release of intracellular calcium ions (*via* inositol 1, 4, 5-tris phosphate). Membrane-associated, activated protein kinase Cα catalyzes the phosphorylation of target proteins, including ZO-1 and myosin 1C, as well as polymerization of soluble G-actin in F-actin. This polymerization results in actin filament rearrangement and subsequent displacement of proteins (including ZO-1) from the junctional complex. As result, intestinal TJs become looser, which increases the paracellular transport of luminal molecules[35].

Zonulin is over-expressed in tissues and sera of subjects affected by autoimmune diseases, including CD[35]. *In vitro* studies showed that increased zonulin release in the small intestine can be triggered by both gluten peptides[38,39] and enteric bacteria[40].

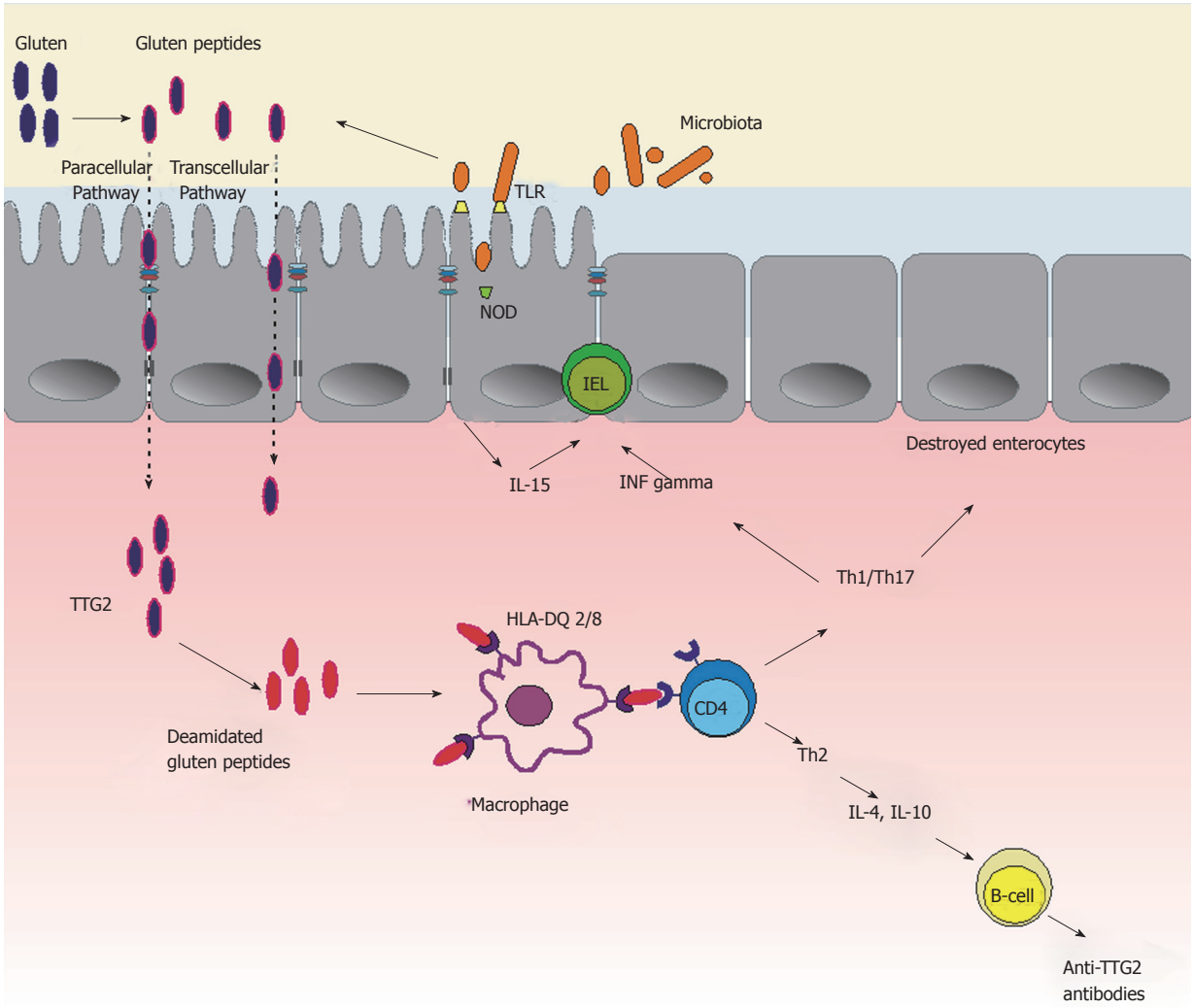

Figure 3 Schematic illustration of celiac disease pathogenesis. Microbiota dysbiosis activates innate immunity resulting in pro-inflammatory changes, which leads to IEL infiltration and epithelial barrier disruption. This ultimately results in an increased paracellular and transcellular transfer of immunogenic gluten peptides and activation of adaptive pro-inflammatory Th1/Th17 pathways, leading to villous atrophy and production of autoantibodies against intestinal TTG2. HLA: Human leucocyte antigen; IEL: Intraepithelial lymphocyte; IL: Interleukin; INF: Interferon; NOD: Nucleotide-binding oligomerization domain; Th: T helper; TLR: Toll-like receptor; TTG2: Tissue transglutaminase 2.

Zonulin secretion has been demonstrated to be independent of either the species or the virulence of the microorganisms tested[40]. However, recently an association of low serum zonulin levels with lower quantities of *Bacteroidaceae* and *Veillonellaceae* and higher quantities of *Faecalibacterium* has been found in overweight pregnant women[41]. Thus, this *in vivo* study suggests that zonulin release could be affected by changes in gut microbiota composition.

Recently, epithelial polarity regulators, especially the Par-3 protein, have been reported to be likely involved in regulating TJ permeability[42]. Par-3 and other proteins regulating cell polarity, such as Par-6 and atypical protein kinase C, form the apical polarity complex that orchestrates the formation of apical junctional complex. In addition, Par-3 located in the junctional complex together with ZO-1 and catenins is able to affect TJs by rearranging the actin cytoskeleton. Schumann *et al*[43] in 2012 found a reduced level of Par-3 and a defect in performing lateral exclusion of Par-3 in the epithelial cells of CD patients. In this context, genetic studies on non-HLA gene candidates associated with CD seem to be very interesting. Wapenaar *et al*[44] in 2008 found two candidate genes: Par-3 and Magi2, encoding the proteins regulating of epithelial polarity. However, this study involved a homogenous Dutch population, and further genome-wide association studies did not confirm this association[45].

DYSFUNCTION OF EPITHELIAL JUNCTIONS IN CD PATIENTS

One of the first studies on the structure of epithelial junctions using freeze-fracture electron micrographs presented severely altered TJs with strand discontinuities and a reduced number of strands in

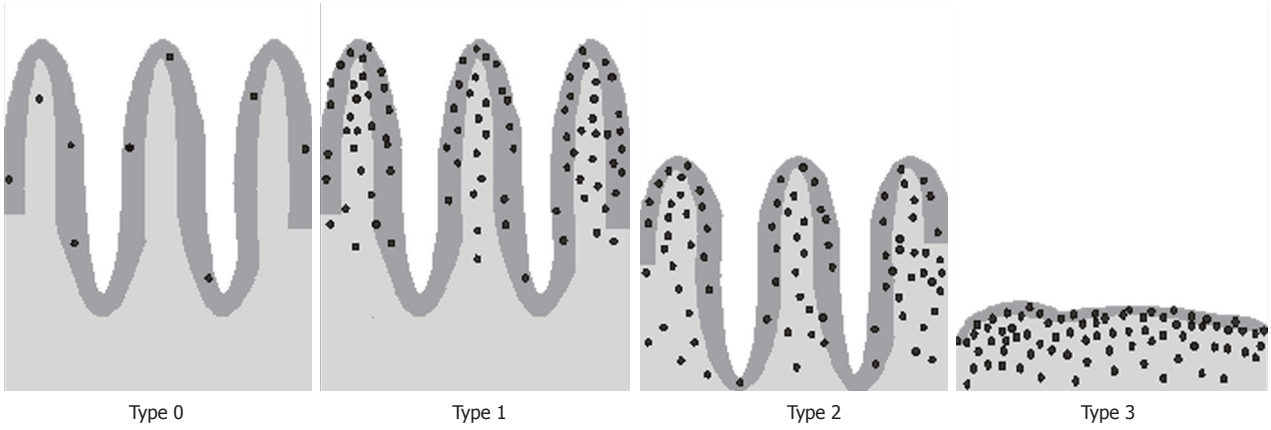

Figure 4 A schematic illustration of progressive histopathological changes in the small intestine according to the modified Marsh-Oberhuber grading scale. Type 0: Normal mucosa with IEL count < 25 per 100 enterocytes; Type 1: Normal mucosa with an increased IEL count; Types 2 and 3 show increased IEL counts and lymphocytes in the lamina propria. IELs are presented as black dots. IEL: Intraepithelial lymphocyte.

children with active CD[46]. GFD improved these abnormalities, but only partially - strand numbers were restored to normal at the surface, but remained low in the crypts. The recent transmission electron microscopy analyses on duodenal biopsies of CD patients also showed changes in TJ ultrastructure: dilatation (saccular or fusiform) and destruction of pentalaminar structures[47]. Interestingly, ultrastructural abnormalities of TJs were also found in asymptomatic and serologically negative first-degree relatives of CD patients[48].

Furthermore, over-expression of occludin and the pore-forming protein claudin-2 was demonstrated in CD patients, as well as an under-expression of pore-sealing proteins claudin-3 and 4, and scaffold protein ZO-1[47,49,50]. After introduction of a GFD, normalization of claudin expression was observed. No improvement after GFD introduction was reported in about 3% of the patients with refractory CD, whose mucosa undergoes a constant inflammatory process[51]. Other studies indicated a subcellular localization and downregulation of claudin 4 and claudin 5 in refractory CD patients[52].

Alterations in AJ structure were also reported. The expression of E-cadherin and β catenin - proteins required for TJ formation - was shown to be reduced in the duodenal epithelium of children with CD. Ciccocioppo *et al*[50] in 2006 showed that a lack of ZO-1 phosphorylation in active CD led to TJ disruption. The authors suggested that non-phosphorylated ZO-1 was unable to detach from β-catenin and to connect with occludin. It was also found that a higher phosphorylation of β-catenin was responsible for the absence of membranous E-cadherin. On the other hand, highly phosphorylated β-catenin was unable to connect with E-cadherin, which, in turn, could bind to the αEβ7-integrin of IELs. However, the levels of both E-cadherin and β-catenin returned to normal following GFD introduction[53,54]. Interestingly, a recent study by Mishra *et al*[48] in 2015 indicated the presence of altered ZO-1 and occludin expression not only in active CD patients but also in asymptomatic and serologically negative first-degree relatives of CD patients.

Fasano *et al*[35] in 2000 tried to explain the increased expression of zonulin found in CD patients. Some studies suggested that gliadin, by binding to the proinflammatory chemokine CXCR3 receptor on the intestinal epithelium, initiates the release of zonulin, which induces cytoskeleton rearrangement, ZO-1 and occludin down-regulation, leading to disruption of TJ integrity and finally to an increase in epithelial permeability[38,55]. Thus, the receptor CXCR3 could be involved in early TJ dysfunction, preceding the immune cascade of events observed in CD patients. Recently, Bondar *et al*[55] in 2014 showed that CXCL10 - a ligand for CXCR - is over-expressed in the small intestine of CD patients and strongly activated by poly I:C (an experimental model of viral infections) and IL-15 in non-CD controls. Thus, it cannot be excluded that the CXCR3/CXCL10 axis activated by infectious agents may play a role in initiating gluten-induced inflammatory processes in the small intestinal mucosa.

Overall, the presented results show that epithelial barrier impairment occurring in CD patients can play an important role in CD development. Because epithelial function is regulated by microorganisms colonizing the intestines[56], there is a hypothesis that dysbiosis, *i.e*. disturbances in both the quantity and composition of the gut microbiota, is a critical factor for the activation of innate immunity, leading to epithelial barrier dysfunctions.

GUT MICROBIOTA: THE MAINSTAY OF EPITHELIAL AND IMMUNE HOMEOSTASIS

The microbiota colonizing the gut after birth reaches the pattern found in adults within 2-3 years of life. Eventually, the human intestine is colonized with more than 1000 species categorized into subgroups

of phyla, classes, orders, families and genera, with *Firmicutes* and *Bacteroidetes* constituting the most abundant phyla[57]. The number of bacteria in the gut microbiota is similar to the number of cells making up the human body[58], and microbiota genes (microbiome) outnumber those in the human genome by approximately 100-fold. This complex microbial community adjusts the immune system, protects the body against pathogens, harvests nutrients and energy from the diet, and ferments non-digestible carbohydrates.

Extensive studies in germ-free (GF) animals, *i.e.* animals deprived of the gut microbiota, have demonstrated an indispensable role of microbiota in shaping the local mucosal gut-associated lymphoid tissue as well as systemic immunity[59,60]. In contrast to conventionally raised (CV) mice, GF mice have hypoplastic Peyer's patches and decreased number of both IgA-secreting plasma cells and lymphocytes located in the lamina propria. Colonization of GF animals with components of the gut microbiota induces production of secretory immunoglobulins A (sIgA). sIgAs are natural antibodies that constitute the first line of defense by reacting with a wide spectrum of microorganisms and toxic molecules, which directly affects the composition of the gut microbiota[61]. Experimental data have shown that sIgAs cooperate with innate defense factors to reinforce the epithelial barrier[62].

Epithelial barrier integrity also depends on homeostatic regulatory mechanisms, including mucosal induction of regulatory T (Treg) cells, and the gut microbiota plays a decisive role in this process[63]. According to some reports, gut-colonizing commensals are responsible for differentiation of effector T helper (Th) 1, Th17, and Treg cells responsible for Th1/Th2/Th17 homeostasis[64]. Colonization of GF mice with components of conventional microbiota also induced the recruitment and activation of IELs, some of which (especially γδ IELs) were reported to be involved in epithelial cell generation and differentiation[65,66]. Thus, the gut microbiota seems capable of protecting the epithelium and strengthening its barrier function[59].

Recently, using transmission electron microscopy, we found ultrastructural differences of enterocytes and epithelial junctions in GF mice, CV or specific pathogen-free (SPF) mice, and mice inoculated with a mixture of *Lactobacillus* strains obtained from stools of healthy children[61]. Brush borders of GF-mouse enterocytes were irregularly arranged and exhibited decreased numbers of cytoskeletal microfilaments and a lack of elongation into the terminal web. The AJ region was significantly broader and shorter in GF animals compared both with that in CV mice and in mice colonized with *Lactobacillus* strains. Consistent with other reports[67,68], we observed that the gut microbiota and *Lactobacillus* strains significantly increased the expression of TJ proteins: occludin and ZO-1[61]. On the other hand, there is experimental evidence that certain components of the gut microbiota, such as *Escherichia coli, Klebsiella pneumoniae* and *Streptococcus viridans,* are able to increase gut permeability[69].

The gut microbiota interacts with the host *via* pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) expressed on the surface of epithelial and dendritic cells. Recognition of specific microbial structures, called microorganism-associated molecular patterns, by PRRs induces signaling cascades that eventually result in immune response activation and the production of cytokines responsible for intestinal barrier strengthening (*e.g.,* TGF-β and IL-10) or weakening (*e.g.,* IL-15, TNF-α and IFN-γ)[70]. Alterations in TLR4 and TLR2 expression, as well as functional single-nucleotide polymorphisms in the genes expressed upon TLR4 activation, have also been associated with CD[45,71,72].

Interestingly, epithelial barrier function may be controlled indirectly by the intestinal metabolome, *e.g.,* gut microbiota metabolites in the form of low-molecular weight chemical intermediates[73]. Soluble dietary fibers (such as fructans, pectin, inulin and xylans) and resistant starches can be actively fermented by commensal microbiota in the human colon, producing biologically active short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate. These SCFAs are the main metabolites produced by gut-colonizing bacteria and a major source of energy for intestinal epithelial cells[74]. Acetate and propionate are predominantly produced by bacterial species of the phylum *Bacteroidetes*, whereas butyrate is primarily produced by those of the phylum *Firmicutes*. SCFAs serve as specific activators of orphan G-protein-coupled receptors, such as GPR43 and GPR41, predominantly expressed in intestinal epithelial cells[75,76]. GPR43 deficiency leads to expansion of *Firmicutes* in the gut microbiota and consequently raises fecal SCFAs and plasma acetate levels. Indoles, produced from tryptophan by various Gram-positive and Gram-negative intestinal bacteria, and acetate, produced by *Bifidobacterium* strains, enhance epithelial defense functions and suppress intestinal inflammation[77-79]. Microbe-derived SCFAs also have an impact on terminal differentiation of CD4+ Th cells[80].

The gut microbiota is responsible not only for immune homeostasis and epithelial barrier function, but also can have direct impact on gluten digestion in the intestinal tract. There is evidence that certain bacterial strains isolated from feces, *e.g.*, *Bifidobacterium* and *Bacteroides fragilis*, are capable of digesting immunogenic gliadin peptides, which are rich in proline residues but resistant to human enzymes[81,82].

GUT MICROBIOTA AND METABOLOME IN CD

Several studies addressed the phenomenon of gut dysbiosis in CD patients with active untreated disease and those on a GFD. Fecal analyses in untreated CD patients showed an imbalance in the composition of intestinal microbiota characterized by an increase in the number of *Bacteroides* species and reduced numbers of *Bifidobacterium* species[83-86]. In addition, CD patients, both untreated and treated with a GFD, demonstrated a lesser diversity of *Bacteroides* species in biopsy samples of the duodenal microbiota in comparison with controls[87]. The numbers of *Escherichia coli* and *Staphylococcus* bacteria were also higher in fecal and biopsy specimens of untreated CD children than in controls[88]. *Escherichia coli* strains from CD children carried a higher number of virulence genes than those from healthy children. Nadal *et al*[89] in 2007 reported a significantly lower ratio of harmless Gram-positive bacteria (*Lactobacillus* and *Bifidobacterium*) to potentially harmful Gram-negative bacteria (*Bacteroides/Prevotella* and *Escherichia coli*) in CD patients compared to controls, with no distinction between active and inactive CD. The numbers of bacteria of *Streptococcus* and *Prevotella* genera were found to be lower both in adults and children with untreated CD in comparison with healthy controls.

The disturbances in intestinal microbiota composition found in CD patients have been associated with changes in the metabolome[90]. Metabolic profiles of serum, urine and feces in celiac patients revealed a significantly altered profile of volatile organic compounds (*e.g.,* phenols and ketones), SCFAs and amino acids (*e.g.,* proline, methionine, histidine and tryptophan)[91,92]. CD patients were also characterized by higher urine levels of certain gut microbiota-derived metabolites, such as indoxyl sulfate, meta-[hydroxyphenyl] propionic acid and phenylacetylglycine, which were associated with untreated CD[92]. Interestingly, metabolic abnormalities found in celiac patients and "potential" celiac patients (*i.e.* individuals with a positive antibody test but no evidence of intestinal damage) were similar, indicating that CD-related dysmetabolism/dysbiosis precedes the intestinal damage[93].

Only a few serum metabolites can help differentiate between potential and overt CD; none of these metabolites are related to energy metabolism. Glycolysis appears to be somehow impaired in potential CD patients, just as is the case in overt CD patients. This is consistent with the hypothesis that the gut microbiota of CD patients is altered or contains specific species with their distinctive microbial metabolome. Schirmer *et al*[94] in 2016 reported that TNF-α and IFN-γ production was associated with specific microbial metabolic pathways: palmitoleic acid metabolism and tryptophan degradation to tryptophol.

Low doses of pro-inflammatory cytokines, such as IFN-γ, were shown not to affect TJ protein expression but to activate bacterial endocytosis by epithelial cells[95]. This process is dependent on extracellular signal-regulated kinase Cζ and ADP-ribosylation factor-6 signaling[96]. Thus, some commensal bacteria might interact with certain intracellular PRRs, namely, nucleotide-binding oligomerization domain (NOD)-like receptors, and activate epithelium-derived pro-inflammatory cytokines and free radicals that may cause secondary TJ damage[96,97]. An increased activity and expression of inducible nitric oxide synthase in human duodenal enterocytes has been reported in CD patients[97].

Thus, dysbiosis, which can follow viral or bacterial infections or antibiotic therapy, may activate innate immunity leading to pro-inflammatory changes, with the resulting IEL infiltration, epithelial barrier disruption, and increased transfer of immunogenic gluten peptides, which in turn activate inflammation leading to CD development (Figure 3).

ROLE OF IELs IN EPITHELIAL BARRIER HOMEOSTASIS

The typical histopathological presentation of CD is small intestinal enteropathy characterized by an increase in IELs, crypt hyperplasia, and villous atrophy. The changes develop gradually over time. The increased number of IELs is one of the earliest signs of CD[98] and may herald the impending disease[99]. Histological changes in the small intestine can be graded using the Marsh classification[100] modified by Oberhuber[101] (Figure 4). The Marsh-Oberhuber classification includes four categories of CD-associated lesions: infiltrative (type 1), infiltrative-hyperplastic (type 2), flat-destructive (type 3) and atrophic-hypoplastic (type 4)[101].

Irrespective of the type of changes found in CD patients, an increase in the number of IELs is considered to be the most sensitive histopathological marker of CD. The upper limit of normal for IELs in duodenal or jejunal mucosa is 25 IELs per 100 enterocytes. An IEL count between 25 and 29 is considered to be "borderline intraepithelial lymphocytosis", and 30 or more means "pathological lymphocytosis" in the duodenum[103,104]. IELs are classified into two major subgroups based on their phenotypical and functional characteristics: one bears the αβ T-cell receptor (αβ-IEL), while the other bears the γδ T-cell receptor (γδ-IEL). When it comes to the typical composition of the small-intestinal IEL population, approximately 75% of it consists of CD8-positive αβ T cells, 10% constitute CD4-positive αβ T cells, and 15% constitute γδ T cells which are CD4- and either CD8- or CD8+[5].

In CD sensitive patients, gluten exposure causes rapid activation of both αβ-IELs and γδ-IELs[105],

while a GFD lowers both αβ-IEL and γδ-IEL counts; however, lowering of the latter IEL subtype takes months or even years[106]. It is believed that CD8+ αβ-IELs represent the effector T cell subset that mediates epithelial cell destruction (after IL-15 up-regulation) and, ultimately, induces villous atrophy in CD. The role of γδ-IELs in CD pathogenesis remains unclear.

A recent study showed that IEL expansion can be modulated by the host microbiota. Mice deficient in NOD2 (receptors recognizing bacterial molecules)[107] exhibited a significant reduction in IEL counts and IL-15 expression in the epithelium, with the residual IELs displaying reduced proliferation and increased apoptosis. Moreover, *Lactobacillus* strains were able to decrease the number of IELs activated by TLR3 after an experimentally induced viral infection (poly I:C). They also significantly reduced the levels of pro-inflammatory cytokines, such as TNF-α and IL-15, and increased serum and intestinal regulatory IL-10 levels[108]. Finally, the immunomodulatory capacity of *Lactobacilli* helped significantly reduce intestinal tissue damage.

The data above indicate that IEL homeostasis is controlled by commensal microbiota, which affects cytokine production by epithelial cells *via* PRR activation. Moreover, increased IEL counts in CD patients, which lead to epithelial barrier disturbances, may be primarily induced by microbiota dysbiosis.

ROLE OF THE GUT MICROBIOTA PROGRAMMING IN CD DEVELOPMENT

Recent research has shown that early bacterial colonization may affect the risk of developing CD later in life. This phenomenon is called microbial programming[109]. There is evidence indicating that the pioneer microbiota of the neonatal gut is essential for gut maturation as well as for metabolic and immunologic programming[109,110]. Establishment of the human gut microbiota is a complex, stepwise process. The composition of microbiota within the 1st year of life is characterized by low diversity, high instability, and high inter-individual variation[111]. By the age of 2-3 years, the microbiota becomes stable, more diverse, and resembles that found in adults, with *Firmicutes* and *Bacteroidetes* as the predominant phyla. Gut microbiota formation after the birth is dependent on different environmental factors, such as the mode of delivery, breast or formula feeding, or antibiotic therapy[111].

Although the evidence that the perinatal environment influences CD development is still only circumstantial[112], there have been studies showing that cesarean sections and antibiotic treatment in infancy increased the risk of CD[113-115]. There is also evidence that colonization of the gut with microorganisms may be dependent on genetic factors[116,117]. The hypothesis that gut microbiota composition is affected by host genes has been confirmed by studies in twins, showing that fecal microbiota of monozygotic twins was much more similar than that of dizygotic twins[118]. Recent microbiome analyses performed on 22 infants demonstrated that certain HLA genes predisposing to CD could affect microbiota composition[119]. The infants at high genetic risk of CD, *i.e.* those with an HLA-DQ2 genotype, showed a higher proportion of *Firmicutes* and *Proteobacteria* and lower proportion of *Actinobacteria* than those at low genetic risk. At the genus level, the gut microbiota of high-risk infants had a significantly lower proportion of *Bifidobacterium* and unclassified *Bifidobacteriaceae* and a higher proportion of *Corynebacterium*, *Gemella*, *Clostridium*, unclassified *Clostridiaceae*, unclassified *Enterobacteriaceae* and *Raoultella*. Sellitto *et al*[120] in 2012 reported an overall lack of bacteria of the phylum *Bacteriodetes*, with a high abundance of *Firmicutes*, in infants genetically predisposed to CD compared with microbiota composition of low-risk infants. Those differences were stable until 2 years of age.

As CD is strongly associated with HLA genes - almost 100% individuals with CD are carriers of alleles encoding HLA-DQ2/DQ8 molecules - these findings suggest that children with the CD risk genotype have a different microbiota profile than those without genetic predisposition. However, it must be emphasized that about 25%-30% of the general population exhibits the same HLA genotypes as CD patients[121]. In addition, there are also non-HLA genes associated with CD.

CONCLUDING REMARKS AND FUTURE STRATEGIES

Although gluten is necessary in order to activate the processes leading to CD, there is evidence that an imbalance in the gut microbiota and intestinal epithelium can precede the specific gluten-dependent immune response. Under certain conditions affecting the intestinal microbiota, *e.g.,* after infections or antibiotic therapy, an increased translocation of dietary macromolecules (including gluten peptides) *via* the opening of epithelial junctions triggers a cascade of events in genetically susceptible individuals, leading to overt CD. Microbiota disturbances are observed not only in untreated CD patients, but also in potential CD patients and those following a GFD as well as in infants at high genetic risk of CD. The microbial fingerprint associated with CD is likely dependent on specific genetic factors, including (but not exclusively) the HLA-DQ2/-DQ8 genotype. Future strategies should include prospective, birth cohort studies involving comprehensive genome, microbiome and metabolome analyses. Such an approach could help identify a "CD-specific" microbial/metabolic fingerprint, which would

become the target for both primary prevention and management of CD.

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ORIGINAL ARTICLE

Basic Study

Pregnane X receptor and constitutive androstane receptor modulate differently CYP3A-mediated metabolism in early- and late-stage cholestasis

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Abstract

AIM

To ascertain whether cholestasis affects the expression of two CYP3A isoforms (CYP3A1 and CYP3A2) and of pregnane X receptor (PXR) and constitutive androstane receptor (CAR).

METHODS

Cholestasis was induced by bile duct ligation in 16 male Wistar rats; whereas 8 sham-operated rats were used as controls. Severity of cholestasis was assessed on histological examination of liver sections, and serum concentrations of albumin, AST, ALT, GGT, ALPK and bilirubin. Gene and protein expressions of PXR, CAR, CYP3A1 and CYP3A2 were assessed by means of qRT-PCR and Western blot, respectively. Alterations in CYP3A activity were measured by calculating the kinetic parameters of 4-OH and 1'-OH-midazolam hydroxylation, marker reactions for CYP3A enzymes.

RESULTS

The mRNA and protein expression of CYP3A1 increased significantly in mild cholestasis ($P < 0.01$). At variance, mRNA and protein expression of CYP3A2 didn't change in mild cholestasis, whereas the expression and activity of both CYP3A1 and CYP3A2 decreased dramatically when cholestasis became severe. Consistently with these observations, the nuclear expression of both PXR and CAR, which was measured because they both translocate into the cell nucleus after their activation, virtually disappeared in the late stage of cholestatic injury, after an initial increase. These results indicate that early- and late-stage cholestasis affects CYP3A-mediated drug metabolism differently, probably as consequence of the different activation of PXR and CAR.

CONCLUSION

Early- and late-stage cholestasis affects CYP3A-mediated drug metabolism differently. PXR and CAR might be targeted therapeutically to promote CYP3A-mediated liver detoxification.

Key words: Cholestasis; CYP3A; Drug metabolism; Pregnane X receptor; Constitutive androstane receptor

Core tip: We demonstrated that early- and late-stage cholestasis affects CYP3A-mediated metabolism differently, probably as consequence of the different activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR). As a consequence, cholestatic patients may have an altered drug metabolism: in the early stage due to the induction of CYP3A enzymes; and in the late stage due to the high deposition of fibrotic liver and consequent hepatocyte loss. Secondly, since PXR activation is known to induce alternative hepatic export routes and detoxification enzymes, the induction of these cellular pathways with PXR and/or CAR agonists could be exploited as a therapeutic strategy for the management of cholestatic diseases.

Gabbia D, Dalla Pozza A, Albertoni L, Lazzari R, Zigiotto G, Carrara M, Baldo V, Baldovin T, Floreani A, De Martin S. Pregnane X receptor and constitutive androstane receptor modulate differently CYP3A-mediated metabolism in early- and late-stage cholestasis. *World J Gastroenterol* 2017; 23(42): 7519-7530 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7519.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7519

INTRODUCTION

Cholestasis can be defined as a bile flow impairment such that insufficient amounts of bile reach the duodenum[1]. Cholestatic liver diseases comprise a heterogeneous group of disorders resulting from bile duct injury caused by genetic defects, mechanical obstruction, toxins, or immune system dysregulation, and giving rise to bile and liver tissue damage. Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are the two main classes of cholestatic disease, and chronic cholestasis and liver inflammation are their main pathophysiological components[2]. Common clinical manifestations and pathogenic features of cholestatic disease include the responses of cholangiocytes and hepatocytes to injury. In an early stage, the accumulation of potentially cytotoxic bile acids (BAs) in the liver can lead to hepatocellular damage; this is followed by inflammation, which causes hepatic cell death, biliary fibrosis and liver cirrhosis[3]. To escape the deleterious effects of high concentrations of BAs, the liver triggers an adaptive response to cholestasis, activating a complex network of nuclear receptors (NRs) that tightly regulate the BA transporters to maintain proper BA homeostasis. The BAs act as agonists/activators of the NRs, which are ligand-activated transcription factors regulating a broad array of key hepatic processes, such as hepatobiliary excretory function, hepatic glucose and lipid metabolism, inflammation, regeneration, fibrosis, and tumorigenesis[4]. In addition to BAs, there are other biliary constituents that can activate the NRs, such as bilirubin. The NRs include the constitutive androstane receptor (CAR), and the pregnane X receptor (PXR), which control the expression of CYP3A

drug-metabolizing enzymes. PXR and CAR act as xenobiotic sensors, as one of their main functions is to regulate the expression of enzymes and transporters involved in xenobiotic elimination. These NRs are also involved in controlling hepatic processes closely related to the progression of cholestatic diseases, such as BA homeostasis, lipid metabolism, fibrosis and inflammation[5]. Changes in CAR and PXR expression have already been identified in the course of cholestatic liver disease, but different effects have been documented depending on the etiology of the cholestasis. In particular, a pronounced increase in PXR and CAR expression was documented in patients with obstructive cholestasis[5], as opposed to a moderate reduction of their expression in primary biliary cholangitis[6]. In an animal model of cholestasis, it was demonstrated that PXR has a protective effect, reducing inflammation and fibrosis[7].

There is general consensus that liver disease impairs various pathways of hepatic drug metabolism. Both *in vitro* and *in vivo* studies have shown a decrease in CYP3A activity in cholestatic liver disease, but a clear demonstration of the mechanism(s) responsible for it is still lacking[8-13]. To our knowledge, no studies published to date simultaneously analyzed CYP3A enzyme expression and activity and the activation of NRs responsible for their transcriptional control in different stages of cholestatic disease. CYP3A1 and CYP3A2 are regarded as the metabolically most important CYP3A isoforms in male rats. The former is the isoform mainly susceptible to induction, while the latter is the isoform expressed at the highest constitutive level[14,15]. CYP3A enzymes are the most abundant CYPs in human beings, and the most important enzymes in terms of drug metabolism, because they have a key role in the first-pass and systemic metabolism of many drugs[16,17].

The aim of the present study was to analyze the gene and protein expression, and the enzymatic activity of CYP3A1 and CYP3A2, as well as the nuclear expression of CAR and PXR. For this purpose, we used a validated animal model of cholestasis based on the bile duct ligation technique in animals rigorously stratified by degree of liver injury.

MATERIALS AND METHODS

Reagents

Midazolam, 4-hydroxymidazolam (4OH-MDZ), nicotinamide adenine dinucleotide 2′-phosphate (NADPH), dimethylsulfoxide (DMSO), 40% acrylamide solution, sodium dodecyl sulfate (SDS), and Tween 20 were purchased from Sigma-Aldrich Italy (Milan, Italy). 1′-hydroxymidazolam (1′OH-MDZ) was purchased from SPIBio Bertin Pharma (Montigny le Bretonneux, France). Ultrapure water was obtained with Pure-Lab Option Q apparatus (Elga Lab Water, High Wycombe, United Kingdom). Sucrose, Tris and $MgCl_2$ were purchased from Applichem (Chicago, IL, United States). HPLC-grade methanol was purchased from Scharlau (Barcelona, Spain). Rabbit polyclonal anti-PXR, rabbit polyclonal anti-CAR, mouse anti-CYP3A1, rabbit anti-CYP3A2 and HRP-conjugated anti-mouse IgG antibodies were obtained from Abcam (Cambridge, United Kingdom). HRP-conjugated anti-rabbit IgG were obtained from Millipore (Billerica, MA, United States), and HRP-conjugated anti-goat IgG from Jackson Immuno Research (West Grove, PA, United States). Rabbit polyclonal anti-calnexin, rabbit polyclonal anti-GAPDH and goat polyclonal anti-acetyl histone H3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

Animal care and use statement

The procedures involving the animals were managed in accordance with national and international laws and policies (Directive 2010/63/EU on the protection of animals used for scientific purposes). The study design was approved by the Ethics Committee of University of Padova, and by the Italian Ministry for the care and use of laboratory animals (Prot. no. 24, 2015). Rats were kept in standard conditions (24 ℃, 12 h/12 h day/night cycle), and they have *ad libitum* access to food and water. Protocols involving animals were designed to minimize their pain, and each treatment was performed under isoflurane anesthesia.

Animal treatments

Cholestasis was induced in 16 male Wistar Kyoto rats (Charles River, Boston, MA, United States) by bile duct ligation and resection, as previously described[18,19]. Eight sham-operated rats were used as control animals. Eight bile-duct-ligated (BDL) animals were sacrificed two weeks after surgery to obtain a model of mild cholestasis, and the other 8 four weeks after surgery to obtain a model of severe liver injury. Blood was collected at the time of their sacrifice by cardiac puncture to perform biochemical liver function tests: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALKP), γ-glutamyl transferase (GGT), serum albumin, total and conjugated bilirubin. After sacrificing the animals, their livers were promptly removed and weighed, and a piece was excised, fixed in 4% formalin and embedded in paraffin for histological examination. Bile acid concentration was measured on liver tissue omogenates by means of the Bile Acid Assay kit obtained from Sigma-Aldrich Italy (Milan, Italy), following manufacturer's instructions. The remaining liver tissue was washed in a buffer containing 50 mmol/L Tris, 150 mmol/L KCl, 2 mmol/L EDTA, (pH 7.4), frozen and stored at -80 ℃.

Histological examination

Two 4-micron sections were cut from the paraffin-

embedded liver tissue, and stained with hematoxylin-eosin and fuchsin-picric acid (van Gieson staining), as previously described[20,21]. Images were obtained with a Leica SCN400 slide scanner. The Ishak system was adopted to quantify the extent of liver damage, scoring livers from 0 to 6 by severity of fibrosis[22].

Determination of mRNA levels by qRT-PCR

Total RNA was extracted from liver tissue after homogenization and purified with the SV Total RNA Isolation System (Promega Corporation, Madison, WI, United States). Liver tissue was homogenized in lysis buffer and mRNA was purified by means of silica-gel-based columns, according to the manufacturer's instructions. A DNAse treatment was used during RNA extraction to prevent genomic DNA contamination. Purified RNA was eluted in a final volume of 100 μL RNAse-free water. Aliquots were stored at -80 ℃ until use. qRT-PCR was carried out with the commercial One Step SYBR PrimeScript RT-PCR Kit (Takara, Mountain View, CA, United States). The reaction was carried on in a volume of 10 μL as recommended by the manufacturer, using the PCR primers listed in Table 1. The qRT-PCR thermal program was run as follows: 15 min at 50 ℃ and 2 min at 95 ℃ for the reverse transcription, 40 cycles of 15 s at 95 ℃ and 60 s at 60 ℃ for the PCR reaction, and then 15 s at 95 ℃, 15 s at 55 ℃ and 15 s at 95 ℃ for the dissociation curve. During the exponential phase, the fluorescence signal threshold was calculated, and the cycle threshold (C_t) was ascertained. The Ct values were used to calculate the relative mRNA expression, according to the mathematical quantification model proposed by Pfaffl[23]. All samples were tested in triplicate and β-actin was used as the housekeeping gene.

Preparation of hepatic nuclear fraction

Nuclear and cytoplasmic fractions were obtained according to the method described by Dimauro *et al*[24] with minor modifications. Briefly, 150 mg of liver tissue was allowed to thaw in STM buffer containing 250 mmol/L sucrose, 50 mmol/L Tris-HCl pH 7.4, 5 mmol/L $MgCl_2$ and Complete Protease Inhibitor Cocktail (Roche, Milan, Italy). The tissue was then homogenized for 1 min at 1000 rpm with an IKA T25 Ultra-Turrax disperser. The homogenate was maintained in ice for 30 min, vortexed and centrifuged at 800 *g* for 15 min. The pellet was resuspended in STM buffer, vortexed and then centrifuged at 500 *g* for 15 min. The supernatant was discarded and the pellet was washed in STM buffer, vortexed and centrifuged at 1000 *g* for 15 min. The resulting pellet, containing the nuclei, was resuspended in NET buffer, containing 20 mmol/L HEPES, 1.5 mmol/L $MgCl_2$, 0.5 mol/L NaCl, 0.2 mmol/L EDTA, 20% glycerol, 1% Triton-X-100 and Complete Protease Inhibitor Cocktail. It was vortexed for 15 seconds and maintained in ice for 30 min. Then, it was sonicated twice with a Soniprep 150 MSE (London, United Kingdom) and centrifuged at 9000 *g* for 30 min at 4 ℃. The resulting supernatant was the "nuclear fraction".

Preparation of hepatic microsomal fraction

Microsomal fractions were obtained as reported previously by Floreani *et al*[21]. The pellet containing the microsomal fraction was resuspended in 250 mmol/L sucrose, aliquoted and stored at -8 ℃. The protein content of the nuclear and microsomal fractions was measured with a commercially available kit (Thermo Fisher BCA Protein Assay kit, Rockford, IL, United States) using bovine serum albumin for the calibration curve.

Western blot analyses

Western blot analyses to ascertain the protein expression of CYPs and the nuclear protein expression of PXR and CAR were performed using 30 μg per lane of nuclear or microsomal fractions, as previously described[20]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 8% polyacrylamide gels in reducing-denaturing conditions, and proteins were transferred to a 0.45 μm nitrocellulose membrane (BioRad, Hercules, CA, United States). Anti-PXR, anti-CAR, anti-CYP3A1 and anti-CYP3A2 primary antibodies were used to detect PXR, CAR, CYP3A1 and CYP3A2 proteins in the hepatic fractions, as previously described by De Martin *et al*[20]. The signal intensity of the immunoreactive bands was analyzed with the Quantity One software (Bio-Rad Laboratories, Hercules, CA, United States), and normalized to that of the acetyl-histone H3 or the calnexin bands, for the nuclear or microsomal fractions, respectively.

Measurements of 4- and 1'-midazolam hydroxylation

To analyze the formation of 4OH-MDZ and 1'OH-MDZ, microsomal preparations were incubated with increasing concentrations of midazolam (between 0.5 pmol/L and 20 pmol/L, n = 9) in a total volume of 200 μL, as previously described[20]. Ten μg of microsomal proteins obtained from sham and rats with mild cholestasis were used, and 80 μg obtained from rats with severe cholestasis. The reaction was run for 5 or 10 min at 37 ℃ in 0.1 mol/L KH_2PO_4, adding 0.5 mmol/L of NADPH, and then the reaction was stopped by adding 100 μL of cold methanol. The samples were centrifuged at 20000 *g* for 10 min and the supernatants were analyzed by HPLC using a Shimadzu system equipped with a UV detector. The two metabolites were separated chromatographically by means of a Kinetex EVO C18 column (5 μm, 150 mm × 4.6 mm, Phenomenex, Castel Maggiore, Italy). The mobile phase was water and acetonitrile (67:33). Isocratic elution was conducted with a constant flow of 1 mL/min for 15 min. The UV detector was set up at 220 nm, eluting 4OH-MDZ after 4.7 min, and

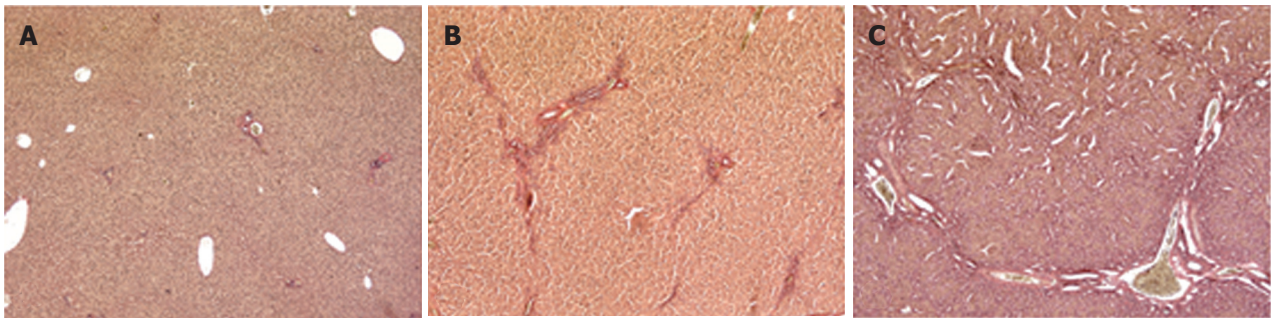

Figure 1 Histological analyses of rat livers. Representative photomicrographs of liver sections taken from sham-operated (A), mildly cholestatic (B) and severely cholestatic rats (C), stained with van Gieson to detect liver fibrosis (magnification × 5).

Table 1 Primer sequences used in the qRT-PCR analysis and NCBI reference sequences

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	RefSeq
CYP3A1	CCATCACGGACACAGAAATG	CTTTCCCCATAATCCCCACT	NM013105
CYP3A2	AGTGGGGATTATGGGGAAAG	CAATGATGGGGGAACATCTCC	NM153312
PXR	CAAATCTGCCGTGTATGTGG	GTTTCATGGCCCTTCTGAAA	NM052980
CAR	GAGGAAAGACATGATACTGTCAG	GTCTGGATGAGCTCTTTCTGCT	NM022941
β-actin	GCCACCAGTTCGCCATGGA	TTCTGACCCATACCCACCAT	NM031144

1'OH-MDZ after 5.2 min. The 4OH-MDZ and 1'OH-MDZ were quantified with standard calibration curves obtained with authentic 4OH-MDZ and 1'OH-MDZ at concentrations ranging from 1 to 50 nmol/mL (n = 8), processed in exactly the same way as the samples obtained from the kinetic experiments. The calibration curves were linear in this concentration range (r^2 = 0.99), the lowest value in the range representing the quantification limit of the assay. Both inter- and intra-assay CVs for the 4OH-MDZ and 1'OH-MDZ determinations (n = 5) were lower than 5% at 1 nmol/mL, and lower than 3% at 50 nmol/mL. MDZ 4- and 1′-hydroxylase activity was expressed as pmol of 4OH-MDZ and 1'OH-MDZ produced per mg of protein per min.

Kinetic and statistical analyses

The F-test was used to judge the best-fitting kinetic model for 4- and 1′-hydroxylation of midazolam (hyperbolic Michaelis-Menten model or Michaelis-Menten model with substrate inhibition). Kinetic parameters were estimated by non-linear regression analysis of untransformed initial velocity data (GraphPad Prism software) using the appropriate equation. The following kinetic parameters were calculated: Vmax (maximum velocity of the reaction), and Km (substrate concentration yielding 50% of Vmax).

Statistical analyses were performed with the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States). Unless specified otherwise, the data are presented as mean values ± SD. The experimental results were compared by one-way analysis of variance (ANOVA) or Student's t-test, as appropriate. In the case of statistically significant differences (α = 0.05), the analysis of variance was followed by the Newman-Keuls *post-hoc* test. A P value < 0.05 was considered statistically significant.

RESULTS

Biochemical findings and histology of rat livers

The animal model of cholestasis was obtained by ligating the common bile duct[25]. The presence of cholestasis was confirmed after sacrificing the animal on the basis of plasma biochemical tests and histological examination. The latter revealed a normal liver architecture in the sham-operated animals (Figure 1A). The livers from rats sacrificed after 2 wk showed mild cholestasis (Figure 1B, Ishak score 1-3), and fibrous septa. Rats sacrificed after 4 wk showed severe cholestasis with bridging fibrosis surrounding cirrhotic nodules, and the original liver structure completely destroyed (Figure 1C, Ishak score 4-6). The degree of liver dysfunction was also assessed on the basis of serum biochemistry (albumin, AST, ALT, total and conjugated bilirubin, alkaline phosphatase and γGT), as shown in Table 2. The rats with mild cholestasis had the same serum albumin levels as the healthy control animals, whereas the levels were significantly lower in the rats with severe cholestasis (P < 0.05 *vs* control and mildly cholestatic rats). All other serum markers of liver injury were significantly increased in rats with severe cholestasis by comparison with either the controls or the animals with mild cholestasis. Liver bile acid concentration increased as liver function worsens (252 ± 32 nmol/g in sham-operated *vs* 404 ± 51 nmol/g in mildly cholestatic *vs* 515 ± 62 nmol/g liver tissue in severely cholestatic rats).

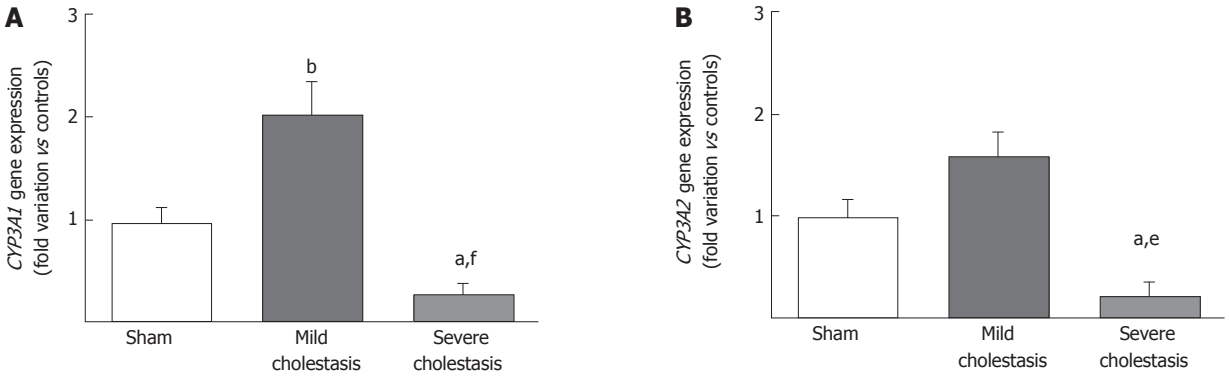

Figure 2 mRNA expression of CYP3A isoforms in rat livers. Gene expression of CYP3A1 (A) and CYP3A2 (B). Results are mean ± SEM, obtained from 8 rats of each group. [a]P < 0.05, [b]*P* < 0.01 *vs* sham-operated rats; [e]*P* < 0.01 and [f]*P* < 0.001 *vs* rats with mild cholestasis.

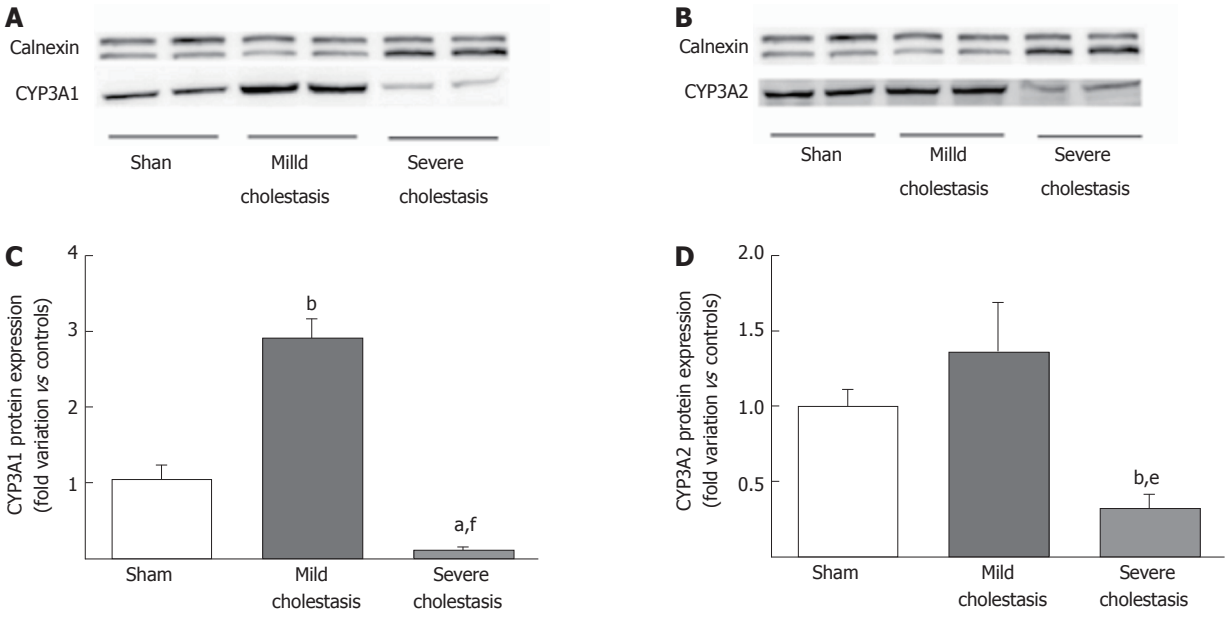

Figure 3 Microsomal protein expression of CYP3A isoforms in rat livers. A representative Western blot experiment showing CYP3A1 (A) and CYP3A2 (B) protein expression in microsomal fractions obtained from sham-operated and cholestatic rats. Densitometric analysis of CYP3A1 (C) and CYP3A2 (D) protein bands. Results are mean ± SEM obtained from 8 rats of each group. Each experiment was conducted in triplicate. [a]*P* < 0.05, [b]*P* < 0.01 *vs* sham rats; [e]*P* < 0.01, [f]*P* < 0.001 *vs* mildly cholestatic rats.

mRNA and protein expression of CYP3A1 and CYP3A2

The mRNA expressions of CYP3A1 and CYP3A2 are shown in Figure 2A and B. We found CYP3A1 gene expression significantly increased in mild cholestasis (*P* < 0.01 *vs* controls), while CYP3A2 gene expression tended to increase with respect to controls, but not to any significant degree. In contrast, there was a significant drop in the gene expression of both CYP3A1 (*P* < 0.05 *vs* controls, and *P* < 0.001 *vs* rats with mild cholestasis) and CYP3A2 (*P* < 0.05 *vs* controls, and *P* < 0.01 *vs* rats with mild cholestasis) when cholestasis became severe.

Figure 3 shows the protein expression of the two CYP3A isoforms (assessed on liver microsomal fractions). Like the mRNA, CYP3A1 protein expression increased significantly in mild cholestasis (*P* < 0.01 *vs* controls), then decreased significantly in severe cholestasis (*P* < 0.05 *vs* controls, and *P* < 0.001 *vs* rats with mild cholestasis). CYP3A2 protein expression only decreased when cholestasis became severe without first showing any significant increase in mild cholestasis (*P* < 0.05 *vs* controls, and *P* < 0.01 *vs* rats with mild cholestasis).

Kinetic analysis of 4- and 1'-hydroxylation activities

Midazolam hydroxylation in positions 1' and 4 was used as a marker reaction to assess CYP3A1 and CYP3A2 enzyme activity in the rat liver microsomal fractions[26,27]. As shown in Figure 4, both reactions followed the classical Michaelis-Menten kinetic model. Table 3 shows the kinetic parameters of the midazolam hydroxylation reactions in the three groups of rats. For both reactions, there was a slight, statistically insignificant increase in Vmax in the rats

Table 2 Biochemical liver function parameters and Ishak scores

	Sham-operated rats	Mildly cholestatic rats	Severely cholestatic rats
Albumin (g/L)	36.1 ± 3.3	35.8 ± 3.8	31.2 ± 3.7[a,d]
ALT (U/L)	43.7 ± 8.1	44.2 ± 8.8	115.3 ± 32.4[c,f]
AST (U/L)	92.2 ± 41.6	103.5 ± 33.0	402.7 ± 136.6[c,f]
γGT (U/L)	0.1 ± 0.6	0.5 ± 0.5	52.4 ± 34.8[b,e]
ALKP (U/L)	133.9 ± 47.1	150.8 ± 38.0	320.1 ± 107.2[c,f]
Conjugated bilirubin (mmol/L)	0.8 ± 0.3	0.7 ± 0.3	133.1 ± 56.9[c,f]
Total bilirubin (mmol/L)	1.1 ± 0.6	1.0 ± 0.3	151.2 ± 64.1[c,f]
Ishak score	0	1-3 (1)	4-6 (5)

The results are given as mean ± SD obtained from 8 rats of each group. Ishak scores are given as range (median). [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$ *vs* sham-operated rats; [d]$P < 0.05$; [e]$P < 0.01$; [f]$P < 0.001$ *vs* mildly cholestatic rats.

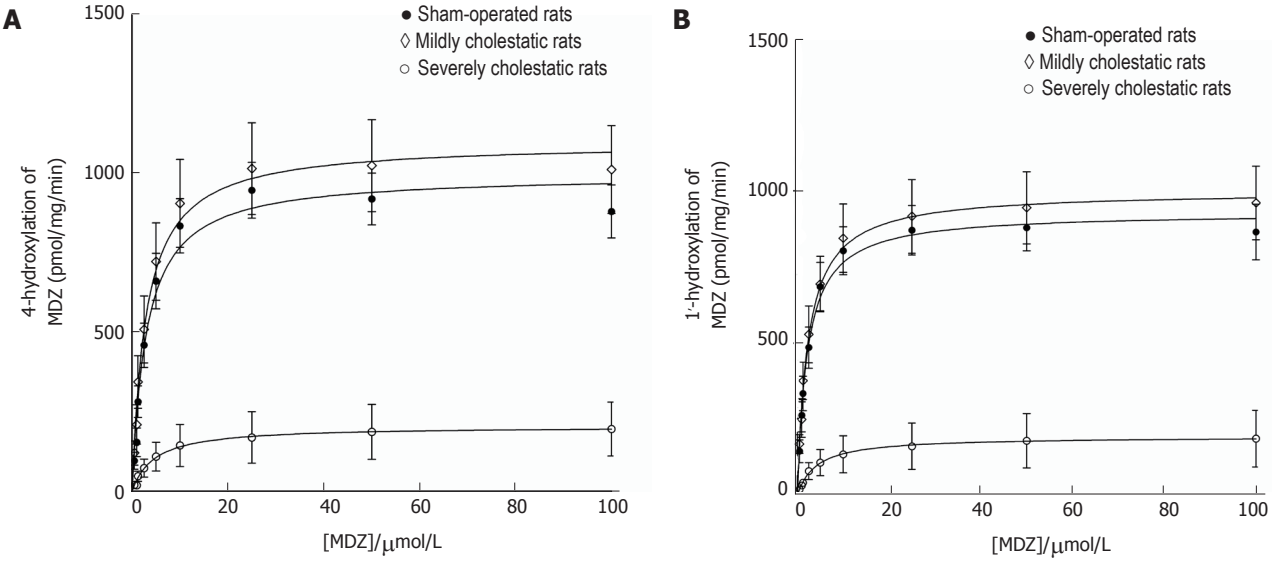

Figure 4 Enzymatic activity of CYP3A in microsomes obtained from sham-operated, mildly and severely cholestatic rats. Kinetics of 4- and 1'- hydroxylation activities of liver microsomes obtained from sham-operated, mildly cholestatic and severely cholestatic rats. Results are means ± SEM of data obtained from 8 rats per group. For each rat, enzymatic activity was tested in duplicate.

with mild cholestasis, while the Vmax of both reactions decreased in the animals with severe cholestatic injury ($P < 0.001$ *vs* controls and rats with mild cholestasis). In both hydroxylation reactions, there were no significant inter-group differences in the Km values.

Expression of nuclear receptors PXR and CAR

To see whether cholestasis influences the expression of CYP3A enzymes by modifying the nuclear levels of the two NRs mainly responsible for regulating their transcription, we measured PXR and CAR mRNA and protein nuclear expression (because they both translocate into the cell nucleus after their activation). As shown in Figures 5 and 6, a significant increase in PXR mRNA and protein expression was observed in the rats with mild cholestasis ($P < 0.001$), whereas a significant reduction was evident in those with severe cholestasis ($P < 0.05$ and $P < 0.001$ *vs* controls and rats with mild cholestasis, respectively). The mRNA expression of CAR rose in mild cholestasis too ($P < 0.05$), and so did its nuclear protein expression, and they both dropped significantly in severe cholestasis ($P < 0.05$ *vs* mild cholestasis).

DISCUSSION

In this study we investigated whether cholestasis affects the gene and protein expression, and the enzymatic activity of CYP3A1 and CYP3A2 enzymes, as well as the activation of CAR and PXR, the nuclear receptors controlling their transcription. For this purpose, we used a validated animal model of cholestasis induced by bile duct ligation, and rigorously stratified the animals on the basis of the severity of their liver injury. We found that mRNA and protein expression of CYP3A1 increased significantly in mild cholestasis ($P < 0.01$), whereas the expression and activity of both CYP3A1 and CYP3A2 decreased dramatically when cholestasis became severe. Alterations of the V_{max} of 4-OH and 1'-OH-midazolam hydroxylation (marker reactions of CYP3A activity) were also identified. Consistently with these findings, the nuclear expression of both PXR and CAR rose initially, then virtually disappeared in the late stage of cholestatic injury.

Table 3 Kinetic parameters for 4- and 1'-hydroxylation activities of microsomal preparations obtained from sham-operated and cholestatic rats

	4-OH MDZ		1'-OH MDZ	
	Vmax (pmol/mg/min)	Km (μmol/L)	Vmax (pmol/mg/min)	Km (μmol/L)
Sham-operated rats	1009.9 ± 269.4	3.4 ± 1.1	946.7 ± 292.2	2.5 ± 0.9
Mildly cholestatic rats	1116.0 ± 333.7	3.5 ± 1.4	1017.0 ± 317.4	2.8 ± 1.6
Severely cholestatic rats	218.4 ± 88.1[c,f]	6.1 ± 4.7	200.1 ± 68.7[c,f]	4.3 ± 2.6

The results are reported as mean ± SD of 8 rats per group. [c]$P < 0.001$ *vs* sham-operated rats; [f]$P < 0.001$ *vs* mildly cholestatic rats.

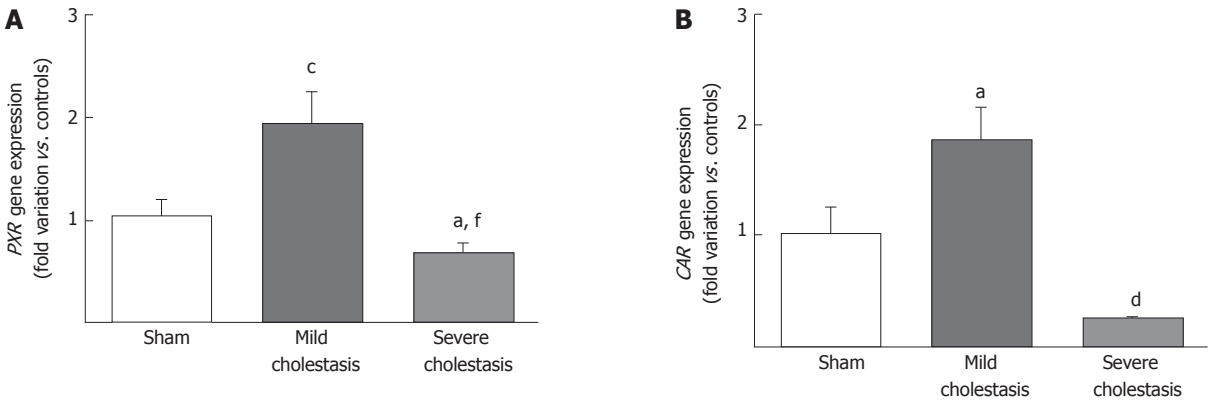

Figure 5 mRNA expression of nuclear receptors in rat livers. Pregnane x receptor (A) and constitutive androstane receptor (B) gene expression in sham-operated and cholestatic rats reported as fold variations compared with sham rats. Results are mean ± SEM obtained from 8 rats in each group. [a]$P < 0.05$, [c]$P < 0.001$ *vs* sham rats; [d]$P < 0.05$, [f]$P < 0.01$ *vs* mildly cholestatic rats.

Liver disease is known to impair various pathways of hepatic drug metabolism. Animal and clinical *in vivo* studies have shown that drug metabolism due to the 3A subfamily of CYP enzymes is significantly altered in severe liver disease[28,29]. *In vitro* studies have shown a decrease in CYP3A activity in cholestatic liver disease[30], though the mechanism(s) behind it have not been clarified.

Various studies, as reviewed in Chen *et al*[31] for instance, focused on the NRs controlling the expression of drug-metabolizing enzymes, analyzing their regulation of gene transcription. Since these events are crucial in the detoxification and elimination of the potentially toxic biliary constituents accumulating in cholestasis, NRs represent attractive targets of pharmacotherapy for cholestatic disorders. CAR and PXR control the expression of CYP3A, and are known to facilitate adaptation to the higher intracellular bile acid concentrations of cholestasis by upregulating alternative hepatic export routes (MRP3 and MRP4), and inducing detoxification enzymes (SULTs, UGTs and CYPs)[5]. In the present study, we examined how different degrees of cholestasis influenced the expression of CYP3A1 and CYP3A2, the main CYP3A isoforms involved in drug metabolism in the rat liver. We demonstrated that cholestasis-induced changes in their expression levels correlate with the observed changes in nuclear PXR and CAR expression, which are probably due in turn to the fact that they are activated by compounds, such as BAs, which increase in the cholestatic liver[5,7,32]. Our finding that only the mRNA and protein levels of CYP3A1, but not CYP3A2, increased significantly in mild cholestasis is consistent with the significant increase in nuclear PXR and CAR expression, and probably due to CYP3A1 being much more susceptible to induction[15]. The significant increase in CYP3A1 mRNA and protein expressions in the early stages of cholestasis prompts a slight, but statistically insignificant increase in the CYP3A-mediated metabolism of midazolam, probably because the constitutive expression of CYP3A1 in the rat liver is much lower than that of CYP3A2[20], and its protein expression is only doubled in rats with mild cholestasis. It is worth noting that a protective role of PXR had already been identified in an animal model of cholestasis, in which this NR was able to modulate inflammation and fibrosis[7]. Indeed, hepatic damage from bile acid accumulation was increased in $PXR^{-/-}$ mice, and, on the basis of gene expression analyses, it has been suggested that, in response to cholestasis, PXR repressed and induced the expression of the hepatic membrane transporters OATP-C and OATP2 (OATP1B1), respectively[33]. Accordingly, Xie *et al*[34], demonstrated that the PXR agonist PCN could not reduce lithocholic acid (LCA)-induced toxicity in $PXR^{-/-}$ mice. Indeed, Saini and collaborators[35] have demonstrated that CAR activation is both necessary and sufficient to confer resistance to the hepatotoxicity of LCA. Our findings support these observations, suggesting that this protective role of PXR is lost in the late stages of

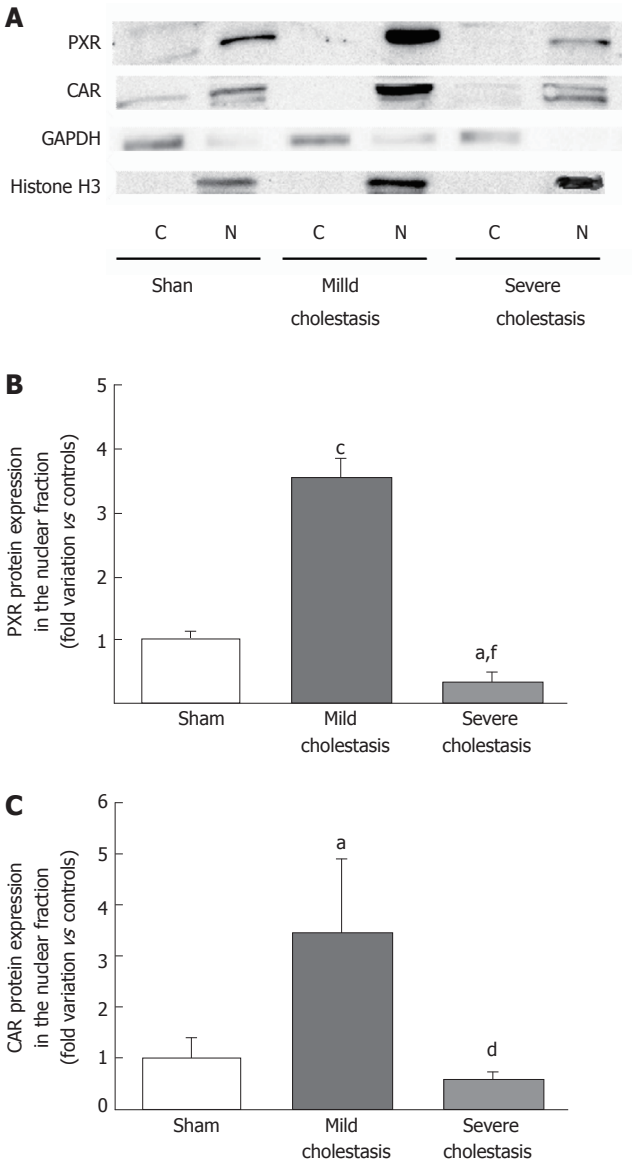

Figure 6 Nuclear protein expression of nuclear receptors in rat livers. Representative Western blot membrane showing pregnane x receptor and constitutive androstane receptor protein expression in the cytoplasmic and nuclear fractions of sham and cholestatic rats (A). GAPDH and Histone H3 were used to assess the purity of the cytoplasmic and nuclear fractions, respectively. Densitometric analysis of proteins in the nuclear fraction representing the PXR (B) and CAR (C) nuclear expression. Results are expressed as mean ± SEM obtained from 8 rats in each group. $^{a}P < 0.05$, $^{c}P < 0.001$ *vs* sham rats; $^{d}P < 0.05$, $^{f}P < 0.001$ *vs* mildly cholestatic rats.

cholestasis due to a downregulation of its expression. This would also be the cause behind the associated decline in drug-metabolizing activity. The changes in CYP3A enzymes can be explained by bearing in mind that BAs start to accumulate in the liver right from the beginning of cholestatic disease, activating the PXR and CAR receptors. This prompts an increase in the expression of transporters and enzymes, such as CYP3A1, to promote the elimination of endogenous and exogenous substances. This compensatory mechanism is lost when liver function deteriorates because the associated reduction in NRs gives rise to a dramatic decrease in both CYP3A isoforms, with a consequent further accumulation of hepatic BAs. There is a well-known cross-talk operating between these NRs[36,37], but the molecular mechanisms behind it remain to be clarified. It has recently been observed that some NRs share the same response elements in transactivation of their target genes. This is the mechanistic molecular base for the so called "cross-talk of NRs"[38]. We know that PXR cross-talks with other NRs (in particular, CAR and FXR) to regulate intermediate metabolism through the trans-activation and trans-repression of genes controlling cholesterol, bile acid, bilirubin, glucose and lipid homeostasis[38]. FXR, CAR and PXR share many regulatory effects and their functionality in the context of regulation of liver detoxification and bile acid metabolism is largely overlapping. Studies performed in animal models of cholestasis have already shown that FXR and PXR agonists show overlapping activities[39]. On the other hand, it has been shown that FXR functions as a CAR antagonist in regulating the expression of the basolateral transporter MRP4[40]. It has also been suggested that PXR may play a role in the regulation of CYP7A1, the rate-limiting enzyme of cholesterol catabolism and bile acid formation, which is transcriptionally regulated by FXR[41].

On the basis of the results obtained in this study, we can hypothesize that PXR and CAR work synergistically to maintain CYP3A induction in the early stages of cholestasis, and their detoxification function fails when the liver dysfunction becomes severe. This finding can have two clinical consequences. For start, cholestatic patients may have an altered drug metabolism: in the early stage due to the induction of CYP3A enzymes; and in the late stage due to the high deposition of fibrotic liver and consequent hepatocyte loss. This effect could be particularly relevant in humans because a single isoform, *i.e.,* CYP3A4, is responsible for metabolizing more than 50% of the drugs used in medical practice[42]. Secondly, since PXR activation is known to induce alternative hepatic export routes (MRP3 and MRP4) and detoxification enzymes (SULTs, UGTs and CYPs), the induction of these cellular pathways with PXR and/or CAR agonists could be exploited as a therapeutic strategy for the management of cholestatic diseases.

In conclusion, our findings clearly demonstrate that early- and late-stage cholestasis affects CYP3A-mediated drug metabolism differently. Since changes in the BA detoxification routes play a pivotal part in cholestatic liver injury, the regulation of PXR and CAR could be targeted therapeutically with the aim of ameliorating cholestatic liver injury by regulating

CYP3A-mediated metabolism of BA.

ARTICLE HIGHLIGHTS

Research background

Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are the two main classes of cholestatic disease, and chronic cholestasis and liver inflammation are their main pathophysiological components[2]. To escape the deleterious effects of high concentrations of bile acids (BAs), a peculiar feature of cholestatic disease, the liver triggers an adaptive response to cholestasis, activating a complex network of nuclear receptors (NRs) that tightly regulate the BA transporters to maintain proper BA homeostasis. Among liver NRs, both the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR), which control the expression of CYP3A drug-metabolizing enzymes, play a major role in these adaptive responses. PXR and CAR act as xenobiotic sensors, as one of their main functions is to regulate the expression of enzymes and transporters involved in xenobiotic elimination. These NRs are also involved in controlling hepatic processes closely related to the progression of cholestatic diseases, such as BA homeostasis, lipid metabolism, fibrosis and inflammation[5]. Changes in CAR and PXR, and CYP3A expression have already been identified in the course of cholestatic liver disease, but different effects have been documented depending on the etiology and severity of the cholestasis. The aim of this study was to analyze expression and enzymatic activity of CYP3A1 and CYP3A2, as well as the nuclear expression of CAR and PXR in a validated animal model of cholestasis rigorously stratified on the basis of the degree of liver dysfunction.

Research motivation

Both *in vitro* and *in vivo* studies have shown a decrease in CYP3A activity in cholestatic liver disease, but a clear demonstration of the mechanism(s) responsible for it is still lacking. To our knowledge, no studies published to date simultaneously analyzed CYP3A enzyme expression and activity and the activation of NRs responsible for their transcriptional control in different stages of cholestatic disease. CYP3A enzymes are the most abundant CYPs in human beings, and the most important enzymes in terms of drug metabolism, because they have a key role in the first-pass and systemic metabolism of many drugs. On the basis of these considerations, the aim of this study was to analyze expression and enzymatic activity of CYP3A1 and CYP3A2, as well as the nuclear expression of CAR and PXR. For this purpose, we used a validated animal model of cholestasis based on the bile duct ligation technique in animals rigorously stratified by degree of liver injury.

Research objectives

In this study we investigated whether cholestasis affects the gene and protein expression, and the enzymatic activity of CYP3A1 and CYP3A2 enzymes, as well as the activation of CAR and PXR, the nuclear receptors controlling their transcription. Our results let us hypothesize that PXR and CAR work synergistically to maintain CYP3A induction in the early stages of cholestasis, and their detoxification function fails when the liver dysfunction becomes severe.

Research methods

The procedures involving the animals were managed in accordance with national and international laws and policies (Directive 2010/63/EU on the protection of animals used for scientific purposes). The study design was approved by the Ethics Committee of University of Padova, and by the Italian Ministry for the care and use of laboratory animals (Prot. no. 24, 2015). Gene and protein expressions of PXR, CAR, CYP3A1 and CYP3A2 were assessed by means of qRT-PCR and Western blot, respectively. Alterations in CYP3A activity were measured by calculating the kinetic parameters of marker reactions for CYP3A enzymes. Statistical analyses were performed with the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States). The experimental results were compared by one-way analysis of variance or Student's *t*-test, as appropriate. In the case of statistically significant differences ($\alpha = 0.05$), the analysis of variance was followed by the Newman-Keuls *post-hoc* test. A P value < 0.05 was considered statistically significant.

Research results

On the basis of the results obtained in this study, we could hypothesize that PXR and CAR work synergistically to maintain CYP3A induction in the early stages of cholestasis, and their detoxification function fails when the liver dysfunction becomes severe. The mechanism by which cholestasis affects "cross-talk" between PXR, CAR and other NRs in the liver remains to be described in detail.

Research conclusions

The findings of this study can have two clinical consequences. For start, cholestatic patients may have an altered drug metabolism: in the early stage due to the induction of CYP3A enzymes; and in the late stage due to the high deposition of fibrotic liver and consequent hepatocyte loss. This effect could be particularly relevant in humans because a single isoform, *i.e.*, CYP3A4, is responsible for metabolizing more than 50% of the drugs used in medical practice. Secondly, since PXR activation is known to induce alternative hepatic export routes (MRP3 and MRP4) and detoxification enzymes (SULTs, UGTs and CYPs), the induction of these cellular pathways with PXR and/or CAR agonists could be exploited as a therapeutic strategy for the management of cholestatic diseases.

Research perspectives

On the basis of the results obtained in this study, we hypothesized that cholestatic patients may have an altered drug metabolism and suggested the induction of liver detoxification by means of PXR and/or CAR agonists as a therapeutic strategy for the management of cholestatic diseases. To test the first hypothesis, clinical studies analyzing the pharmacokinetic parameters of cholestatic patients stratified by degree of liver injury can be performed after the administration of selected drugs. Preclinical studies analyzing the effects of the administration of PXR/CAR agonists on the cholestatic liver are currently in progress in our laboratory.

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ORIGINAL ARTICLE

Basic Study

Induction of precocious intestinal maturation in T-cell deficient athymic neonatal rats

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Abstract

AIM

To investigate whether gut maturation could be induced precociously in an athymic T-cell deficient neonatal rat model.

METHODS

Fourteen day-old athymic (nude) rats (NIH-*Foxn*1^{rnu}) were gavaged with either phytohaemagglutinin - a lectin from red kidney beans (PHA); trypsin - a protease (Prot); or water - vehicle (control) as a single dose on one day or once a day for 3-day. The nude rats were either nurtured by their mothers or cross-fostered by conventional foster dams of the Sprague-Dawley strain from days 3-5 after birth. At 17 d of age, 72 h after administration of the first treatment, intestinal macromolecular permeability was tested *in vivo*, prior to euthanasia, after which blood and gut organs were sampled.

RESULTS

Provocation with both, PHA and protease, resulted in increased gut growth and maturation in nude rat pups

independent of nursing. Foetal-type enterocytes were replaced by non-vacuolated adult-type enterocytes in the distal small intestine epithelium. Decreased intestinal macromolecular permeability (gut closure) was observed, with reduced permeability markers (BIgG and BSA, $P < 0.001$) in circulation. Increased pancreatic function, with an increased trypsin to protein ratio in pancreas homogenates, was observed independent of nursing in the nude pups. Immunostaining showed the presence of a few $CD3^+$-cells in the intestinal mucosa of the nude pups. The number of $CD3^+$-cells remained unaltered by provocation and no differences were observed between the nursing sets. Growth and vitality of the nude pups were dependent on nurturing, since cross-fostering by conventional dams increased their macromolecular absorptive capacity (BSA, $P < 0.05$), as well as their passive immunity (RIgG, $P < 0.05$).

CONCLUSION

Precocious gut maturation can be induced by enteral provocation in athymic rat pups, similarly to in euthymic pups, thus showing an independence from thymus-derived T-cells.

Key words: Gut; Intestinal permeability; Passive immunity; Pancreas; Immunoglobulin G

Core tip: The rat is born with an immature gut and is thus a suitable model to study gut development. Enteral provocation with phytohaemagglutinin or protease induces precocious gut maturation in conventional (euthymic) rats. It has been suggested that T-lymphocytes are required for gut digestive maturation. The current study showed that precocious gut maturation could be induced by enteral provocation in athymic nude rats similar to that which occurs in euthymic rats. The few intestinal mucosal $CD3^+$-T-cells that were observed in the athymic nude rats appeared to be unaffected by enteral provocation. The intestinal absorptive capacity in nude pups was enhanced when nurtured by conventional (immunocompetent) foster dams.

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INTRODUCTION

Rodents are an altricial mammalian species that are born highly immature and undergo extensive postnatal development[1,2] making the neonatal rat a suitable model to study gastrointestinal (GI) development and its coordination with changes in the luminal dietary and microfloral milieu[3,4]. During the third postnatal week, the weaning period in the rat, the GI tract shows enhanced growth and undergoes vast maturation[2].

In the distal small intestine (SI), the immature enterocytes with large supranuclear vacuoles, featuring high endocytic and intracellular digestive capacities[5], are replaced by adult-type non-vacuolated enterocytes, lacking these properties. In the proximal SI, the immature enterocytes expressing the neonatal-Fc-receptor (FcRn), involved in transcytosis of milk-borne IgG, are exchanged for low FcRn expression mature enterocytes[6,7]. These SI changes result in a decreased intestinal permeability (gut closure) and absorption of maternal IgG ceases[8]. The gut digestive capacity increases during weaning with characteristic changes in enterocyte brush-border enzymes and pancreatic enzymes secretion[1,9].

The gut immune system is also immature and naïve at birth in the rat[10], and maternal passive immunity is transferred to the young, *e.g.*, milk-borne antibodies and immune cells[11,12]. At weaning, which is apparently coordinated with digestive maturation, dietary and microbial changes stimulate the immune system through the recruitment of immune cells to the gut mucosa[13] and up-regulation of pro-inflammatory cytokines, resulting in what is referred to as controlled "physiological" inflammation[14].

We have previously shown that gut maturation can be induced precociously in suckling rats by enteral provocation with phytohaemagglutinin (PHA) - a lectin[15,16], or proteases[17], mimicking the naturally occuring processes at weaning. Furthermore, provocation with PHA had effects on the thymus and the recruitment of $CD3^+$ T-lymphocytes to the gut mucosa[16]. In fact, it has been suggested that natural GI development at weaning is dependent on T-cell activation in rats[18].

Consequently, the current study aimed to investigate the impact of thymus-derived T-lymphocytes on GI maturation, using an athymic, T-cell deficient (nude), rat strain[19], in combination with our experimental model of induced precocious gut maturation by enteral provocation in suckling rats[15-17].

MATERIALS AND METHODS

Animals

The experiment was approved by the local Malmö-Lund Ethical Review Committee for Animal Experimentation and conducted in accordance with the European Community regulation concerning the protection of experimental animals (2010/63/EU). The protocol number is M169-14. The experiments were carried out using rats (*Rattus norvegicus*) of the athymic (nude) strain (NIH-*Foxn1*^{mu}, Charles River Laboratories International Inc.) and conventional (euthymic) rats of the Sprague-Dawley (SD) strain (SPRD Han, Taconic

M&B, Denmark). The rats were bred and kept in the departmental facility under specific pathogen-free conditions (20 ± 1℃, 50% ± 10% relative humidity, 12:12 h light-dark cycle). Pregnant dams were moved to individual cages (polycarbonate) with aspen wood bedding (Beekay B & K Universal AB, Sweden), enriched with paper-nesting material (Sizzle-pet, Lillicobiotech). Rats had free access to water and a rodent chow (R36, Lactamin). Parturition date was denominated as day 0 and all nude rat pups litters were kept with the dams during the experiments and a 7 cm wall extender was used to prevent the pups from reaching the chow whilst still nursing from their mothers.

Experimental design

Experiments were performed in a split-litter mode in two different nursing sets of nude rats, either nursed by their mother (Nude/Nude) or cross-fostered from the third postnatal day by conventional dams (Nude/SD). Enteral provocation was performed as previously described[15-17]. In short, 14 day-old nude rats were gavaged *via* a stomach tube with either porcine pancreatic trypsin (Novo) - a protease (Prot), or with phytohaemagglutinin (PHA) purified from red kidney beans (*Phaseolus vulgaris*) - a lectin[15]. Both were administered as a single dose (Prot, 1 mg/g b.wt; PHA, 0.1 mg/g b.wt) or once a day for three days (Protx3, 0.6 mg/g b.wt; PHAx3; 0.05 mg/g b.wt). Control pups received the vehicle water in matching volumes (0.01 ml/g b.wt).

Intestinal in vivo permeability test

At the end of the experiment on day 17, intestinal macromolecular permeability was assessed. A marker cocktail solution containing bovine serum albumin (BSA, Sigma, 1.25 mg/g b.wt) and bovine immunoglobulin G (BIgG, Sigma, 0.25 mg/g b.wt) was administered to the rats via a stomach tube and blood samples were collected 3 h later.

Euthanasia and organ collection

On day 17, the animals were weighed and anesthetized using a subcutaneous injection of a mixture of ketamine (Ketalar®, Pfizer, United States; 0.17 mg/g b.wt.) and azaperone (Stresnil®, Janssen Pharmaceutica, Belgium; 0.03 mg/g b.wt.) prior to sample collection. To ensure deep anaesthesia the rats' eyelid and withdrawal reflexes were assessed. After opening the thorax, blood was collected by cardiac puncture in EDTA-containing syringes. The blood was then centrifuged at 3000 × *g* for 15 min at + 4℃ and plasma was obtained and stored at -20℃ until further analysis. The pancreas was then dissected out, weighed and stored at -70℃. The SI was divided into proximal and distal halves, the luminal content flushed out with ice-cold saline and SI length and weights were measured. Samples from the middle part of each SI half were fixed in 40 g/L phosphate buffered formaldehyde for 24 h and further embedded in paraffin. Spleen, liver, stomach and caecum were also weighed.

Histology and immunohistochemistry

SI sections were deparaffinised and stained with haematoxylin and eosin using standard procedures. Morphometry of at least 20, appropriately oriented, villi and crypts from each rat was analysed using ImageJ software (http://imagej.nih.gov/ij). For immunohistochemical analysis of $CD3^+$-cells, SI sections were deparaffinised and subjected to antigen retrieval by microwaving (2 × 8 min, 750 W) in 10 mmol/L Na-Citrate buffer (pH 6.0). Endogenous peroxidase was blocked with Peroxidized1, while background was reduced with Background Punisher (Biocare Medical, Llc.). Incubation with the primary antibody, rabbit-monoclonal anti-CD3 (1:100, SP7, Abcam), was done overnight at + 4℃. Detection of $CD3^+$-cells was performed using an HRP-Polymer Detection kit (MACH4 Universal; Biocare Medical, Llc.), according to the manufacturer's instructions and the sections were counter-stained with haematoxylin. As a negative control for the unspecific binding of the HRP-Polymer detection kit, sections incubated with the antibody diluent, 10 g/L BSA in PBS were included.

Plasma analyses

The BSA marker concentration was measured by electroimmunoassay[20], using rabbit anti-BSA as the precipitating antibody and purified BSA as the standard, while the BIgG concentration was determined by single radial immunodiffusion using rabbit anti-BIgG as the precipitating antibody (Sigma-Aldrich) and purified BIgG as the standard[21], as previously described[8]. Passive immunity transfer was measured as rat plasma IgG level, which was quantified by single radial immunodiffusion using rabbit anti-rat-IgG (DAKO A/S, Denmark) as the precipitating antibody and purified rat IgG (Miles Laboratories, INC., United States) as the standard[21].

Pancreatic analyses

The pancreata were homogenized in ice-cold 0.2 mol/L Tris-HCl buffer + 0.05 mol/L $CaCl_2$, pH 7.8 (1:10 wt/vol) using a glass homogenizer, and then centrifuged at 15000 × *g* for 20 min at + 4℃. After activation with enteropeptidase (Sigma-Aldrich), trypsin activity, in the supernatant of the pancreatic homogenates was determined spectrophotometrically using a microplate modification[22] of the method described by Fritz *et al*[23] and benzoyl-DL-arginine-4-nitroanilide (BAPNA, Sigma-Aldrich) as the substrate. The trypsin activity in units (U) was recalculated as the amount of enzyme that catalyses 1 μmol of substrate per minute. The protein concentration in the supernatants was determined using the Lowry

method[24] with a modification for 96-well microplates and using purified BSA (Sigma Chemicals) as the standard.

Statistical analyses

Statistical analysis was carried out using Prism 7 (http://www.graphpad.com). Comparisons between Nude/Nude treatment and controls were performed using an unpaired *t*-test and multiple *t*-test with Sidak post-test. Comparisons within Nude/SD, were performed using a one or two-way ANOVA and Dunnett's multiple comparison post-test. Comparison of control groups in different nurturing sets were performed either using an unpaired *t*-test or a one-way ANOVA and Tukey's multiple comparison post-test. All differences were considered significant when $P < 0.05$.

RESULTS

Body and organ weights

Gavage with PHA had no significant effects on the body weight of nude, mother-fed pups (Nude/Nude) 3 d after treatment. Significantly increased body weights were observed in the fostered, nude pups (Nude/SD) that were treated repeatedly with PHA or protease (PHA/Protx3) compared to control littermates gavaged with water (Table 1).

Administration of PHA to Nude/Nude pups and PHA or protease to Nude/SD pups stimulated SI growth with increased weights of both SI regions observed compared to that of controls. No significant differences in SI length and stomach and caecum weights were observed in the rats that received PHA and/or protease compared to controls. Significantly increased liver weights ($P \leqslant 0.03$) were observed in treated Nude/SD pups, while liver weights in the Nude/Nude pups were decreased ($P = 0.06$). Spleen weight was not significantly affected by treatment or nurturing.

Body weights of Nude/Nude pups appeared generally lower than the Nude/SD pups, even though no significance was found, possibly due to the high variation between the Nude/Nude pups. The relative weights of the proximal SI ($P = 0.039$) and liver ($P = 0.0001$) were significantly increased in Nude/Nude control pups compared to Nude/SD controls.

Pancreas

Gavage with PHA or protease did not affect pancreatic weight, but increased pancreatic protein and trypsin contents (Table 1). Pancreatic protein and trypsin contents were significantly increased in all treated Nude/SD pups ($P \leqslant 0.04$ and $P \leqslant 0.0004$, respectively) compared to controls, except for in the Nude/SD pups that were repeatedly treated with protease (Protx3), while no effect was observed in the Nude/Nude pups. The relative trypsin to protein pancreatic content was significantly increased in all treated nude pups nurtured either by their mother (Nude/Nude, *P* except for in the pups that were repeatedly treated with protease (Protx3).

Intestinal morphology

Significantly increased crypt depth ($P = 0.036$) was observed in the proximal small intestine of PHA-treated Nude/Nude pups, while in the Nude/SD pups both crypt depth (PHA and PHAx3, $P \leqslant 0.0068$) and villi height were significantly increased after PHA treatments (PHA and PHAx3, $P \leqslant 0.01$) (Table1). In the distal SI, only crypt depth was significantly increased by both treatments in Nude/SD rats ($P \leqslant 0.02$), resulting in a significantly decreased villi height to crypt depth ratio in the PHA ($P = 0.023$) and Protx3 ($P = 0.048$) groups of Nude/SD rats. No significant effects were observed in villi width.

In the distal SI, the proportion of non-vacuolated (adult-type) epithelial cells appearing from the villi base following treatments (Figure 1) was measured to assess maturational status. The proportion of non-vacuolated (adult-type) epithelial cells was significantly increased after all treatments in the nude pups, independently of their nurturing ($P \leqslant 0.017$), except for in the Protx3 group ($P = 0.08$), compared to control pups.

Intestinal permeability

Significantly decreased plasma concentrations of both permeability markers, BIgG and BSA, were observed in all treatment groups compared to controls gavaged with water ($P = 0.0001$), except for the BSA levels in the Protx3 Nude/SD pups ($P = 0.07$) (Figure2). The plasma concentration of the cumulative permeability marker of milk-borne IgG absorption, RIgG, was significantly decreased following a single treatment with PHA and protease ($P \leqslant 0.003$) compared to control pups in the Nude/SD group that were gavaged with water (Figure 2C).

The impact of maternal nurturing (athymic-nude *vs* euthymic-SD) was assessed by comparing the intestinal permeability of the control nude pups. The foster-nurtured (Nude/SD) pups displayed significantly higher BSA permeability compared to those nurtured by their mother (Nude/Nude) ($P = 0.01$), while no such difference was observed for BIgG permeability ($P = 0.39$) (Figure 2A and B). The nude pups nurtured by their mother (Nude/Nude) displayed significantly lower plasma RIgG concentrations compared to those nurtured by foster dams (Nude/SD, $P = 0.02$) or conventional pups (SD/SD, $P = 0.0004$), while no differences in plasma RIgG concentration were observed between cross-fostered nude rats (Nude/SD) and conventional rat pups (SD/SD) (Figure 2C).

Intestinal CD3 immunostaining

Nude rat pups displayed very few $CD3^+$-cells in the SI mucosa, mainly associated with the epithelium, possibly intraepithelial lymphocytes (IEL), and some

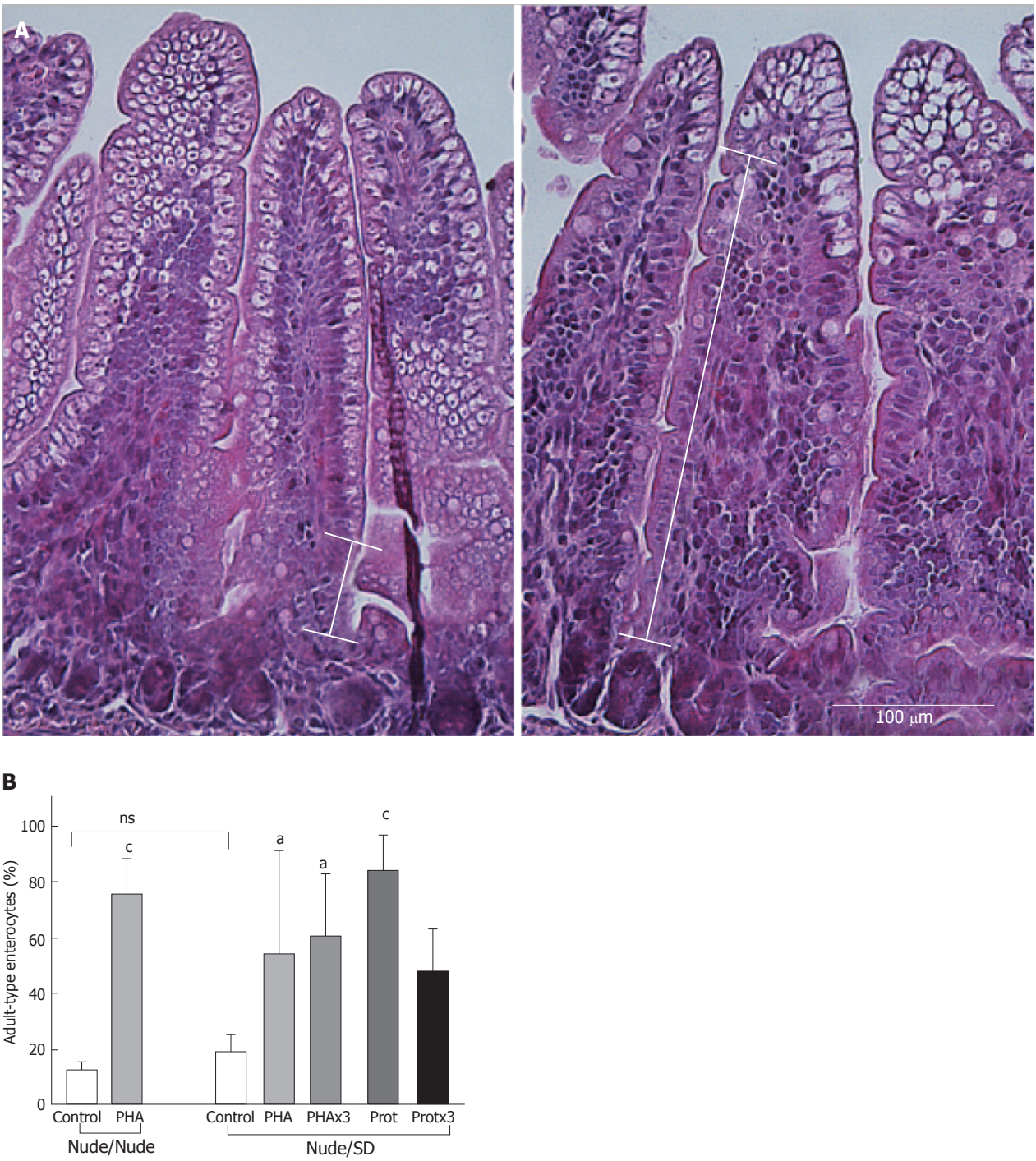

Figure 1 Epithelial maturation in the distal small intestine expressed as adult-type epithelium replacing the foetal-type vacuolated epithelium. A: Photomicrograph (× 200, scale bar 100 μm) of H&E stained distal small intestine representative of a rat after PHA gavage (right) as compared to a control rat (left), with white bar connectors showing the portion of adult-type epithelium along the villus. B: Degree of intestinal epithelial maturation (%) in nude 14 day old rat pups treated by gavage with a kidney bean lectin - PHA (n = 4) or water (Control n = 3) and reared by their own mothers (Nude/Nude) and nude rat pups gavaged with PHA (n = 5) once or once a day for 3 d (PHAx3 n = 7), a protease once (Prot n = 4) or once a day for 3 days (Protx3 n = 7) or water (Control n = 5) and fostered by conventional SD dams (Nude/SD). Data presented as mean ± SD and differences were considered significant when $P < 0.05$. Significant differences between groups within nurturing groups (Nude/Nude or Nude/SD) indicated with $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$ or non-significant (ns).

= 0.001) or by foster dams (Nude/SD, $P \leqslant 0.013$), except for in the pups that were repeatedly treated with protease (Protx3).

Intestinal morphology

Significantly increased crypt depth (P = 0.036) was observed in the proximal small intestine of PHA-treated Nude/Nude pups, while in the Nude/SD pups both crypt depth (PHA and PHAx3, $P \leqslant 0.0068$) and villi height were significantly increased after PHA treatments (PHA and PHAx3, $P \leqslant 0.01$) (Table1). In the distal SI, only crypt depth was significantly

increased by both treatments in Nude/SD rats ($P \leq 0.02$), resulting in a significantly decreased villi height to crypt depth ratio in the PHA ($P = 0.023$) and Protx3 ($P = 0.048$) groups of Nude/SD rats. No significant effects were observed in villi width.

In the distal SI, the proportion of non-vacuolated (adult-type) epithelial cells appearing from the villi base following treatments (Figure 1) was measured to assess maturational status. The proportion of non-vacuolated (adult-type) epithelial cells was significantly increased after all treatments in the nude pups, independently of their nurturing ($P \leq 0.017$), except for in the Protx3 group ($P = 0.08$), compared to control pups.

Intestinal permeability

Significantly decreased plasma concentrations of both permeability markers, BIgG and BSA, were observed in all treatment groups compared to controls gavaged with water ($P = 0.0001$), except for the BSA levels in the Protx3 Nude/SD pups ($P = 0.07$) (Figure2). The plasma concentration of the cumulative permeability marker of milk-borne IgG absorption, RIgG, was significantly decreased following a single treatment with PHA and protease ($P \leq 0.003$) compared to control pups in the Nude/SD group that were gavaged with water (Figure 2C).

The impact of maternal nurturing (athymic-nude *vs* euthymic-SD) was assessed by comparing the intestinal permeability of the control nude pups. The foster-nurtured (Nude/SD) pups displayed significantly higher BSA permeability compared to those nurtured by their mother (Nude/Nude) ($P = 0.01$), while no such difference was observed for BIgG permeability ($P = 0.39$) (Figure 2A and B). The nude pups nurtured by their mother (Nude/Nude) displayed significantly lower plasma RIgG concentrations compared to those nurtured by foster dams (Nude/SD, $P = 0.02$) or conventional pups (SD/SD, $P = 0.0004$), while no differences in plasma RIgG concentration were observed between cross-fostered nude rats (Nude/SD) and conventional rat pups (SD/SD) (Figure 2C).

Intestinal CD3 immunostaining

Nude rat pups displayed very few $CD3^+$-cells in the SI mucosa, mainly associated with the epithelium, possibly intraepithelial lymphocytes (IEL), and some rarely scattered as villus *lamina propria* lymphocytes (LPL) (Figure 3). No differences were observed between PHA-treated, protease-treated and control nude pups (data not shown) or between nursing sets. In contrast, in euthymic SD rats, 7 to 28-d old, increased amounts of $CD3^+$-cells were observed in the SI mucosa (age-matched 17-d old control SD/SD shown in Figure 3).

DISCUSSION

The current study investigated, whether precocious gut maturation could be induced in nude rat pups inherently lacking a functional thymus (T-cell deficient)[25], as previously shown in the conventional euthymic rat model[15,17].

Gavage of PHA and/or protease had a growth promoting effect in the nude rat pups, resulting in significantly increased body weight, especially in the rat pups that received the treatments more than once. Moreover, increased villi height was observed in the proximal SI segment and increased crypt depth was observed in both the proximal and distal SI segments in the treated nude pups, indicating increased crypt cell proliferation. The villi height/crypt depth ratio decreased in most of the treatment groups, denoting the structural transition which occurs during the maturation process[1]. In addition, SI maturation was confirmed by the change in enterocyte phenotype in the distal SI, with immature vacuolated enterocytes being replaced by adult-type non-vacuolated ones[15,17].

Although no treatment effect was observed with regards to pancreas growth, increased trypsin content was observed after enteral provocation in the nude pups, indicating maturation of the digestive function, which as previously suggested is possibly involved in the initiation of intestinal maturation [17].

The intestinal barrier function was assessed using the *in vivo* permeability test, which indicated cessation of the absorption of both macromolecular markers (BSA and BIgG) in PHA- and protease-treated nude pups, consistent with "gut closure"[8,15-17]. BSA, which is assumed to be absorbed mainly through nonspecific endocytosis, decreases the intestinal passage co-inciding with the replacement of the immature highly endocytotic vacuolated epithelial cells in the distal SI. BIgG is allegedly transported by FcRn-mediated transcytosis in the proximal SI[8] where the epithelial expression of FcRn is reduced during natural and induced maturation in rat pups[6,7].

Provocation with PHA and protease (single dose) in nude pups reared by immunocompetent foster-dams resulted in significantly lower plasma concentrations of the cumulative marker for passive immunity transfer, RIgG, compared to that observed in the corresponding control rat pups. Thus indicating the progressive replacement of the foetal-type gut epithelium by the mature-type gut epithelium with a lowered expression of FcRn over time[7].

Taken together, these results indicate that enteral provocation with PHA or protease can induce precocious gut maturation in athymic neonatal rats resulting in similar changes to those previously shown in euthymic rat pups or seen during natural weaning[15-17,26]. Previous studies in immunodeficient mice have shown that during the peri- and post-weaning periods, recruitment of lymphocytes to the intestinal epithelia elicited a transcriptional response in the enterocytes but did not have effects on crypt proliferation[27]. The treatments used in the current study were administered using two

Table 1 Effects of luminal provocation with phytohaemagglutinin or protease on growth and precocious gut maturation in suckling 17 d old nude rats nurtured either by their mothers or by conventional foster dams

	Nude/Nude					Nude/SD							
	Control		PHA		Control	PHA		PHAx3		Prot		Protx3	
Number of individuals (n)[3]	9-10		11		5	5		8		4		7	
Body weight (g)	23.03 ± 9.26	ns[4]	23.64 ± 7.80	ns[5]	26.62 ± 1.56	26.88 ± 0.81	ns[5]	28.30 ± 1.69	a[5]	27.78 ± 1.04	ns[5]	28.63 ± 1.28	b[5]
Organs weight (mg/g2.wt6)													
Stomach	6.5 ± 0.9	ns	7.0 ± 0.6	ns	6.1 ± 0.5	7.0 ± 0.3	ns	6.8 ± 0.5	ns	6.3 ± 0.4	ns	6.2 ± 0.2	ns
SI length (cm)	2.1 ± 0.7	ns	2.2 ± 0.5	ns	1.8 ± 0.1	1.9 ± 0.0	ns	1.9 ± 0.1	ns	2.0 ± 0.1	ns	1.8 ± 0.1	ns
Proximal SI	17.0 ± 1.9	$P < 0.05$	22.0 ± 1.8	c	15.0 ± 0.6	22.8 ± 1.5	d	22.0 ± 1.1	d	22.8 ± 1.1	c[1]	18.7 ± 1.3	d
Distal SI	13.9 ± 1.4	ns	15.7 ± 1.4	a	13.2 ± 0.4	16.4 ± 1.0	d	18.0 ± 1.2	d	17.7 ± 1.1	d	16.0 ± 1.3	d
Caecum	2.4 ± 0.6	ns	3.0 ± 0.4	ns	2.0 ± 0.2	2.9 ± 0.5	ns	2.7 ± 0.3	ns	2.8 ± 0.4	ns	2.8 ± 0.3	ns
Spleen	4.4 ± 0.6	ns	4.4 ± 0.8	ns	4.4 ± 0.4	4.4 ± 0.2	ns	4.5 ± 0.2	ns	5.4 ± 0.3	ns	4.5 ± 0.2	ns
Liver	38.8 ± 3.2	$P < 0.05$	36.5 ± 2.1	a	34.9 ± 1.2	36.3 ± 1.6	a	36.4 ± 1.3	b	37.0 ± 0.8	b	36.2 ± 1.1	b
Pancreatic function:													
Pancreas (mg/g b.wt)	3.3 ± 0.8	ns	3.8 ± 0.4	ns	2.7 ± 0.3	3.3 ± 0.3	ns	3.3 ± 0.3	ns	3.5 ± 0.3	ns	3.3 ± 0.3	ns
Trypsin (U/mg wet wt[7])	2.8 ± 1.5	ns	5.5 ± 1.2	c	2.8 ± 1.1	8.5 ± 1.7	d	6.5 ± 0.9	d	6.8 ± 0.9	d	3.5 ± 0.9	ns
Protein (μg/mg wet wt[7])	70.2 ± 24.0	ns	64.3 ± 15.5	ns	65.4 ± 8.4	97.3 ± 10.7	c	92.8 ± 16.0	c	85.3 ± 6.9	a	74.5 ± 5.2	ns
Trypsin (U/mg Protein)	43.1 ± 19.1	ns	91.8 ± 34.5	b	42.3 ± 17.0	89.5 ± 25.5	c	70.7 ± 10.7	a	79.5 ± 8.5	b	47.6 ± 13.2	ns
Morphometry:(n)	-3		-4		-5	-5		-7		-4		-7	
Proximal SI: Villi height	375 ± 55	ns	440 ± 21	ns	394 ± 23	457 ± 24	b	452 ± 20	a	394 ± 47	ns	418 ± 34	ns
Villi width	117 ± 16	ns	123 ± 16	ns	107 ± 10	113 ± 10	ns	105 ± 12	ns	113 ± 17	ns	120 ± 8	ns
Crypt depth	70 ± 3	ns	83 ± 7.0	a	61 ± 4.0	81 ± 7.0	b	81 ± 13	b	64 ± 6	ns	68 ± 8	ns
VH/CD	5.4 ± 0.6	ns	5.3 ± 0.4	ns	6.5 ± 0.6	5.7 ± 0.8	ns	5.7 ± 0.9	ns	6.2 ± 0.7	ns	6.2 ± 0.6	ns
Distal SI: Villi height	362 ± 11	ns	356 ± 14	ns	365 ± 58	370 ± 27	ns	426 ± 52	ns	374 ± 31	ns	379 ± 35	ns
Villi width	99 ± 4	ns	100 ± 11	ns	91 ± 12	91 ± 13	ns	97 ± 8.0	ns	91 ± 9	ns	91 ± 7.0	ns
Crypt depth	68 ± 5	ns	72 ± 3.0	ns	56 ± 6.0	71 ± 7.0	b	72 ± 6.0	b	69 ± 7	a	69 ± 7.0	b
VH/CD	5.3 ± 0.3	$P < 0.05$	4.9 ± 0.2	ns	6.5 ± 0.5	5.3 ± 0.7	a	6.0 ± 1.0	ns	5.5 ± 0.2	ns	5.5 ± 0.2	a

[1]Nude 14 d old rat pups were treated by gavage with PHA or water (Control) and reared by their own nude mothers (Nude/Nude); [2]Nude 14-day-old rat pups gavaged with PHA once or once a day for 3 days (PHAx3), a protease once (Prot) or once a day for 3 d (Protx3) or water (Control) and fostered by conventional Sprague-Dawley (SD) dams (Nude/SD); [3]Data presented as mean ± SD; [4]Statistical differences between treatment groups compared to control group within the same nursing set; [5]Statistical differences between control groups from different nursing sets (Nude/Nude compared to Nude/SD). $P < 0.05$ or non-significant (ns); [6]Organ weights expressed per body weight (mg/g b.wt); [7]Pancreatic trypsin activity (U/mg) and protein content expressed per pancreatic wet weight (μg/mg). [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.0001$ or non-significant (ns). PHA: phytohaemagglutinin.

different approaches based on previously published results showing that PHA and Protease induced precocious gut maturation were dose-dependent[15-17]. The lower dose treatment that was repeated (once a day for three days) serves as an experimental simulation of the naturally occurring processes at weaning, where there is a gradual transition from a milk-based to a solid diet. Alternatively, the single administration of a higher treatment dose could be considered similar to an abrupt stressful event, such as maternal separation from the dam, which requires a rapid mechanism of adaptation involving accelerated maturation.

Maternal effects were observed on the overall vitality of the rat pups. Since the nude rats that were nurtured by nude mothers showed a general decrease in growth with signs of undernourishment, they were transferred to immunocompetent foster dams, allowing us to perform experiments in larger sets of littermates. The results showed that nude rat pups nursed by nude mothers displayed lower absorption of the BSA marker, however no effects were observed in the absorption of the BIgG marker. The difference in absorption between the two marker molecules could be due to the different routes of transport, unspecific (BSA) *vs* receptor-mediated endocytosis (BIgG). The passive immunity transfer, as seen by the accumulation of plasma RIgG, was decreased in nude rats nursed by nude mothers compared to

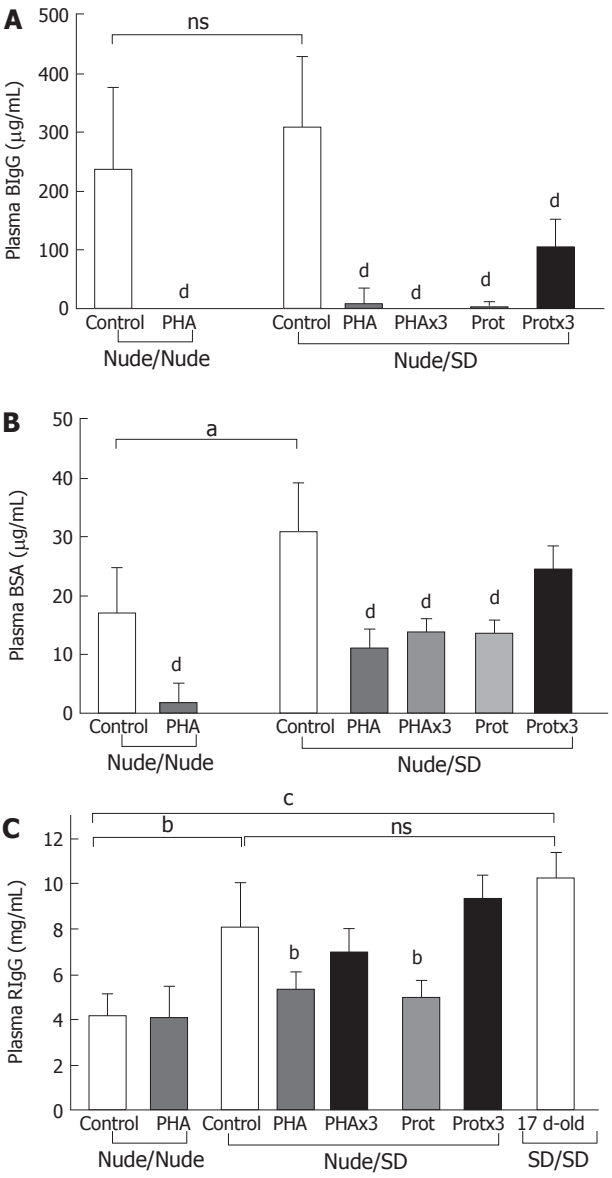

Figure 2 Intestinal *in vivo* permeability. Plasma concentrations 3 h after gavage of the marker molecules: A: Bovine IgG (BIgG); B: Bovine serum albumin (BSA) in nude 17-day-old suckling rats after luminal provocation; C: Transfer of passive immunity *via* milk as plasma concentrations of immunoglobulin G (RIgG) in nude suckling 17-day-old rats after luminal provocation and compared to 17-day-old suckling conventional pups. Nude 14-day-old rat pups treated by gavage with PHA (n = 11) or water (Control, n = 8) and reared by their own mothers (Nude/Nude), and nude rat pups gavaged with PHA (n = 5) once or once a day for 3 d (PHAx3, n = 7), a protease once (Prot, n = 4) or once a day for 3 d (Protx3, n = 7) or water (Control, n = 5) and fostered by conventional SD dams (Nude/SD). Data presented as mean ± SD and differences were considered significant when $P < 0.05$. Significant differences between groups within nurturing groups (Nude/Nude or Nude/SD) and between control groups from different nursing sets indicated with $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$ or non-significant (ns).

those fostered by conventional dams. Hence, maternal milk from immunocompetent dams provided higher levels of passive immunity (IgG) while simultaneously improving the unspecific intestinal absorptive capacity (BSA) of the nude pups during the suckling period, resulting in better growth and survival. It has previously been shown that immunodeficient mice fostered by wild-type dams had increased pre-weaning mRNA expression of intestinal FcRn[28], despite this we did not observe differences in BIgG absorption.

Consequently, differences in the absorptive capacity due to foster-nursing indicated that milk factors from conventional dams promoted gut growth and delayed gut functional maturation in nude rat pups. Thus, suggesting that the immunocompetence of the dam, maternal milk content and composition, and passive immunity transfer, influence the timing of gut maturation even though it is a genetically programmed process. This would imply that nude dams would provide pups with immunodeficient milk lacking the capacity to supress gut maturation.

In conventional rats mucosal $CD3^+$-cells can be found early after birth and increase in number at weaning[13], whereas they are not present until after weaning in mice[28] and until 4-6 mo of age in the athymic rats[25]. However, the current study showed the presence of low amounts of $CD3^+$ T-cells already in neonatal nude rats. These cells could be of maternal origin, translocated from the milk into the intestinal mucosa[11,12,19], or they could be developed outside of the thymus, in the SI[29]. Since neither cross-fostering, nor enteral provocation as seen in euthymic rats[16] seemed to increase the number of $CD3^+$-cells, the extra-thymic development of these mucosal $CD3^+$-cells would be more probable. Nonetheless, phenotypic differences between intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) have been described[4] and further characterisation of the intestinal $CD3^+$-IELs found in nude rats should be studied.

The recruitment of thymus-derived T-cells to the intestinal mucosa has been shown to be essential for gut maturation[18,19] while kept immature by regulatory T-cells until weaning, as recently shown in mice[14]. The present study shows that gut maturation is independent of thymus-derived T-cells, but since the immune system in neonatal nude rats can adjust to the thymus deficiency[30] further studies are needed to elucidate the possible involvement of extra-thymic $CD3^+$-T-cells, such as δ-T-cells or other immune cells, in gut maturation[31].

In conclusion, the study showed a novel role of maternal milk in the regulation of the macromolecular absorptive capacity in immunodeficient athymic rat pups. Furthermore, despite the hypothesis on the direct role of T-cells in gut development, this study showed that induced gut maturation after enteral provocation does not depend on thymus derived T-cells, although an influence of rare mucosal extra-thymic $CD3^+$-cells cannot be excluded.

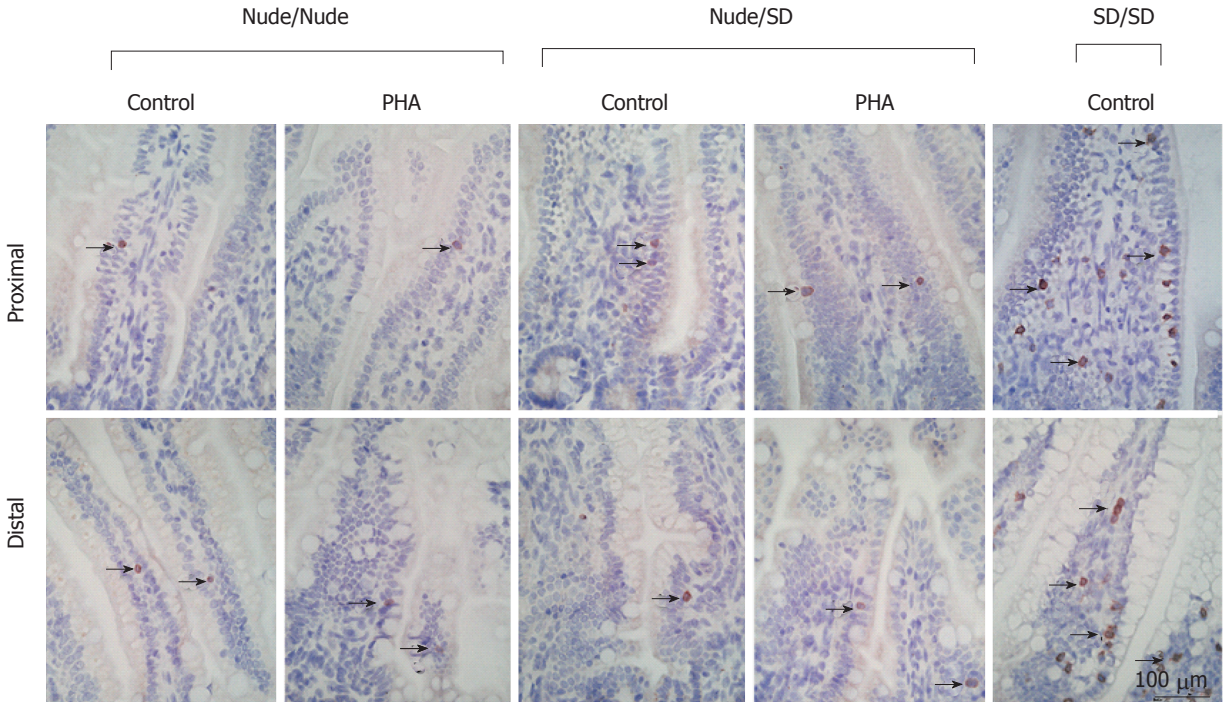

Figure 3 CD3$^+$ cells in the intestinal mucosa of nude rats. Immunostaining of the proximal and distal small intestine in nude 17-day-old suckling rats after luminal provocation with PHA at 14 d of age or water-gavage (controls), nurtured by their mothers (Nude/Nude) or by conventional foster SD dams (Nude/SD) as compared to 17-day-old suckling conventional SD pups (SD/SD). Representative photomicrographs (× 200, scale bar 100 μm) of immunostained CD3$^+$-cells in the small intestine. Positive cells indicated by arrows. PHA: phytohaemagglutinin.

COMMENTS

Background

In the distal small intestine (SI), the immature enterocytes with large supranuclear vacuoles, featuring high endocytic and intracellular digestive capacities, are replaced by adult-type non-vacuolated enterocytes, lacking these properties. In the proximal SI, the immature enterocytes expressing the neonatal-Fc-receptor (FcRn), involved in transcytosis of milk-borne IgG, are exchanged for low FcRn expression mature enterocytes. These SI changes result in a decreased intestinal permeability (gut closure) and absorption of maternal IgG ceases. The gut digestive capacity increases during weaning with characteristic changes in enterocyte brush-border enzymes and pancreatic enzymes secretion.

Research frontiers

The current study investigated, whether precocious gut maturation could be induced in nude rat pups inherently lacking a functional thymus (T-cell deficient), as previously shown in the conventional euthymic rat model.

Innovations and breakthroughs

The study showed a novel role of maternal milk in the regulation of the macromolecular absorptive capacity in immunodeficient athymic rat pups. Furthermore, despite the hypothesis on the direct role of T-cells in gut development, this study showed that induced gut maturation after enteral provocation does not depend on thymus derived T-cells, although an influence of rare mucosal extra-thymic CD3$^+$-cells cannot be excluded.

Applications

The study showed a novel role of maternal milk in the regulation of the macromolecular absorptive capacity in immunodeficient athymic rat pups. Furthermore, despite the hypothesis on the direct role of T-cells in gut development, this study showed that induced gut maturation after enteral provocation does not depend on thymus derived T-cells, although an influence of rare mucosal extra-thymic CD3$^+$-cells cannot be excluded.

Peer-review

In the current manuscript, the authors reported that athymic (nude) rats gut maturation could be induced by enteral provocation of PHA and trypsin, and independence from thymus-derived T-cells. This is interesting and would gain our knowledge on intestinal maturation. The study was well designed and the manuscript was well organized. It is preferred to determine the disaccharidase activity of the small intestine for better denoting the gut maturation.

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ORIGINAL ARTICLE

Basic Study

Nuclear heat shock protein 110 expression is associated with poor prognosis and hyperthermo-chemotherapy resistance in gastric cancer patients with peritoneal metastasis

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Abstract

AIM

To investigate the significance of heat shock protein 110 (HSP110) in gastric cancer (GC) patients with peritoneal metastasis undergoing hyperthermo-chemotherapy.

METHODS

Primary GC patients (n = 14) with peritoneal metastasis or positive peritoneal lavage cytology who underwent distal or total gastrectomy between April 2000 and December 2011 were enrolled in this study. The patients underwent postoperative intraperitoneal hyperthermo-chemotherapy using a Thermotron RF-8 heating device two weeks after surgery. We analyzed nuclear HSP110 expression in surgically resected tumors using immunohistochemistry. Additionally, the effect of HSP110 suppression on hyptherthermo-chemosensitivity was assessed *in vitro* in the MKN45 GC cell line using the HSP inhibitor KNK437.

RESULTS

HSP110 immnohistochemical staining in 14 GC patients showed that five (35.7%) samples belonged to the low expression group, and nine (64.3%) samples belonged to the high expression group. Progression-free survival was significantly shorter in the HSP110 high-expression group than in the low-expression group (P = 0.0313). However, no significant relationships were identified between HSP110 expression and the clinicopathological characteristics of patients. Furthermore, high HSP110 expression was not an independent prognostic factor in GC patients with peritoneal metastasis (P = 0.0625). HSP110 expression in MKN45 cells was suppressed by KNK437 at the hyperthermic temperature of 43 ℃ *in vitro*. Comparison of MKN45 cell proliferation in the presence and absence of KNK437 at 43 ℃, revealed that proliferation was significantly decreased when HSP110 was inhibited by KNK437. Additionally, HSP110 suppression *via* HSP inhibitor treatment increased cellular sensitivity to hyperthermo-chemotherapy *in vitro*.

CONCLUSION

The expression of nuclear HSP110 in GC patients might be a new marker of chemosensitivity and a therapeutic target for patients who are tolerant to existing hyperthermo-chemotherapies.

Key words: Peritoneal metastasis; Hyperthermia; Hyperthermo-chemotherapy; Heat shock protein 110; Gastric cancer; Drug resistance

Core tip: Gastric cancer remains one of the most common cancers worldwide. Peritoneal dissemination is the most common reason behind gastric cancer (GC) recurrence, and the median survival duration for patients with metastatic and recurrent GC is only 13-16 mo. In our department, we have used intraperitoneal hyperthermo-chemotherapy in patients with advanced rectal cancer and GC with peritoneal metastasis. In this study, we evaluated the significance of heat shock protein 110 (HSP110) expression in GC patients with peritoneal metastasis and assessed the effects of HSP110 suppression on hyperthermo-chemotherapy sensitivity *in vitro*.

Kimura A, Ogata K, Altan B, Yokobori T, Mochiki E, Yanai M, Kogure N, Yanoma T, Suzuki M, Bai T, Kuwano H. Nuclear heat shock protein 110 expression is associated with poor prognosis and hyperthermo-chemotherapy resistance in gastric cancer patients with peritoneal metastasis. *World J Gastroenterol* 2017; 23(42): 7541-7550 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7541.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7541

INTRODUCTION

Despite progress in early diagnosis and improvements in treatment, gastric cancer (GC) is a central cause of cancer-related deaths worldwide. Furthermore, a particularly high GC mortality rate has been reported in Asia[1]. Peritoneal dissemination is the most common reason behind GC recurrence, and the first line of treatment for this disease is chemotherapy. New chemotherapies for metastatic and recurrent GC are being developed. However, the median survival duration for patients with metastatic and recurrent GC is only 13-16 mo[2-7].

Intraperitoneal hyperthermo-chemotherapy is an effective alternative treatment to standard chemotherapy in GC patients with peritoneal dissemination[8-12]. Indeed, hyperthermic intraperitoneal perfusion chemotherapy (HIPEC) with cisplatin combined with an intravenous chemotherapy regimen with paclitaxel, 5-fluorouracil, and leucovorin has improved survival rate and decreased the postoperative recurrence of locally advanced GC[12]. Furthermore, it has been suggested that hyperthermia and 5-fluorouracil act synergistically to promote apoptosis and enhance thermotolerance in the SGC-7901 GC cell line[9]. In our department, we have used intraperitoneal hyperthermo-chemotherapy in patients with advanced rectal cancer and GC with peritoneal metastasis[13-16]. A preliminary non-random study in a small number of patients revealed that patients in the postoperative intraperitoneal hyperthermo-chemotherapy (PIHC) group had a higher survival rate and better prognosis than did the patients in the control group[15].

Heat shock proteins (HSPs) have been characterized as molecular chaperones that prevent the formation of misfolded protein structures[17-19]. HSPs are induced by exposure to the stress condition, including fever,

irradiation and chemicals. HSPs in cancer maintain several oncoproteins homeostasis and promote cancer cell survival by inhibiting apoptosis induction[17,20]. It was reported that overexpression of HSPs might be correlated with poor prognosis in several types of human carcinomas[21-26]. Additionally, it was reported that high levels of various HSP family members are associated with increased chemoresistance in several malignancies[27-29]. Previously, we found that high expression of nuclear HSP110 is associated with cancer progression and poor prognosis in GC patients and that HSP110 suppression increases chemosensitivity in human GC cell lines[17].

However, the clinicopathological significance of HSP110 expression and localization and their relationship with hyperthermo-chemotherapy sensitivity in GC patients are still unclear. Therefore, the purposes of current study were to determine the significance of HSP110 expression in GC patients with peritoneal metastasis and to evaluate the impact of HSP110 inhibition on hyperthermo-chemotherapy sensitivity *in vitro*.

MATERIALS AND METHODS

Patients and samples

The Institutional Review Board of Gunma University Hospital approved this study, and written informed consent was obtained from all patients. From April 2000 to December 2011, 14 GC patients with peritoneal dissemination underwent distal or total gastrectomy for cytoreduction at the Department of General Surgical Science, Gunma University. All patients were diagnosed with peritoneal metastasis (P1) or positive peritoneal lavage cytology (CY1) during surgery. Gastric cancer staging was performed according to the Classification of Gastric Carcinoma (third edition) of the Japanese Gastric Cancer Association[30]. All patients underwent PIHC.

PIHC

Treatment regimens for PIHC were administered as previously described[15]. Patients underwent distal or total gastrectomy with lymph node dissection, and a 19-Fr silicon drain was inserted in the left subphrenic lesion. PIHC was administered to patients diagnosed with P1 or CY1 during surgery. Two weeks after surgery, hyperthermia was induced using 8-MHz radiofrequency capacitive heating equipment (Thermotron RF-8, Yamamoto Vinyter, Osaka, Japan). Physiologic saline (1 L) containing 80 mg/m^2 cisplatin was warmed to 37 ℃ in a dry incubator and introduced as quickly as possible into the peritoneal cavity via a catheter. After PIHC, the catheter was clamped for six hours. A minimum temperature of higher than 40 ℃ was achieved in the abdominal cavity and maintained for 60 min. The treatment was repeated every two weeks for a maximum of four cycles. After completing all PIHC courses, patients took S-1 (Taiho Pharmaceutical Company, Tokyo, Japan) for one year from the date of surgery.

Immunohistochemical staining

Resected surgical specimens were fixed in formalin, embedded in paraffin, cut into 4-μm thick sections, and mounted on glass slides. Immnohistochemical staining was performed as previously reported[17,31]. All sections were deparaffinized in xylene. Sections were dehydrated in alcohol and soaked with 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature to inhibit endogenous peroxidase. The sections were soaked in boiling water, and immersed in Immunosaver (Nishin EM, Tokyo, Japan) at 98 ℃ for 90 min. Non-specific binding sites were blocked by incubating the sections with Serum-Free Protein Block (DAKO, Carpinteria, CA, United States) for 30 min at room temperature. The sections were incubated with a 1:100 dilution of a rabbit monoclonal antibody against HSP110 (GeneTex, CA, United States) for 24 h at 4 ℃. The signal from the primary antibody was visualized using the Histofine Simple Stain MAX-PO (MULTI) (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. The chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB) was applied as a 0.02% solution containing 0.005% hydrogen peroxide in 50 mmol/L ammonium acetate-citrate acid buffer (pH 6.0). All sections were counterstained with Mayer's hematoxylin solution.

The immunohistochemically stained samples were analyzed by two researchers blinded to the patient information. Staining for HSP110 was assessed using the immunoreactive score (IRS) to evaluate the proportion of cells expressing HSP110 and their relative staining intensity as previously described[31,32]. The intensity of nuclear HSP110 staining was graded as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining (Figure 1A). The percentage of nuclear HSP110-expressing GC cells was calculated based on at least 1000 cancer cells in total from five representative areas. The percentage of nuclear HSP110 staining was scored as follows: 0, no staining; 1+, 1%-10%; 2+, 11%-50%; and 3+, 51%-100% (Figure 1A). The IRS was calculated by multiplying the intensity and expression scores to arrive at values of 0, 1+, 2+, 3+, 4+, 6+, or 9+. IRS values of 0, 1+, 2+ and 3+ represent low HSP110 expression, while IRS values of 4+, 6+, and 9+ represent high HSP110 expression.

Cell culture

The human GC cell line, MKN45, was purchased from RIKEN BRC through the National Bio-Resource Project of MEXT, Tokyo, Japan. MKN45 cells were maintained in RPMI 1640 medium (Wako, Osaka, Japan) containing 10% fetal bovine serum and supplemented with 100

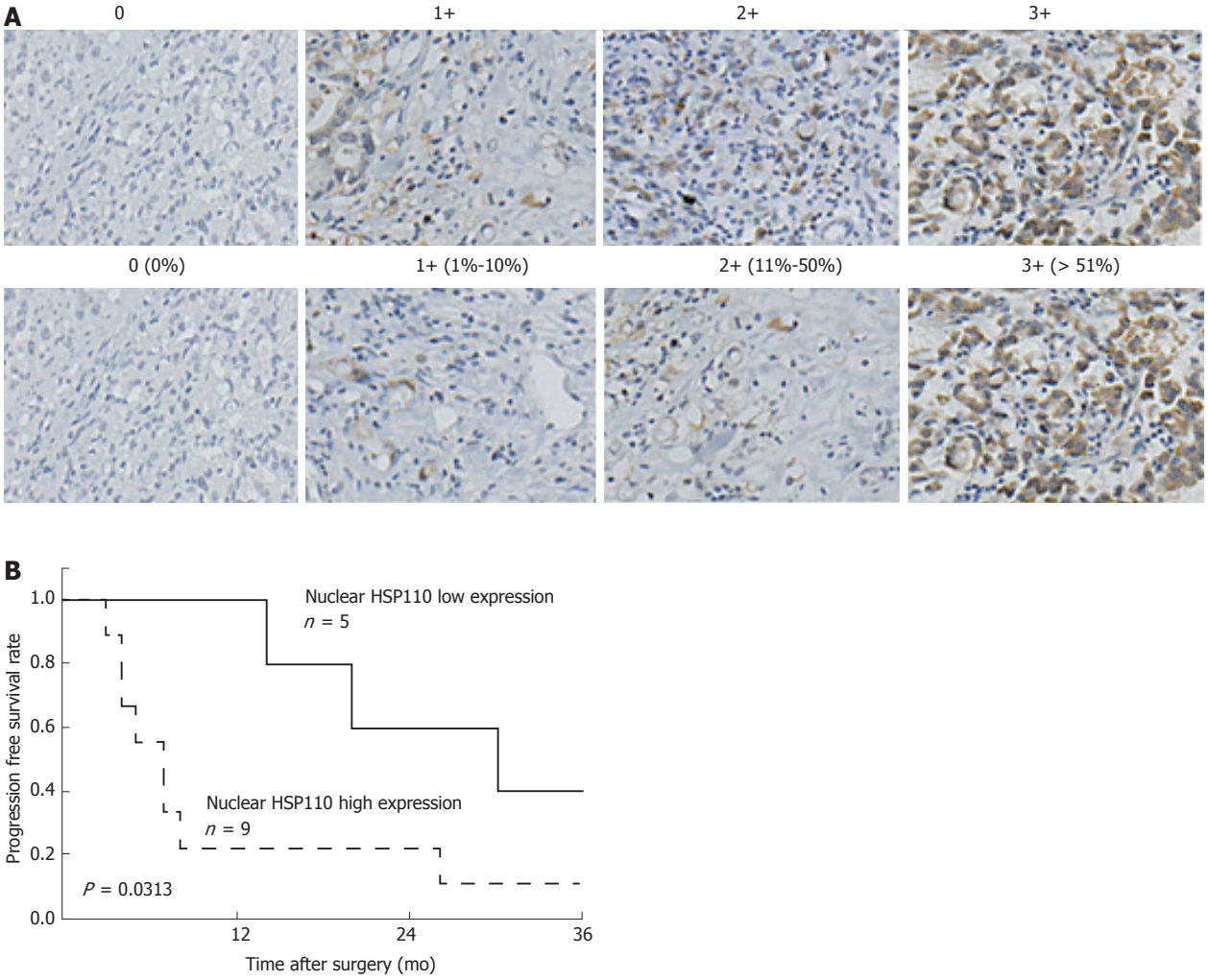

Figure 1 Immunohistochemical staining for heat shock protein 110 in gastric cancer patients. A: Representative images of gastric cancer. Representative images indicating the intensity of nuclear HSP110 staining (upper panel) and the percentage of nuclear-stained gastric cancer cells (lower panel) are shown. B: The three-year progression-free survival curve of 14 gastric cancer patients according to their nuclear HSP110 expression. HSP110: Heat shock protein 110.

units/mL penicillin and streptomycin sulfate (Invitrogen, Carlsbad, CA, United States).

Heat shock protein inhibitor

KNK437 (benzylidene lactam compound; Merck Millipore, Darmstadt, Germany) was used as a heat shock protein inhibitor. KNK437 was dissolved in dimethyl sulfoxide (DMSO) before being added to the culture medium as described[33]. The final concentration of DMSO in the culture medium for each treatment was 0.25% (v/v). The same concentration of DMSO was used as a control. Cells were incubated at 43℃ for 3 h during heat treatment.

Protein extraction and western blot analysis

Western blotting was used to evaluate HSP110 and β-actin expression in MKN45 cells. MKN45 cells were treated with KNK437 at 43℃ for 3 h. Then, total proteins were extracted using the PRO-PREP Protein Extraction Solution Kit (iNtRON Biotechnology, Sungnam, Kyungki-Do, South Korea). Proteins were separated on a 10% polyacrylamide gel and transferred to nitrocellulose membranes using a wet transfer protocol. The membranes were incubated overnight at 4℃ with rabbit monoclonal antibody against HSP110 (1:1000; GeneTex) and β-actin (1:1000; Sigma, St Louis, MO, United States). The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies, and each proteins were evaluated using the ECL Prime Western Blotting Detection System (GE Healthcare, Tokyo, Japan) using Image Quant LAS4000 (GE Healthcare Life Sciences, United Kingdom).

Cell proliferation assay

Cell proliferation was measured with the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). MKN45 were seeded (3000 cells/well) into 96-well plates in 100 μL medium with KNK437 (50 or 100 nmol/L). To generate heat shock, cells were incubated at 43℃ for 3 h before KNK437 (50 or 100 nmol/L) was added. Cell proliferation was assessed at 0, 12, 24, and 36 h. To assess cell proliferation, 10 μL of Cell Counting Kit-8 reagent was added to each well

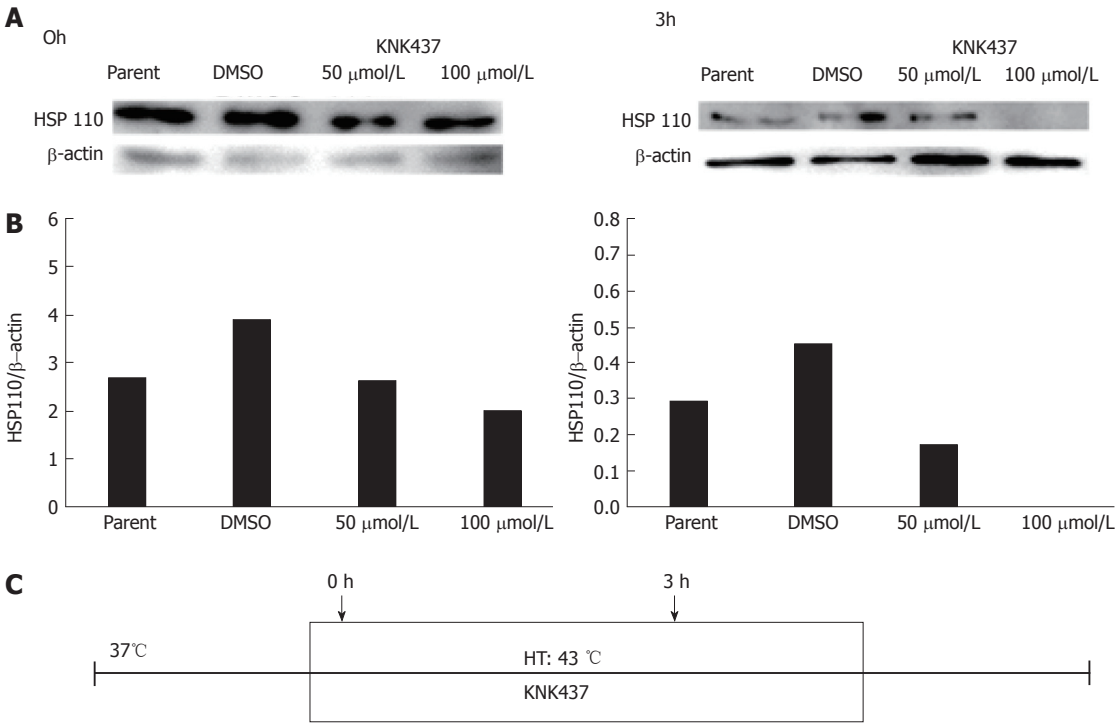

Figure 2 Heat shock protein 110 suppression by KNK437 under hyperthermic conditions. HSP110 expression in MKN45 cells was suppressed by KNK437 under hyperthermic conditions of 43 ℃. A: Western blots of HSP110 and β-actin expression; B: Relative expression of HSP110 (normalized to β-actin). C: Schematic representation of experimental timeline. HSP110: Heat shock protein 110; HT: Heat treatment.

and incubated at 37℃ for 2 h. Next, the absorbance of each well was detected at 450 nm using an xMark Microplate Absorbance Spectrophotometer (Bio Rad, Hercules, CA, United States).

Chemosensitivity assay

Cell Counting Kit-8 was used to evaluate the sensitivity of MKN45 cells to hyperthermo-chemotherapy with cisplatin. MKN45 cells were plated (approximately 10000 cells per well) into 96-well plates in 100 μL of medium with KNK437 (50 or 100 nmol/L) before cisplatin exposure. The cells were subjected to heat shock at 43℃ for 3 h; then, 10 μL of Cell Counting Kit-8 reagent was added, and the cells were incubated for 2 h at 37℃. The absorbance of each well was evaluated at 450 nm using an xMark Microplate Absorbance Spectrophotometer. Then, the cells were treated with various concentrations of cisplatin (0, 0.1, 1 or 10 μmol/L) for 48 h. As control, the sensitivity of the cells to chemotherapy with cisplatin in the absence of heat treatment was evaluated in the same manner.

Statistical analysis

Statistically significant differences were analyzed with Student's *t*-test for continuous variables and the chi-square test for categorical variables. The data for continuous variables are expressed as the mean ± SE of the mean. Survival curves were calculated according to the Kaplan-Meier method and analyzed with the log-rank test. Univariate and multivariate analysis by the Cox proportional hazards model was used to identify prognostic factors. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with JMP software (version 12; SAS Institute Inc., Cary, NC, United States).

RESULTS

The clinical significance of nuclear HSP110 expression in GC patients with peritoneal metastasis

We evaluated the expression of nuclear HSP110 in 14 GC patients by immunohistochemical staining. Five (35.7%) samples belonged to the low-expression group, and nine (64.3%) samples belonged to the high-expression group. The relationships between nuclear HSP110 expression with the clinicopathological features of 14 GC patients are shown in Table 1. There were no significant relationships between nuclear HSP110 expression and clinicopathological features of the GC patients. However, the three-year progression-free survival rate was significantly lower in the high HSP110 expression group than that in the low-expression group ($P = 0.0313$; Figure 1B). The results of univariate analyses of clinicopathological factors affecting progression-free survival rates after surgery are shown in Table 2. The relative risk for all factors including HSP110 expression was greater than 1. However, none of the results were statistically significant. Univariate regression analysis revealed that high HSP110 expression was not an independent

Table 1 Relationship between clinicopathological characteristics of gastric cancer patients and nuclear heat shock protein 110 expression *n* (%)

Factors	HSP110 expression in gastric cancer patients (n = 14)		
	Low (n = 5)	High (n = 9)	P value
Age (mean ± SE)	55.2 ± 3.8	59.3 ± 2.8	0.4025
Sex,			
Male	2 (22.2)	7 (77.8)	0.1575
Female	3 (60.0)	2 (40.0)	
Histology			
Well, Moderate	1 (33.3)	2 (66.7)	0.9227
Muc, Poor, Signet	4 (36.4)	7 (63.6)	
Depth			
sm, mp, ss	0 (0.0)	0 (0.0)	
se, si	5 (35.7)	9 (64.3)	
Lymph node metastasis			
Absent	0 (0.0)	2 (100.0)	0.2549
Present	5 (41.7)	7 (58.3)	
Lymphatic invasion			
Absent	1 (33.3)	3 (66.7)	0.4797
Present	4 (40.0)	6 (60.0)	
Venous invasion			
Absent	4 (40.0)	6 (60.0)	0.5967
Present	1 (25.0)	3 (75.0)	
Peritoneal lavage cytology			
Negative	1 (25.0)	3 (75.0)	0.5967
Positive	4 (40.0)	6 (60.0)	
Peritoneal metastasis			
Absent	2 (50.0)	2 (50.0)	0.4805
Present	3 (30.0)	7 (70.0)	

Well: Well-differentiated; Moderate: Moderately differentiated; Muc: Mucinous; Poor: Poorly differentiated; Signet: Signet ring cells; sm: Submucosa; mp: Muscularis propria; ss: Subserosa; se: Serosa exposed; si: Serosa infiltrating.

prognostic factor in GC patients (P = 0.0625; Table 2).

HSP110 expression in GC cell lines

We previously detected HSP110 expression in MKN7, MKN45, and MKN74 human GC cell lines[17]. In this study, we used the MKN45, which is a poorly differentiated GC cell line, for further analysis. HSP110 expression in MKN45 cells was suppressed by KNK437 at the hyperthermic temperature of 43℃ (Figure 2).

The effect of HSP110 suppression on the proliferation of MKN45 GC cells

Compared to the untreated cells, the proliferation of MKN45 cells at 43℃ was significantly reduced when HSP110 was inhibited by KNK437 treatment. However, the same analysis performed at 37℃ revealed no significant difference in the proliferation of MKN45 cells grown in the presence or absence of KNK437 (Figure 3A).

Effect of HSP110 suppression on hyperthermo-chemosensitivity in GC cell lines

At 43℃, the cisplatin sensitivity of MKN45 cells was significantly higher in the KNK437-mediated HSP110 inhibition group than that in the parental or control cell groups. However, when the MKN45 cells were grown at 37℃, there were no significant differences in cisplatin sensitivity among cells treated with KNK437 and parental and control cells (Figure 3B). Furthermore, the therapeutic sensitivity of MKN45 cells treated with cisplatin and KNK437 under hyperthermic conditions was higher than that observed with the other therapeutic combinations (Figure 3C).

DISCUSSION

In this study, we found that high nuclear HSP110 expression in GC patients with peritoneal metastasis undergoing hyperthermo-chemotherapy was associated with poor prognosis and poor progression-free survival. Our studies with MKN45 cells showed that the KNK437-mediated inhibition of HSP110 increased the hyperthermo-chemosensitivity of GC cells *in vitro*. However, there were no significant relationships between nuclear HSP110 expression and the clinicopathological features of the GC patients. Previously, we reported that nuclear HSP110 expression was associated with venous invasion in 210 GC patients[17]. However, we were unable to identify a significant association between HSP110 expression and venous invasion due to the small number of patients included in this study. It is possible that high nuclear HSP110 expression was also associated with venous invasion in the cases in the present study, which may have influenced prognosis. Additionally, our univariate regression analysis showed that high HSP110 expression was not an independent prognostic factor in GC. These results may also be attributed to the small number of patients in the current study.

In this study, we used the HSP inhibitor KNK437 to suppress HSP110 expression in MKN45 GC cells. However, the mechanism by which KNK437 inhibits HSPs is not fully understood. In COLO 320DM (human colon carcinoma) cells, KNK437 was shown to inhibit the acquisition of thermotolerance and the induction of various HSPs including HSP105, HSP70, and HSP40 in a dose-dependent manner[33]. Another study reported that thermotolerance is suppressed by KNK437 through the inhibition of heat-induced accumulation of HSP27 and HSP72 and the induction of p53-independent apoptosis[34]. Moreover, it has been reported that in SCC VII cells, the inhibition of thermotolerance by KNK437 can improve the efficacy of clinical fractionated hyperthermia[35]. Previous reports have linked certain protein expression with hyperthermo-chemosensitivity. Upregulation of miR-218 has been observed in GC patients after cytoreductive surgery and intraperitoneal hyperthermic chemotherapy, and this was shown to increase chemosensitivity to cisplatin[36]. Consistent with this report, our results show that the alteration of various protein levels can affect hyperthermo-chemosensitivity in MKN45 cells *in vitro*. Specifically, this study showed that hyperthermo-chemosensitivity to the intraperitoneal infusion of

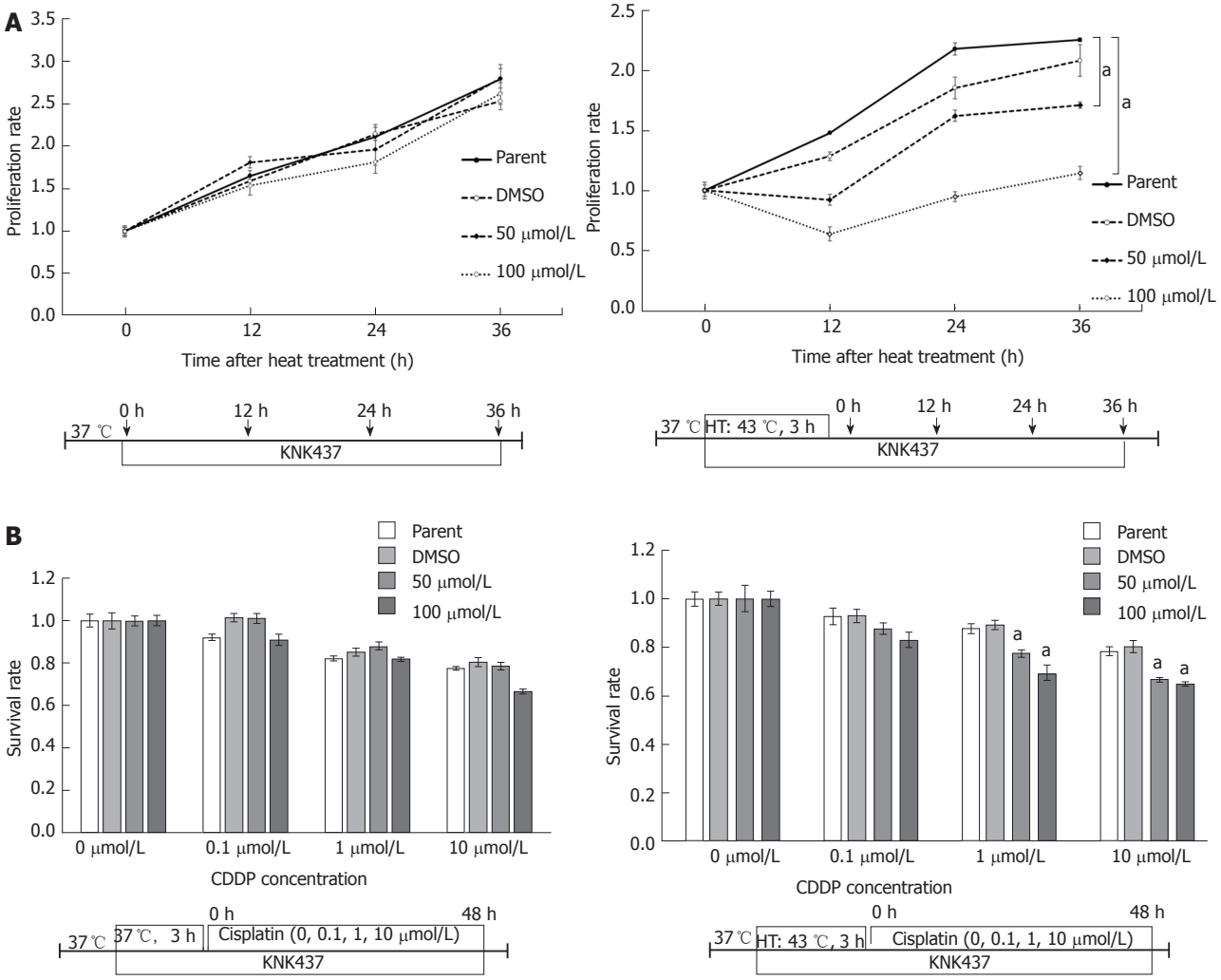

Figure 3 Functional analysis of the MKN45 human gastric cancer cell line treated with KNK437 under hyperthermic conditions. A: Proliferation of MKN45 cells with and without KNK437-mediated HSP110 suppression. The results for normal (37 ℃) (right panel) and hyperthermic (43 ℃) (left panel) conditions are shown. Proliferation of MKN45 cells in the KNK437-mediated HSP110 suppression group (under hyperthermic condition) was significantly lower than that of the parental and control groups ($^aP < 0.05$); B: Cisplatin sensitivity in MKN45 cells in the presence or absence of KNK437-mediated HSP110 suppression. The results for normal (37 ℃) (right panel) and hyperthermic (43 ℃) (left panel) conditions are shown. Cisplatin sensitivity of MKN45 cells under the hyperthermic condition of 43 ℃ was significantly increased by KNK437-mediated HSP110 suppression ($^aP < 0.05$); C: Treatment of MKN45 cells with various therapeutic combinations. Therapeutic sensitivity of MKN45 cells treated with cisplatin and KNK437 under hyperthermic conditions was greater than that of cells with other therapeutic combinations ($^aP < 0.05$). HSP110: Heat shock protein 110; HT: Heat treatment.

Table 2 Univariate analyses of clinicopathological features affecting progression-free survival rates in patients after surgery

Clinicopathological variables	Univariate analysis		
	RR	95%CI	*P* value
Age (< 65 yr/≥ 65 yr)	2.17	0.46-8.33	0.2988
Sex (male/female)	1.62	0.48-6.22	0.4412
Histology (differentiated/undifferentiated)	2.18	0.46-8.33	0.2988
Lymph node metastasis (absent/present)	1.25	0.19-5.02	0.7822
Lymphatic invasion (absent/present)	1.29	0.33-4.29	0.6904
Venous invasion (absent/present)	1.18	0.26-4.13	0.8057
Peritoneal lavage cytology (negative/positive)	1.32	0.38-6.10	0.6766
Peritoneal metastasis (absent/present)	2.28	0.58-15.08	0.2568
HSP110 expression (low/high)	3.40	0.94-16.01	0.0625

HSP110: Heat shock protein 110.

cisplatin was enhanced by HSP110 suppression. Combination therapy with hyperthermo-chemotherapy and HSP110 inhibitors might be a new treatment strategy for GC patients with peritoneal metastasis.

This study has several limitations. First, the sample size was very small because hyperthermochemotherapy is not a common therapy for GC patients with peritoneal metastasis and because only a few institutions perform this therapy. Second, the KNK437 HSP inhibitor used in this study is not specific to HSP110 alone. Hence, further analysis are needed with the specific suppression of HSP110. Third, we assessed only resistance to cisplatin in this study. However, resistance to S-1 might also affect the progression-free survival of GC patients. Finally, we administered cisplatin *via* intraperitoneal infusion. Recently, the effectiveness of the hydrophobic drug paclitaxel has been demonstrated *via* intraperitoneal chemotherapy. In the future, we need to validate the results of combination therapy with an HSP110-specific inhibitor and hyperthermo-chemotherapy with paclitaxel.

In conclusion, nuclear HSP110 expression is associated with poor prognosis in GC patients with peritoneal metastasis who are treated via intraperitoneal hyperthermo-chemotherapy. Therefore, the IRS values related to HSP110 expression might be used as effective biomarkers for the prognoses of GC patients with peritoneal metastasis. Furthermore, HSP110 suppression in the MKN45 GC cell line increased their hyperthermo-chemosensitivity against cisplatin. Taken together, our results show that nuclear HSP110 expression in GC patients with peritoneal metastasis might be a clinically useful biomarker of prognosis and a therapeutic target for patients who are tolerant to existing chemotherapies or hyperthermia.

COMMENTS

Background

Peritoneal metastasis is the most common reason behind gastric cancer (GC) recurrence. Previously, the authors reported the significance of postoperative intraperitoneal hyperthermo-chemotherapy for GC with peritoneal metastasis. The expression of heat shock protein (HSPs) is induced by exposure to stress, including heat. In cancer, HSPs promote the survival of malignant cells by inhibiting the induction of apoptosis. However, the clinicopathological significance of heat shock protein 110 (HSP110) expression, localization, and association with hyperthermo-chemotherapy resistance in GC has not been fully elucidated. Here, the authors evaluated the significance of HSP110 expression in GC patients with peritoneal metastasis who underwent hyperthermo-chemotherapy.

Research frontiers

High levels of HSPs might be correlated with poor prognosis in several types of cancer. Additionally, high levels of various HSP family members have been reported to be associated with increased chemoresistance in several malignancies.

Innovations and breakthroughs

In this study, the authors found that high nuclear HSP110 expression in GC patients with peritoneal metastasis who treated using hyperthermo-chemotherapy was associated with poor prognosis and poor progression-free survival. The KNK437-mediated inhibition of HSP110 increased hyperthermo-chemosensitivity of MKN45 GC cells *in vitro*. Therefore, nuclear HSP110 expression in GC patients with peritoneal metastasis might be a new marker of chemosensitivity and a therapeutic target in patients who are tolerant to existing hyperthermo-chemotherapies.

Applications

This study indicated that nuclear HSP110 expression in GC patients with peritoneal metastasis might be a clinically useful biomarker of prognosis and a therapeutic target for patients who are tolerant to existing chemotherapies or hyperthermia.

Terminology

HSPs have been characterized as molecular chaperones that prevent the formation of misfolded protein structures. HSPs are induced by exposure to the stress condition, including fever, irradiation and chemicals. HSPs in cancer maintain several oncoproteins homeostasis and promote cancer cell survival by inhibiting apoptosis induction.

Peer-review

Interesting and well written paper describing the role of HSP110 in GC with peritoneal metastasis.

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ORIGINAL ARTICLE

Basic Study

Combined treatment of pancreatic cancer xenograft with ^{90}Y-ITGA6B4-mediated radioimmunotherapy and PI3K/mTOR inhibitor

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Author contributions: Aung W designed the research, performed the majority of experiments, analyzed the data and wrote the manuscript; Ukai K, Kouda K and Kurozawa Y provided the anti-integrin $\alpha_6\beta_4$ antibody; Sudo H perfomed the antibody radiolabeling; Sugyo A participated in the animal experiments; Tsuji AB, Higashi T, Furukawa T and Tsuneo S coordinated the research and helped for the manuscript preparation; all authors revised and endorsed the final draft.

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Abstract

AIM

To investigate the therapeutic effect of combined integrin $\alpha_6\beta_4$-targeted radioimmunotherapy (RIT) and PI3K/mTOR inhibitor BEZ235 in a pancreatic cancer model.

METHODS

Phosphorylation of Akt, mTOR, the downstream effectors eukaryotic initiation factor 4E binding protein 1 (4EBP1) and S6 ribosomal protein (S6) were evaluated in BxPC-3 human pancreatic cancer cells treated with Yttrium-90 (^{90}Y) labeled anti-integrin $\alpha_6\beta_4$ antibody (ITGA6B4) and BEZ235 by western blotting. The cytotoxic effect of BEZ235 was investigated using a colony formation assay. Therapeutic efficacy enhancement by oral BEZ235 administration was assessed using mice bearing BxPC-3 xenograft tumors. Tumor volume measurements and immunohistochemical analyses (cell proliferation marker Ki-67, DNA damage marker p-H2AX and p-4EBP1 staining) of tumors were performed for evaluation of combined treatment with ^{90}Y-ITGA6B4 plus BEZ235, or each arm alone.

RESULTS

We found that phosphorylation of Akt (p-Akt), 4EBP1 (p-4EBP1) and S6 (p-S6) was inhibited by BEZ235. Colony formation in BxPC-3 cells was additively suppressed by the combination of ^{90}Y-ITGA6B4 and BEZ235. Pretreatment with BEZ235 before ^{90}Y-ITGA6B4 exposure resulted in significant reduction of cells plating efficiency (PE) (0.54 ± 0.11 *vs* 2.81 ± 0.14 with 185 kBq/mL ^{90}Y-ITGA6B4 exposure, $P < 0.01$; 0.39 ± 0.08 *vs* 1.88 ± 0.09 with 370 kBq/mL ^{90}Y-ITGA6B4 exposure, $P < 0.01$) when 5×10^3 cells per dish were plated. *In vivo*, the combined treatment with ^{90}Y-ITGA6B4 plus BEZ235 enhanced the inhibition of tumor growth and statistically significant differences of relative tumor volume were observed for 27 d after the treatment start date when compared with the ^{90}Y-ITGA6B4 single injection treatment (1.03 ± 0.38 *vs* 1.5 ± 0.15 at Day 27, $P < 0.05$), and for 41 d when compared with the BEZ235 treatment alone (1.8 ± 0.7 *vs* 3.14 ± 1.19 at Day 41, $P < 0.05$). Tumors from treatment groups showed reduction in volumes, decreased Ki-67-positive cells, increased p-H2AX-positive cells and decreased p-4EBP1 expression.

CONCLUSION

The therapeutic efficacy of ^{90}Y-ITGA6B4-RIT can be improved by combining with dual PI3K and mTOR inhibitor, BEZ235, in a pancreatic cancer model suggesting potential clinical application.

Core tip: We examined whether the therapeutic effect of ^{90}Y-labeled anti-$\alpha_6\beta_4$ integrin antibody (ITGA6B4)-mediated radioimmunotherapy (RIT) is improved by dual PI3K/mTOR inhibitor BEZ235 in the treatment of pancreatic cancer xenograft. There is no report about the combined therapeutic effects of RIT and BEZ235 in cancer treatment, though BEZ235 has been tested for its anticancer and potential radiosensitizing effect. Our studies (*in vitro/in vivo*) and results suggest for the first time that it is possible to improve the therapeutic efficacy by combining ^{90}Y-ITGA6B4-RIT and BEZ235 and this combination can be a potential encouraging treatment modality in the future.

Aung W, Tsuji AB, Sudo H, Sugyo A, Ukai Y, Kouda K, Kurosawa Y, Furukawa T, Saga T, Higashi T. Combined treatment of pancreatic cancer xenograft with ^{90}Y-ITGA6B4-mediated radioimmunotherapy and PI3K/mTOR inhibitor. *World J Gastroenterol* 2017; 23(42): 7551-7562 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7551.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7551

INTRODUCTION

Pancreatic cancer is one of the most difficult malignant diseases to cure[1]. Its treatment options are limited including surgery, adjuvant chemotherapy, and radiation therapy and have not given encouraging outcomes so far. A possible solution might emerge from the use of targeted therapy such as radioimmunotherapy (RIT)[2]. RIT involves a selective internal radiation therapy using cytotoxic radionuclides conjugated to tumor-directed antibodies[3]. We recently reported the results obtained from a preclinical study of RIT using a novel monoclonal anti-integrin $\alpha_6\beta_4$ antibody (ITGA6B4) labeled with beta-emitter Yttrium-90 (^{90}Y) (^{90}Y-ITGA6B4). According to our study, although ^{90}Y-ITGA6B4 showed significant anti-tumor effects, the myelotoxicity caused by an overdose was a major challenge to overcome[4]. To counteract this problem, the feasibility of reducing the radiation dose by combining other therapeutic modalities and retaining the same or better therapeutic effect is important. Thus, combining RIT with other chemotherapeutic candidates is one of the options to reduce the radiation dose to normal organs especially bone marrow.

The phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is a critical intracellular signaling pathway involved in regulating cell metabolism and survival, cell cycle

progression, proliferation, adhesion, and migration, particularly during cancer progression, metastasis, and radioresistance[5-10]. Moreover, this pathway is frequently aberrant and activated in cancer cells. Several downstream targets including the serine/threonine kinase Akt that activates mTOR are activated by PI3K. Furthermore, activation of this pathway is known to decrease sensitivity to chemotherapeutics as well as to irradiation (IR)[11,12], resulting in a limited treatment outcome. The PI3K/Akt and mTOR signaling pathways are also frequently dysregulated in pancreatic ductal adenocarcinoma (PDAC)[13]. Meanwhile, BEZ235 (also known as NVP-BEZ235, Dactolisib) is a potent dual pan-class I PI3K and mTOR inhibitor that suppresses PI3K and mTOR kinase activity and has been tested in preclinical studies for many cancers to demonstrate remarkable anticancer effects[14]. This orally administrable inhibitor is the first PI3K/mTOR dual inhibitor to undergo clinical trials[15,16], and already shown promising cytostatic results in breast cancer treatment[17].

Cao *et al*[18] reported that acute oral dosing with BEZ235 strongly suppressed the phosphorylation of protein kinase B (PKB)/Akt in primary human pancreatic cancers grown as orthotopic xenografts. They also showed the inhibition of downstream Thr37/46 eukaryotic initiation factor 4E binding protein 1 (4EBP1) and Ser235/236 S6 ribosomal protein (S6), consistent with the effects of BEZ235 as a dual PI3K/mTOR inhibitor[18]. Matsushima *et al*[19] reported that the phosphorylation of Akt (p-Akt), 4EBP1 (p-4EBP1), and S6 (p-S6) was inhibited in BEZ235-treated MBT-2 murine bladder cancer cells. Similarly, Kuger *et al*[20] reported that inhibiting the PI3K/Akt/mTOR pathway with BEZ235 caused dephosphorylation of the transcription and translation regulators 4EBP1 and S6. Although several studies have demonstrated that BEZ235 could be a potential radiosensitizer to external radiation therapy[21-29], there are currently no reports evaluating BEZ235 as a suitable drug to improve the therapeutic effect of RIT. The present study aimed to investigate whether the combination of RIT with molecular targeting BEZ235 therapy could enhance the therapeutic efficacy.

MATERIALS AND METHODS

Cell culture and drug preparation

Human pancreatic cancer cell line BxPC-3 was purchased from American Type Culture Collection (Manassas, VA, United States) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS, Nichirei Biosciences, Tokyo, Japan), 100 U/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate (Invitrogen, Carlsbad, CA, United States) at 37 ℃ in a humidified atmosphere containing 5% CO_2. NVP-BEZ235 was purchased from Selleck Chemicals (Houston, TX, United States), dissolved in dimethyl sulfoxide (Sigma-Aldrich) and the 2.5 mmol/L stock was stored at -20 ℃. For *in vivo* treatment, it was mixed with the vehicle NMP/polyethylene glycol 300 (10/90, v/v).

Antibody radiolabeling

Human anti-$\alpha_6\beta_4$ monoclonal antibody (IgG_1) was labeled with beta-emitter ^{90}Y, as previously reported[30]. Briefly, the antibody solution and a chelating agent, *N*-[(*R*)-2-amino-3-(*p*-isothiocyanato-phenyl)propyl]-trans-(S,S)-cyclohexane-1,2-diamine-*N*,*N*′,*N*″,*N*‴-pentaacetic acid (CHX-A″-DTPA) (Macrocyclics, Dallas, TX, United States) were mixed at a molar ratio of 1:2.5 and incubated overnight at 37 ℃. The conjugation ratio of DTPA and the antibody was estimated to be 1.3 calculated from the ratio (of ^{111}In-DTPA-antibody to ^{111}In-DTPA) determined by isoelectric focusing. Unconjugated DTPA was removed using a Sephadex G-50 column eluted with 0.1 mol/L sodium acetate buffer (GE Healthcare, Little Chalfont, United Kingdom). Afterward, the DTPA-conjugated antibody (71.2 μg in 0.1 mol/L sodium acetate buffer, pH 6.0) was incubated with a mixture of ^{90}Y-chloride (74 MBq, Eckert & Ziegler Radiopharma GmbH, Berlin, Germany) and 1 mol/L sodium acetate buffer (pH 6.0) for 30 min at room temperature (RT). The radiolabeled antibody was purified using a Sephadex G-50 column (730 × *g* for 2 min). The radiochemical purity as determined by TLC was > 95%. The radiochemical yield was approximately 80%, and the specific activity was approximately 1500 kBq/μg.

Western blot analysis

Western blotting was performed to analyze the proteins of interest from cultured cells. Cancer cells were cultured and treated with medium containing 0.1 μmol/L BEZ235 or DMSO (vehicle) for 1 h. The medium was then discarded and cells were exposed to medium containing ^{90}Y-ITGA6B4 (indicated doses 185 and 370 kBq/mL) in the presence and absence of BEZ235 treatment. At 18 h after incubation, whole-cell lysates were prepared using radioimmunoprecipitation assay buffer (Wako Pure Chemical Industries, Osaka, Japan) with protease inhibitor cocktail. Total protein concentration was measured using the NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). Protein samples (45 μg) were separated on a 4%-20% polyacrylamide gel (ATTO Corporation, Tokyo, Japan) and transferred to an Immobilon-P membrane (Millipore, Billerica, MA, United States). The following antibodies: anti-human phospho-Akt (Ser473) (D9E) monoclonal antibody, anti-human phospho-4EBP1 (Thr37/46) (236B4) monoclonal antibody, anti-human phospho-mTOR (Ser2448) (D9C2) monoclonal antibody, anti-human phospho-S6 Ribosomal protein (Ser235/236)

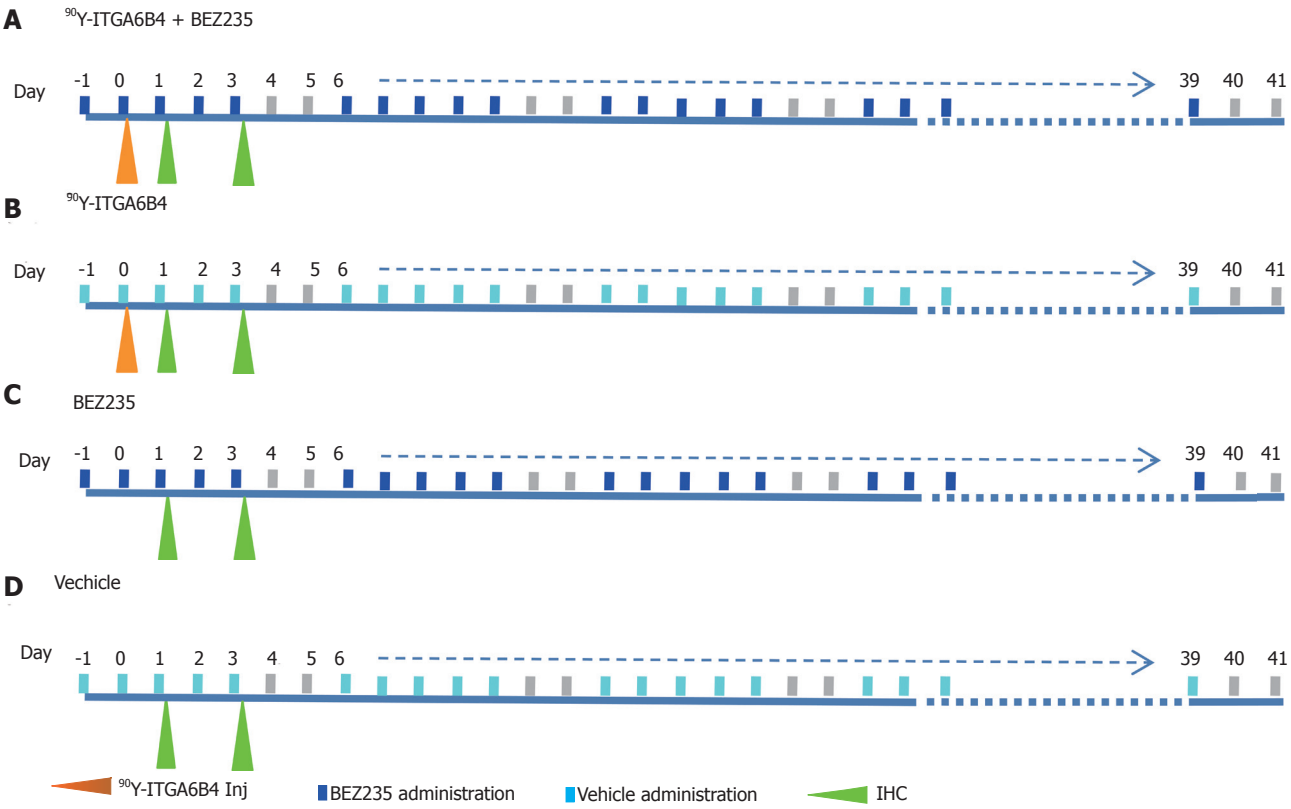


Figure 1 Experimental treatment scheme. Group A: ^{90}Y-ITGA6B4 + BEZ235; Group B: ^{90}Y-ITGA6B4; Group C: BEZ235; Group D: Vehicle.

polyclonal antibody, and anti-human GAPDH monoclonal antibody were purchased from Cell Signaling technology (Danvers, MA, United States). Anti-human Akt1 (C-20) polyclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, United States). These were used as primary antibodies. Horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody purchased from GE Healthcare (Little Chalfont, United Kingdom) was used as the secondary antibody. Immunoreactive bands were visualized using the Enhanced Chemiluminescence Plus western blotting detection system (GE Healthcare).

Colony formation assay

Cells (10, 5, 2.5 × 10^3 cells/dish) were plated in triplicate onto 60-mm dishes. After overnight incubation, exponentially growing cells were treated with the medium containing 0.1 μmol/L μmol BEZ235 or DMSO (vehicle) for 1 h. The medium was then discarded and adherent cells were exposed to medium containing ^{90}Y-ITGA6B4 (indicated doses 185 and 370 kBq/mL) in the presence and absence of BEZ235 treatment for 24 h. The medium was then replaced with drug-free medium and the cells were cultured for 7 d for colony formation. At the indicated time point, cells were fixed and stained with Gentian violet and the grown colonies (clusters of > 50 cells) were counted. Plating efficiencies (PE) were determined as (number of colonies counted/number of cell inoculated) × 100.

Mouse pancreatic tumor xenograft model

All animal experiments were performed in accordance with the animal experimentation protocol approved by the Animal Care and Use Committee of National Institute of Radiological Sciences. Nude mice (7-wk-old female BALB/cA Jcl-nu/nu mice) were obtained commercially from CLEA, Shizuoka, Japan. They were housed in a restricted access room and acclimatized to standard laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, free access to food and water). Subcutaneous tumors were generated by injecting a suspension of 5 × 10^6 BxPC-3 cells in 100 μL RPMI medium mixed with BD Matrigel matrix (BD Biosciences, Bedford, MA, United States) into the right thigh of nude mice.

In vivo tumor treatment study

When the subcutaneous tumors in mice reached approximately 10 mm at the longest diameter, the xenograft tumor-bearing mice were randomly assigned to 4 groups (n = 10 for each group) for the treatment study. Experimental treatment was performed according to the shown scheme (Figure 1). Group A received daily oral administration of BEZ235 35 mg/kg, 5 d/wk for a 6-wk schedule with a single administration of ^{90}Y-ITGA6B4 (2.8 MBq) following 1 h of the second BEZ235 dose, group B received a single administration of ^{90}Y-ITGA6B4 (2.8 MBq), group C received daily oral

administration of BEZ235 35 mg/kg, 5 d/wk for a 6-wk schedule, and group D received no treatments except oral vehicle administration. Intragastric gavage administration of BEZ235 was carried out with conscious mice, using disposable flexible gavage needles (1 inch length, 1.25 ball diameter). Each quantity of the injected antibody was adjusted to 20 μg by the addition of intact unlabeled antibody. According to previous pharmacokinetic studies, mice were injected with ^{90}Y-ITGA6B4 at 1 h following BEZ235 administration because within this time frame, BEZ235 achieves effective intra-tumoral concentrations[15]. To observe the tumor response, tumor volumes of mice (n = 6 for each group) were measured twice a week throughout the experiment using calipers, and were approximated using the equation: volume (mm^3) = [length (mm)] × [width (mm)] ^{2}/2. Relative tumor volume was calculated as the volume on the indicated day divided by the volume on the day treatment began.

Immunohistochemical analysis

On Day 1 and Day 3 after ^{90}Y-ITGA6B4 administration, 2 mice of each group (n = 2) were euthanized by cervical dislocation under anaesthesia (Isoflurane). Tumor tissue specimens were extirpated, fixed in 4% paraformaldehyde, and embedded in paraffin. Tumor specimens obtained from untreated mice were used as control. Paraffin-embedded tissue sections were cut (5 μm), rehydrated, and subjected to antigen retrieval. Ki-67 staining of sections was performed using an anti-human Ki-67 polyclonal antibody (Dako Denmark, Glostrup, Denmark), as previously described[31]. Phospho-Histone H2AX (p-H2AX) and Phospho-4EBP1 (p-4EBP1) staining were also detected using anti-human p-H2AX (Ser139) (20E3) monoclonal antibody and anti-human p-4EBP1 (Thr37/46) (236B4) monoclonal antibody (Cell Signaling technology), respectively. To detect the apoptotic tumor cells, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining was performed with an ApopTag Peroxidase In situ Apoptosis Detection Kit (Millipore Corporation, Temecula, CA, United States). Each slide was observed using the Olympus BX43 microscope system (Olympus, Tokyo, Japan). For quantitative and statistical analysis, we counted the number of Ki-67-positive cells observed in three random fields of view at 200× magnification in 2 different tumors sections from each group (n = 6). Similarly, p-H2AX-positive cells were also counted.

Statistical analysis

All results were expressed as mean ± SD. Significant differences between groups were determined by Student's t-test (Excel, Microsoft, Redmond, WA, United States). Two-tailed unpaired t-test was used for comparisons of relative tumor volume, Ki-67 positive cells and p-H2AX-positive cells. Two-tailed paired t-test was used for comparisons of PE. P-values < 0.05 were considered significant.

RESULTS

BEZ235 downregulates the PI3K/Akt signaling pathway in BxPC-3 pancreatic cancer cells

The effects of BEZ235 on the PI3K/Akt signaling pathway were determined by western blotting using BxPC-3 cells. Cells were treated with or without 0.1 μmol/L BEZ235 for 1 h before exposure to ^{90}Y-ITGA6B4. Total cell lysates were prepared after 18 h of ^{90}Y-ITGA6B4 treatment. Akt1 expression and phosphorylation of Akt (p-Akt) were increased at 18 h after exposure to 185 or 370 kBq/mL ^{90}Y-ITGA6B4. These increased expression levels were attenuated by pretreatment with 0.1 μmol/L BEZ235. Similarly, phosphorylation of 4EBP1 (p-4EBP1) and S6 (p-S6), which are Akt and mTOR downstream effectors, was inhibited by BEZ235 (Figure 2). No obvious changes in mTOR expression and phosphorylation were noted in our experiments (data not shown).

BEZ235 augments the cytotoxic effect exerted by ^{90}Y-ITGA6B4 treatment

In the colony formation assay, 24 h exposure of BxPC-3 cells to ^{90}Y-ITGA6B4 (185 or 370 kBq/mL) apparently resulted in radiation dose-dependent inhibition of cell survival and proliferation, which was evidenced by the reduced size and number of colonies and by the decreased PE. Moreover, pretreatment with 0.1 μmol/L BEZ235 for 1 h before ^{90}Y-ITGA6B4 exposure resulted in significant reduction of colony formation and PE to a greater degree than in the single-drug treatment. For example, combined treatment with BEZ235 and ^{90}Y-ITGA6B4 (185 kBq/mL) to cells (5 × 10^3 cells/dish) showed 90.9% reduction of PE from that of control while ^{90}Y-ITGA6B4 treatment alone showed 52.5% reduction of PE from that of control. All relevant PE were compared in Figure 3 (P < 0.01). These results indicate that combination of BEZ235 and RIT markedly exaggerates the cytotoxic activity.

BEZ235 inhibits the growth of BxPC-3 xenografts and potentiates ^{90}Y-ITGA6B4 mediated RIT

The anti-tumor effect of ^{90}Y-ITGA6B4 with or without BEZ235 was evaluated using a BxPC-3 xenograft tumor model. Tumor growth was significantly delayed by ^{90}Y-ITGA6B4 (2.8 MBq) treatment alone for 58 days following the treatment start date compared with that in vehicle treatment (control). Tumor growth was also significantly delayed by BEZ235 (35 mg/kg, 5 d/wk for 6 wk) treatment alone for 23 d compared with that in control. Combined treatment with ^{90}Y-ITGA6B4 plus BEZ235 enhanced the inhibition of tumor growth and statistically significant differences were observed for 27 d after the treatment start date when compared with the ^{90}Y-ITGA6B4 single injection treatment, and

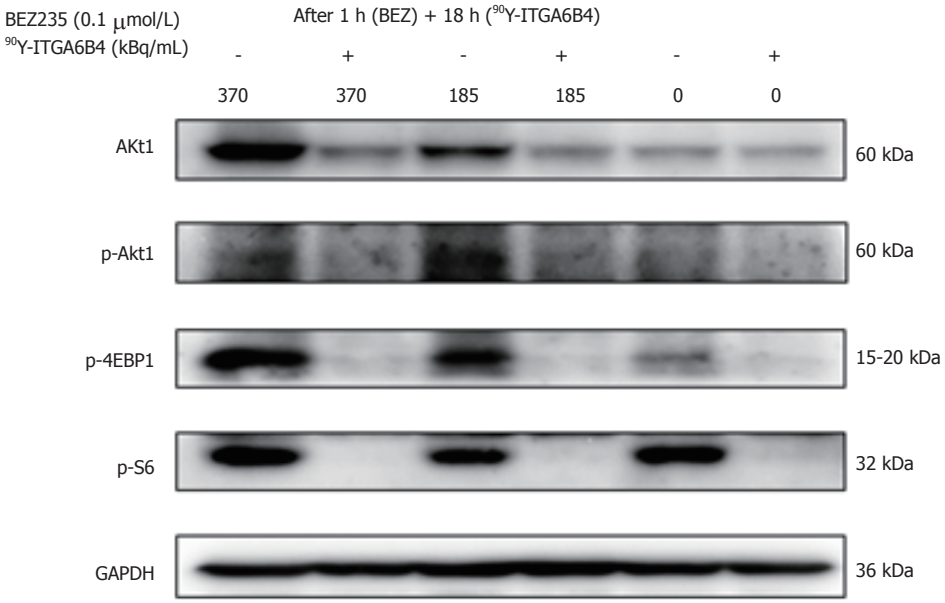


Figure 2 Effect of BEZ235 and ^{90}Y-ITGA6B4 on PI3K/Akt signaling. Expression levels of selective proteins associated with the PI3K/Akt pathway were determined at 18 h after ^{90}Y-ITGA6B4 exposure (185 or 370 kBq/mL). Representative western blot analyses are shown. Akt1 expression and Akt phosphorylation (p-Akt) likely induced by ^{90}Y-ITGA6B4 were inhibited upon BEZ235 treatment. Concordantly, phosphorylation of 4EBP1 (p-4EBP1) and S6 (p-S6), both Akt and mTOR downstream effectors, was markedly inhibited by BEZ235 pretreatment.

Table 1 Comparison of the number of Ki-67 positive cells and p-H2AX-positive cells in tumor sections from xenografted mice treated with the vehicle control, BEZ235, ^{90}Y-ITGA6B4, or ^{90}Y-ITGA6B4 + BEZ235

Group	Ki-67-positive cells		p-H2AX-positive cells	
	Day 1	Day 3	Day 1	Day 3
Vehicle (control)	95.7 ± 11.4	87.0 ± 36.5	2.2 ± 0.8	2.3 ± 1.2
BEZ235	27.3 ± 19.4^{b}	24.8 ± 8.4^{b}	5.2 ± 3.8	4.0 ± 2.2
^{90}Y-ITGA6B4	21.2 ± 11.8^{b}	31.2 ± 18.8^{b}	10.8 ± 2.8^{b,e}	12.7 ± 4.7^{b,d}
^{90}Y-ITGA6B4 + BEZ	13.0 ± 8.8^{b}	7.3 ± 3.6^{a,b,d}	12.3 ± 5.5^{b,c}	19.3 ± 7.0^{b,d}

Values are expressed as mean ± SD, $n = 6$. $^{b}P < 0.01$ *vs* Vehicle, $^{d}P < 0.01$ *vs* BEZ235; $^{a}P < 0.05$ *vs* ^{90}Y-ITGA6B4, $^{c}P < 0.05$ *vs* BEZ235, $^{e}P < 0.05$ *vs* BEZ235.

for 41 d when compared with the BEZ235 treatment alone ($P < 0.05$, Figure 4A). At 27 d after starting the treatment, the combination treatment showed 30.9% or 53.9% further reduction of the tumor volume ratio compared to that attained with RIT alone or BEZ235 alone, respectively. Neither significant average body weight loss (Figure 4B) nor differences in general conditions of mice were observed between the groups throughout the experiment.

Immunohistochemical evaluation of the therapeutic effects of combined ^{90}Y-ITGA6B4 plus BEZ235 treatment or each arm alone

Proportions of cell positive for the cell proliferation marker protein[32] Ki-67 were decreased in tumor sections of mice treated with the combination ^{90}Y-ITGA6B4 plus BEZ235, or each arm alone, when compared with those of the control group. It is well-known that phosphorylation of the histone variant H2AX (p-H2AX) is a highly specific and sensitive molecular marker to monitor both DNA double-strand break (DSB) initiation and resolution, especially during radiation treatment[33]. P-H2AX-positive cells were increased in tumor sections of mice receiving the combined treatment or ^{90}Y-ITGA6B4 treatment alone, when compared with those in the control group. The quantitative results and statistical analysis were summarized in Table 1. Immunohistochemical staining with the p-4EBP1 antibody depicted decreased expression of p-4EBP1 in the combined treatment or each arm alone, when compared with that in the control (Figure 5). On the other hand, TUNEL-positive cells were rare and there was no clear difference in the TUNEL staining patterns among all the groups (Data not shown).

DISCUSSION

Several previous *in vitro* and *in vivo* studies have revealed that BEZ235 could be a potential radiosensitizer in the treatment of various cancers such as fibrosarcoma as well as hypopharyngeal carcinoma[21], prostate carcinoma[22,23], non-small cell lung cancer (NSCLC)[24], glioblastoma[25], colorectal cancer (CRC)[26], and breast cancer[34]. Chen *et al*[26] reported that inactivation of the PI3K/Akt pathway by BEZ235 could enhance radiosensitivity by hindering the PI3K/Akt/mTOR pathway and DNA repair mechanisms. To prevent the radiation induced activation of PI3K/Akt/mTOR

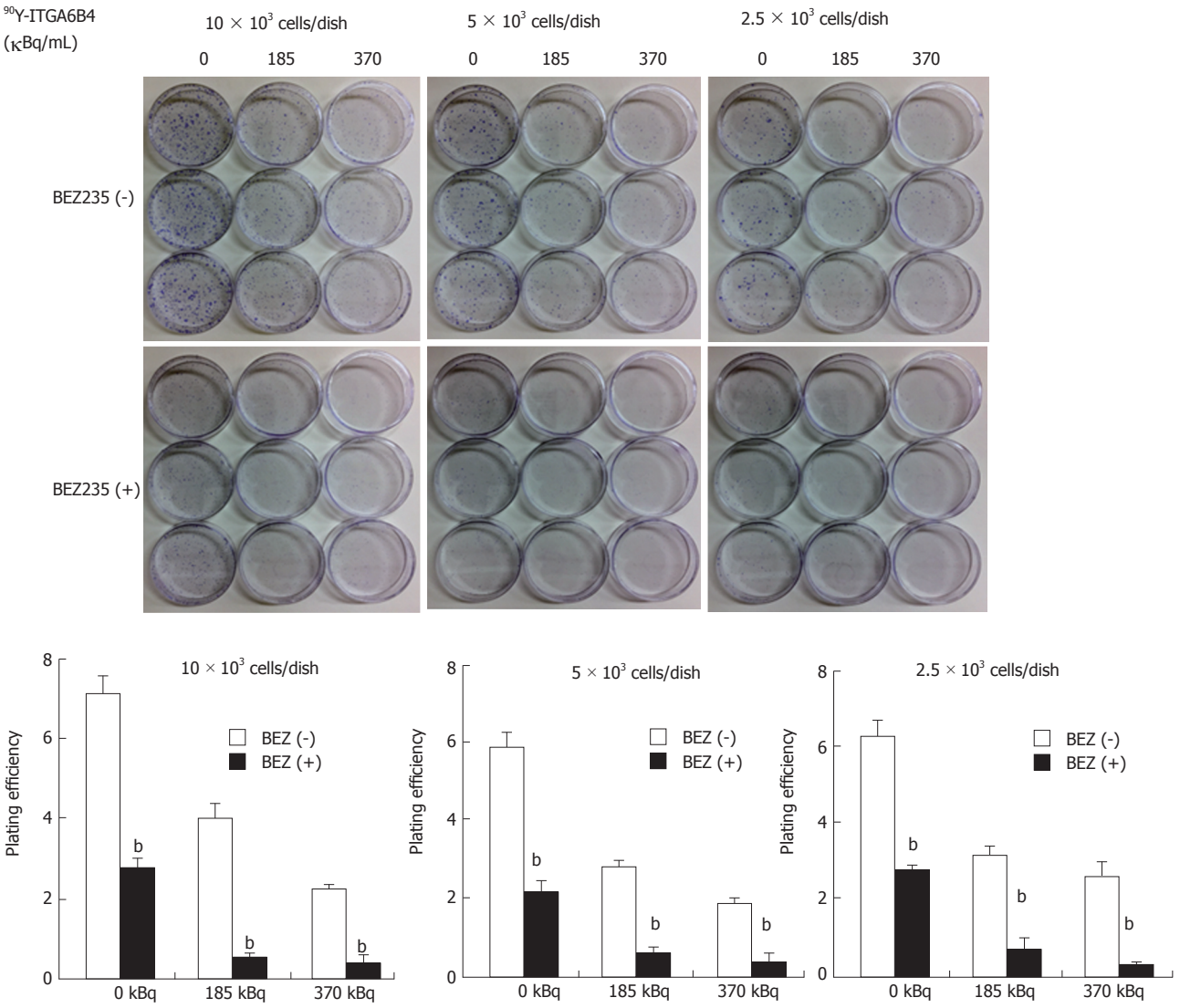


Figure 3 Cytotoxic effect of BEZ235 that augments the cytotoxic activity of RIT in BxPC-3 cells. Treatment with ^{90}Y-ITGA6B4 alone or BEZ235 alone resulted in decreased colony formation ability in cells. Moreover, pretreatment with BEZ235 before ^{90}Y-ITGA6B4 exposure induced stronger inhibition of the colony formation ability than that by ^{90}Y-ITGA6B4 or BEZ235 treatment alone; moreover, plating efficiencies (PE) of cells were significantly different between the BEZ235 (+) and (-) conditions ($^bP < 0.01$). Data represent mean ± SD, $n = 3$.

signaling, they treated the colorectal cancer cells with BEZ235 one hour before the radiation and found that BEZ235 synergistically inhibited cell viability. Their findings are in accordance with those of Kuger *et al*[27] demonstrating that radiosensitivity of glioblastoma cells was enhanced by BEZ235 exposure one hour before irradiation. Some recent studies described that BEZ235 could potently inhibit major DNA damage response kinases, attenuate the repair of irradiation-induced DNA damage, and confer striking tumor radiosensitization in glioblastoma[25,29] .

In contrast to external beam radiation therapy, one of the convincing benefits of RIT is its capacity to strike the primary tumor as well as the systemically metastasizing and residual lesions. However, systemic administration of large amounts of radiolabeled antibodies may cause bone marrow suppression. Small but adequate quantities of radiolabeled antibody that may bind to targets and produce efficient cytotoxic effects are therefore desirable, and new strategies are consequently needed to improve the effectiveness of RIT. We speculated that the combination of RIT and BEZ235 may help reduce the dose of RIT and generate a greater therapeutic response than each arm used alone, with less frequent treatment-related toxicity and increased radiosensitivity. The radiophysical properties of ^{90}Y (β-particles with a maximum emitted energy of 2.28 MeV and emission range of 11 mm, half-life 2.7 d) are a relatively preferable and practical choice for RIT. In our previous study, we conducted RIT with ^{90}Y labeled anti-integrin $\alpha_6\beta_4$ antibody (^{90}Y-ITGA6B4) in a pre-clinical mouse pancreatic cancer model[4]. As the treatment protocol, we used a single administration of ^{90}Y-ITGA6B4 (3.7 MBq), and double administrations of ^{90}Y-ITGA6B4 once-weekly (3.7 MBq × 2) in our previous study[4]. We found significantly reduced tumor

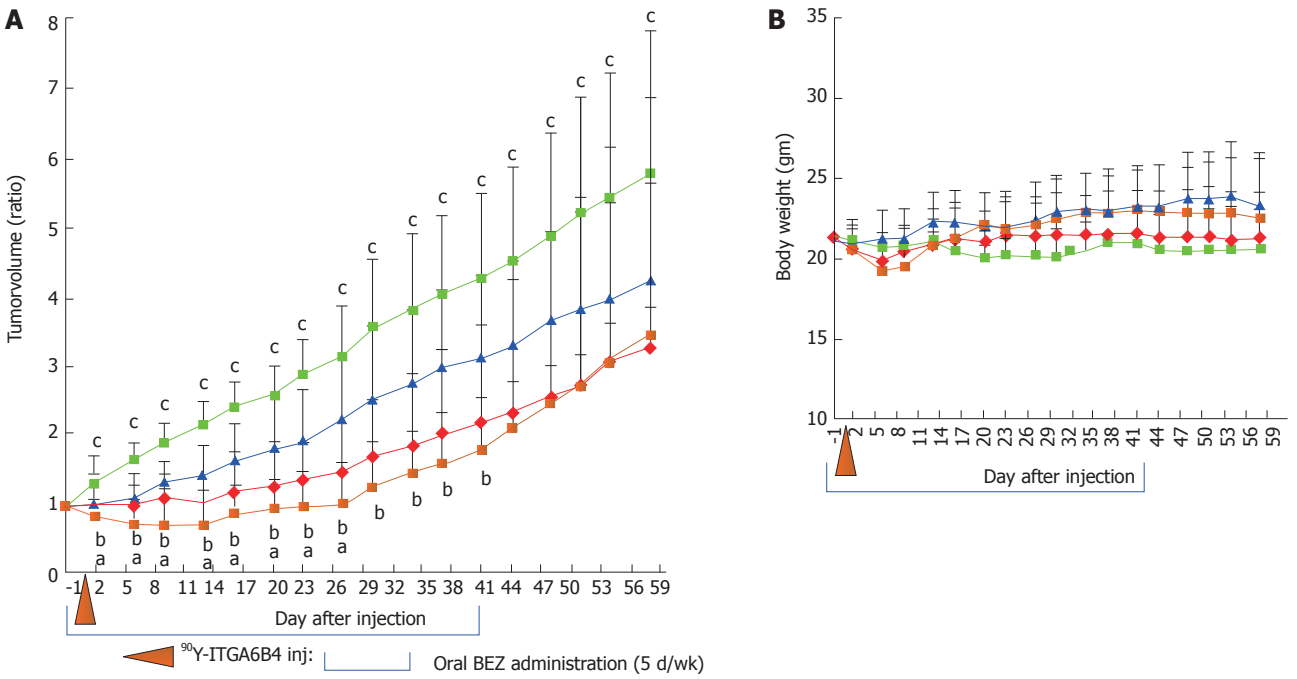
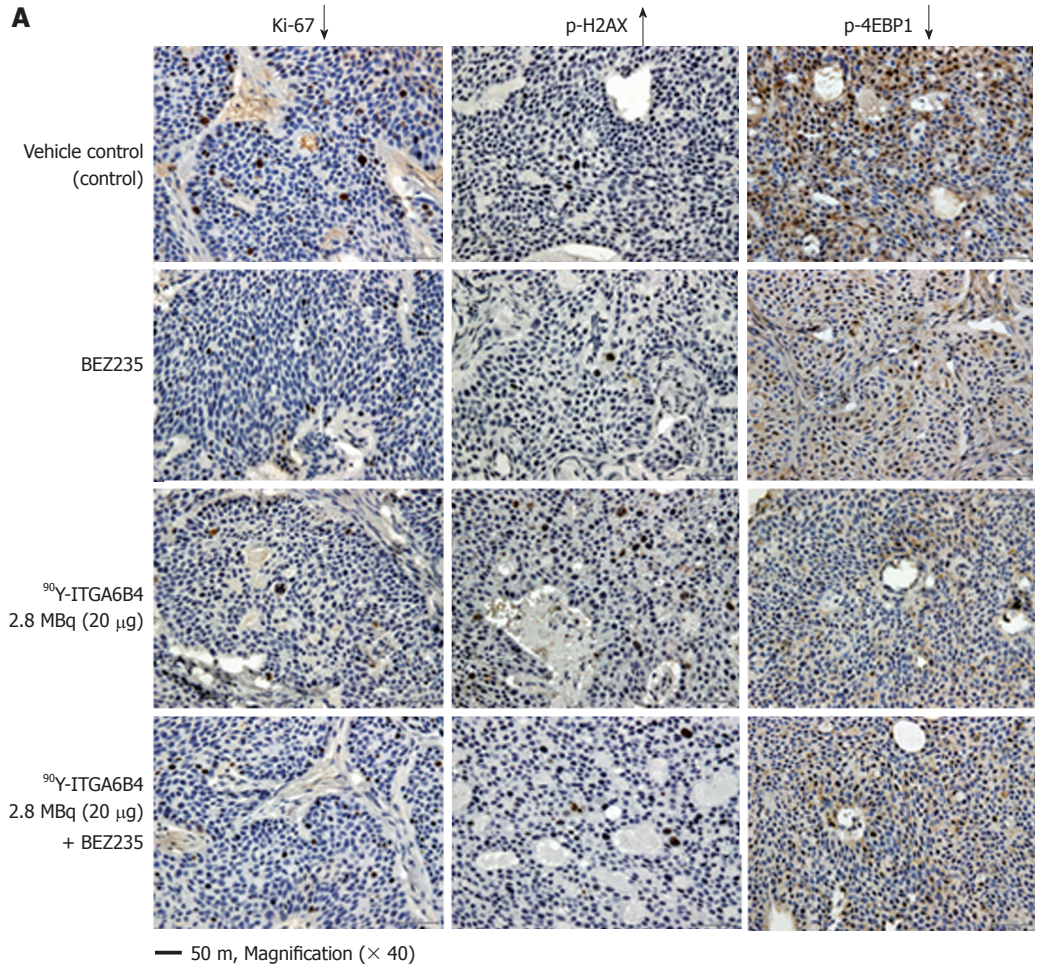


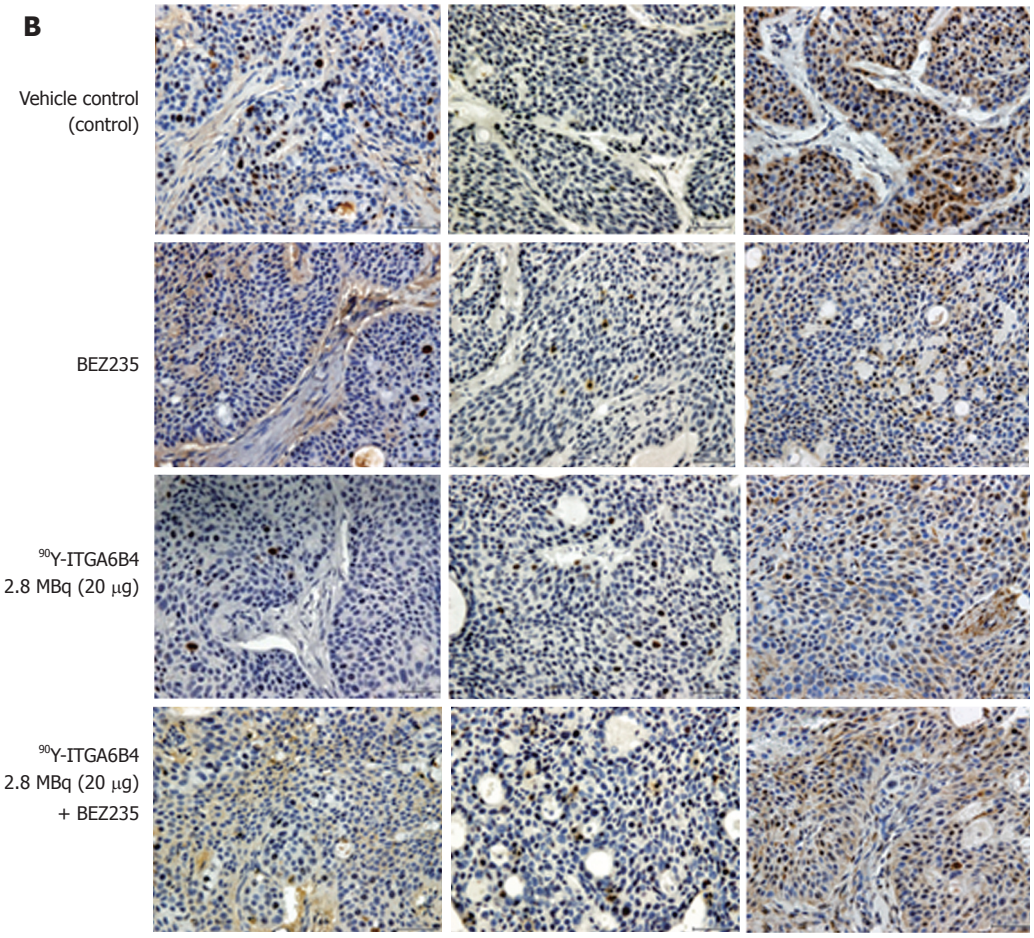
Figure 4 Enhancement of mediated radioimmunotherapeutic effects on BxPC-3 xenografts in combination with BEZ235 treatment. A: Tumor volume changes expressed as the ratio of the volume on the indicated day and the volume on the day treatment began. Compared with the control vehicle administration (light blue squares), single administration of ^{90}Y-ITGA6B4 (2.8 MBq) alone (red diamonds) or oral administrations of BEZ235 alone (35 mg/kg, 5 d/wk for 6 wk) (blue triangles) resulted in impaired tumor growth. When ^{90}Y-ITGA6B4 treatment was administered in conjunction with BEZ235 (orange squares), further reduction in tumor volume ratio was observed indicating enhanced therapeutic effect upon combined treatment. Values represent mean ± SD, $^{a}P < 0.05$ (^{90}Y-ITGA6B4 + BEZ235 *vs* ^{90}Y-ITGA6B4), $^{b}P < 0.05$ (90Y-ITGA6B4 + BEZ235 *vs* BEZ235), $^{c}P < 0.05$ (^{90}Y-ITGA6B4 + BEZ235 *vs* Vehicle, ^{90}Y-ITGA6B4 *vs* Vehicle); B: Average mouse body weight did not differ significantly among all 4 groups. Vertical orange arrowhead indicates the day of 90Y-ITGA6B4 injection. Values represent mean ± SD, $n = 6$.

growth rates in both groups compared with those in the untreated control. However, one mouse from the group receiving the double administrations showed pale skin and few petechiae during the study, and died on day 22 following the first dose. We considered myelotoxicity due to an overdose as the cause of death because analysis of hematological parameters at day 27 after starting the RIT indicated decreased RBC, WBC, and platelet counts in mice treated with double administrations, whereas only a decreased RBC count was observed in mice receiving a single administration. Based on these previous results[4], we chose a single administration dose of ^{90}Y-ITGA6B4 (2.8 MBq) for RIT in this study design. Moreover, we decided to include daily oral administration of BEZ235 35 mg/kg, 5 d/wk over a 6-wk schedule. To the best of our knowledge, this is the first report about the combined therapeutic effects of RIT and BEZ235 in a preclinical pancreatic cancer model.

In the present study, inhibition of the PI3K/Akt/mTOR pathway with BEZ235 was proven by western blotting in BxPC-3 tumor cells upon observing the downregulation of p-Akt, as well as the downstream targets, p-4EBP1 and p-S6. The reduction of p-Akt, p-4EBP1 and p-S6 suggested that a plausible mechanism of the cytotoxic effect of BEZ235 derived from inhibition of the PI3K/Akt/mTOR pathway. The 4EBP1 protein integrates its function at the level of translation regulation[35]. Recently, it has been shown that some kinases phosphorylate 4EBP1 dependent or independent of mTOR, indicating that mTOR may not be the only kinase that phosphorylates 4EBP1[36], and that 4EBP1 is regarded as a point of convergence of various signaling pathways[35]. Phosphorylation of S6 ribosomal protein correlates with an increase in the translation of mRNA transcripts encoding proteins involved in cell cycle progression as well as ribosomal proteins and elongation factors necessary for translation[37]. Upregulation of mRNA translation is effectively involved in sustained cell growth and proliferation[37]. The opposite effect was observed with BEZ235 treatment.

The cytotoxic effect of BEZ235 on BxPC-3 cells was evaluated using the colony formation assay. Exposure to ^{90}Y-ITGA6B4 alone resulted in decreased clonogenicity in cells and BEZ235 pretreatment caused more potent inhibition of colony formation indicated by reduced counts and smaller colonies. Taken together with the results of western blot analysis, BEZ235 might prevent PI3K pathway reactivation and further enhance radiation-induced cell killing. Next, we determined the anti-tumor effect of ^{90}Y-ITGA6B4 with or without BEZ235 treatment in BxPC-3 xenograft tumors through longitudinal measurement of tumor volume and immunohistochemical analyses. We found that a single injection of ^{90}Y-ITGA6B4 (2.8 MBq) alone significantly delayed tumor growth compared with that upon vehicle administration. Furthermore, combined


A
Ki-67
p-H2AX
p-4EBP1
Vehicle control (control)
BEZ235
^{90}Y-ITGA6B4 2.8 MBq (20 μg)
^{90}Y-ITGA6B4 2.8 MBq (20 μg) + BEZ235
50 m, Magnification (× 40)


B
Vehicle control (control)
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^{90}Y-ITGA6B4 2.8 MBq (20 μg) + BEZ235

Figure 5 Ki-67, p-H2AX, p-4EBP1 immunostaining of tumor sections. A: On Day 1 and; B: On Day 3 after administration of 90Y-ITGA6B4 alone or combined with BEZ235, intratumoral proliferation was determined by immunostaining for Ki-67 nuclear antigen. A marked reduction in Ki-67-positive cell numbers was observed in samples from mice treated with 90Y-ITGA6B4 + BEZ235 as well as with 90Y-ITGA6B4 alone and BEZ235 alone, compared with those in the untreated control sample. Meanwhile, increased p-H2AX-positive cell numbers were observed in samples from mice that received 90Y-ITGA6B4 treatment alone or the combined treatment than those in the control. Immunohistochemical analysis for p-4EBP1 showed that phosphorylation of 4EBP1 was decreased in the treatment groups compared with the control group. Tumor section images were acquired at 200× magnification and representative images are shown (scale bar, 50 μm). The quantitative and statistical analysis were summarized in Table 1.

treatment with ^{90}Y-ITGA6B4 plus BEZ235 more strongly and significantly inhibited the tumor growth for 27 d when compared with that in ^{90}Y-ITGA6B4 treatment alone, and for 41 d when compared with that in BEZ235 treatment alone ($P < 0.05$, Figure 4A). To further retain the superior therapeutic efficacy of the combined treatment and to impede tumor regrowth, further fractionated ^{90}Y-ITGA6B4 administration with appropriate timings will be required and will be interesting to address in next study. Concerning toxicity, we can judge that there was no enhancement of toxicity during the combined treatment because body weight loss, obvious abnormal changes in general conditions and death were not observed in the mice receiving treatment (Figure 4B).

Previously, Fokas *et al*[28] has demonstrated that BEZ235 itself causes DNA damage even in nonirradiated cells, as evidenced by moderately increased phosphorylation of the histone variant H2AX, and the enhanced persistence of p-H2AX foci after irradiation, thus attributing radiosensitivity to head and neck and bladder cancer cell lines. In our immunohistochemical analyses, significantly increased DNA damage marker p-H2AX-positive cells were noted in the tumor sections of mice that received ^{90}Y-ITGA6B4 plus BEZ235, or ^{90}Y-ITGA6B4 treatment alone, when compared with those of the control group (Figure 4). In contrast, the proliferation marker nuclear protein Ki-67-positive cells were significantly reduced in treatment groups. Besides, immunohistochemical examination showed decreased phosphorylation of 4EBP1 in the treatment groups. These results suggest that BEZ235 treatment could potentiate the radioimmunotherapeutic effect of ^{90}Y-ITGA6B4 in BxPC-3 tumors. We detected no appreciable induction of apoptotic cells in TUNEL-stained sections, and the staining patterns among groups were not very different at 1 and 3 d after ^{90}Y-ITGA6B4 administration (data not shown). This result is in line with the results of our previous RIT study in which TUNEL assay was conducted at 2 d post-administration[4].

Chen *et al*[26] have reported that irradiation up-regulates the Akt/mTOR signaling pathway including the activation of Akt and mTOR, which were attenuated by BEZ235 pretreatment. Likewise, RIT may also upregulate Akt/mTOR signaling pathway to some extent after administration, but it seems that this activated Akt/mTOR signaling pathway is attenuated by BEZ235 pretreatment. The mechanisms underlying the treatment with BEZ235 in combination with RIT for pancreatic cancer still need to be clarified with a more detailed study covering evidences at multiple times-points during treatment with various dose regimens of both drugs. In the current study, we obtained an insight in to the presumptive mechanism of a combination treatment with PI3K/mTOR inhibitors and RIT as well as its therapeutic effects.

In conclusion**,** our findings imply that it is possible to improve the therapeutic efficacy by combining ^{90}Y-ITGA6B4-mediated radioimmunotherapy with the dual PI3K and mTOR inhibitor, BEZ235, and this combination is a promising treatment option for future pancreatic cancer therapy, though many hurdles remain to be overcome to reach clinical use.

COMMENTS

Background

Pancreatic cancer is one of the most difficult malignant diseases to cure. Its treatment options are limited and have not given encouraging outcomes so far. A possible solution might emerge from the use of radioimmunotherapy (RIT) and other molecular targeting chemotherapeutic candidates. The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is frequently dysregulated and activated in human cancers including pancreatic cancer. Several studies have revealed that BEZ235 (NVP-BEZ235) can inhibit the PI3K and mTOR kinase activity, could be a potential radiosensitizer, and has been used in preclinical studies in many cancers with excellent results of anticancer effects.

Research frontiers

The authors have studied the radioimmunotherapeutic effect and toxicity of Yttrium-90 labeled anti-integrin $\alpha_6\beta_4$ antibody (^{90}Y-ITGA6B4) in a mouse pancreatic cancer model. In the previous study, ^{90}Y-ITGA6B4 showed anti-tumor effects but myelotoxicity caused by an overdose was a major handicap. To overcome this obstacle, combining RIT with other chemotherapeutic candidates is one of the options for reducing the radiation dose and retaining the therapeutic effect.

Innovations and breakthroughs

To the best of our knowledge, there was no report about the therapeutic effects of combined ^{90}Y-ITGA6B4-RIT and BEZ235 in cancer treatment. Thus, The authors examined this effect for the first time in a pre-clinical pancreatic cancer xenograft model.

Applications

These *in vitro* and *in vivo* studies and results suggest that it is possible to improve the therapeutic efficacy by combing ^{90}Y-ITGA6B4-RIT and BEZ235 and this combination can be a potential encouraging treatment option for future pancreatic cancer therapy.

Terminology

The ^{90}Y-ITGA6B4-RIT is a specific treatment option for cancer by cytotoxic radionuclide (beta-emitter Yttrium-90) conjugated to anti-integrin $\alpha_6\beta_4$ monoclonocal antibody (ITGA6B4). The PI3K/Akt/mTOR pathway is a

critical intracellular signaling pathway involved in regulating cell metabolism and survival, cell cycle progression, proliferation, adhesion, and migration, particularly during cancer progression, metastasis, and radioresistance.

Peer-review

It's a well-designed study and answers a good scientific question.

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ORIGINAL ARTICLE

Basic Study

Effects of Hemp seed soft capsule on colonic ion transport in rats

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Abstract

AIM

To investigate the effect of Hemp seed soft capsule (HSCC) on colonic ion transport and its related mechanisms in constipation rats.

METHODS

Sprague-Dawley male rats were randomly divided into three groups: normal group, constipation group and HSSC group. Rats in the constipation and HSSC groups were administrated loperamide 3 mg/kg per day orally for 12 d to induce the constipation model. Then, the HSSC group was given HSSC 0.126 g/kg per day by gavage for 7 d. The normal and constipation groups were treated with distilled water. After the treatment, the fecal wet weight and water content were measured. The basal short-circuit current (*Isc*) and resistance were measured by an Ussing Chamber. Besides the *in vivo* drug delivery experiment above, an *in vitro* drug application experiment was also conducted. The accumulative concentrations of HSSC (0.1 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 2.5 mg/mL, 5.0 mg/mL, 10.0 mg/mL and 25.0 mg/mL) were added to the normal isolated

colonic mucosa and the *Isc* was recorded. Further, after the application of either ion (Cl^- or HCO_3^-) substitution, ion channel-related inhibitor (N-phenylanthranilic acid, glybenclamide, 4,4-diisothiocyano-2,2-stilbenedisulfonic acid or bumetanide) or neural pathway inhibitor [tetrodotoxin (TTX), atropine, or hexamethonium], the *Isc* induced by HSSC was also measured.

RESULTS

In the constipation group, the fecal wet weight and the water content were decreased in comparison with the normal group ($P < 0.01$). After the treatment with HSSC, the fecal wet weight and the water content in the HSSC group were increased, compared with the constipation group ($P < 0.01$). In the constipation group, the basal *Isc* was decreased and resistance was increased, in comparison with the normal group ($P < 0.01$). After the treatment with HSSC, the basal *Isc* was increased ($P < 0.05$) and resistance was decreased ($P < 0.01$) in the HSSC group compared with the constipation group. In the *in vitro* experiment, beginning with the concentration of 1.0 mg/mL, differences in *Isc* were found between the experimental mucosa (with HSSC added) and control mucosa. The *Isc* of experimental mucosa was higher than that of control mucosa under the same concentration (1.0 mg/mL, $P < 0.05$; 2.5-25 mg/mL, $P < 0.01$). After the Cl^- or HCO_3^- removal and pretreated with different inhibitors (cAMP-dependent and Ca^{2+}-dependent Cl^- channels, Na^+-K^+-$2Cl^-$ cotransporter (NKCC), Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger inhibitor), there were differences between experimental mucosa and control mucosa; the *Isc* of experimental mucosa was lower than that of control mucosa under the same concentration ($P < 0.05$). Meanwhile, after pretreatment with neural pathway inhibitor (TTX, atropine, or hexamethonium), there were no differences between experimental mucosa and control mucosa under the same concentration ($P > 0.05$).

CONCLUSION

HSSC ameliorates constipation by increasing colonic secretion, which is mediated *via* the coaction of cAMP-dependent and Ca^{2+}-dependent Cl^- channels, NKCC, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger.

Key words: Hemp seed soft capsule; Constipation; Ion transport; Cl^-; HCO_3^-

Core tip: In this study, we established a constipation model using the application of loperamide and found that Hemp seed soft capsule could improve the symptom of constipation. Further, it was found that the effect of Hemp seed soft capsule might be achieved by increasing colonic secretion, which is mediated *via* the combined action of cAMP-dependent and Ca^{2+}-dependent Cl^- channels, Na^+-K^+-$2Cl^-$ cotransporter, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger. However, the submucosal neurons seemed to not play a key role in the process.

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INTRODUCTION

Constipation is one of the most common gastrointestinal complaints in clinical practice. With the modern changes in living and working environments, constipation maintains a high incidence and greatly influences the life quality of patients. According to an epidemiological survey, the average incidence of constipation in the worldwide general population was 16%[1]. In China, the prevalence rate of constipation was 8.2% in the general population, being higher in the elderly population (18.1%) and pediatric population (18.8%)[2].

As reported, multiple factors (diet, lifestyle, certain medication, organic or functional diseases, *etc*) and various potential pathogeneses (disturbed intestinal motility, sensory and secretion, microbiota, *etc*) contribute to the occurrence and development of constipation. A variety of treatment methods have been adopted for constipation, such as lifestyle or dietary modification, secretagogues and prokinetics[3]. However, the current treatment options are limited due to various side effects, such as diarrhea, melanosis coli and so on[4,5].

In recent years, Traditional Chinese Medicine (TCM) has been reported to be effective in the treatment of constipation[6,7], and Hemp seed soft capsule (HSSC) is one of the safe and effective Chinese herbal medicines[8]. However, the treatment mechanism of HSSC is unclear, which restricts its widespread application. Intestinal secretion is known to play an important role in constipation[9]. Besides, *Semen Cannabis* as the monarch drug of HSSC was reported to possess rich fatty oil and stimulate intestinal mucosa secretion[10]. Hence, in the present study, we aimed to explore whether the regulation of intestinal secretion was a possible mechanism of HSSC in the improvement of constipation.

MATERIALS AND METHODS

Animals

Specific pathogen-free male Sprague-Dawley rats (250 g ± 10 g) were purchased from the Chinese People's Liberation Army Military Medical Academy Experimental Animal Center. Animals were housed in the Institute of Basic Theory, China Academy of Chinese Medical

Sciences. The level Ⅱ-feeding condition maintains the room temperature at 23 ℃-26 ℃, the light /dark cycle of 12h/12h and relative humidity from 45% to 60%. All the animals were given free access to food and water.

Reagents and apparatus

Preparation of the Krebs solution involved NaCl 117 mmol/L, KCl 4.7 mmol/L, $MgCl_2$ 1.2 mmol/L, $NaHCO_3$ 24.8 mmol/L, KH_2PO_4 1.2 mmol/L, $CaCl_2$ 2.56 mmol/L, and glucose 11.1 mmol/L. For preparation of the Krebs solution without Cl^-, the NaCl, KCl, $MgCl_2$ and $CaCl_2$ were replaced by sodium gluconate, potassium gluconate, magnesium gluconate and calcium gluconate, respectively. For preparation of the Krebs solution without HCO_3^-, the $NaHCO_3$ was replaced by NaCl and the solution was buffered with 10 mmol/L HEPES-free acid.

HSSC was acquired from Tianjin Central Pharmaceutical Co., LTD (Tianjin, China). N-Phenylanthranilic acid (DPC; 144509), glybenclamide (G0639), 4,4-diisothiocyano-2,2-stilbenedisulfonic acid (DIDS; D3514), bumetanide (B3023), and tetrodotoxin (TTX; T8024) were obtained from Sigma-Aldrich Co., Ltd (St. Louis, MO, United States). Atropine sulfate monohydrate (A800762) was purchased from Macklin Biochemical Co., Ltd (Shanghai, China). Hexamethonium bromide (H0481) was obtained from TCI Chemicals Co., Ltd (Tokyo, Japan).

Multichannel voltage-current clamp (VCC MC6) was purchased from Physiologic Instruments Corporation (San Diego, CA, United States). The bridge amplifier (ML228) and the recording and analysis system (Power Lab) were purchased from AD Instruments Corporation (New South Wales, Australia).

Animal groups and interventions

Twenty-four rats were randomly divided into three groups: normal group, constipation group and HSSC group. The constipation and HSSC groups were given oral administration of loperamide 3 mg/kg daily for 12 d[11,12]. The control rats were administered with the same volume of distilled water.

After the 12-d loperamide treatment, the rats in the HSSC group were given HSSC by gavage (0.126 g/kg per day). HSSC was water soluble and diluted into aqueous solution. The concentration of the stock solution was 1 g/mL. Whereas, the normal and constipation groups were treated with distilled water (2 mL/100 g per day). The interventions for all the groups were given for 7 consecutive days.

Fecal water content

After the treatment, the rats were placed in metabolic cages, individually. Fecal samples in 24 h were collected, weighed and dried. The weight of the samples before and after drying were made for measuring the fecal water content (%) using the following formula[13,14]: (wet weight - dry weight)/wet weight × 100%.

Tissue preparation

The distal colon (6-7 cm from the anus) was obtained and cut longitudinally along the mesenteric border. Then, the serosal and muscular layers were carefully separated from the mucosal and submucous layers with fine tweezers. The tissue preparations were cut into small sheets, with an area of more than 0.5 cm^2.

Isc measurement after application of HSSC

Isc was the main evaluation index for colonic mucosa secretion. Ussing Chamber was the indispensable instrument for measuring *Isc*, which was conducted after first mounting the tissue preparations in the Ussing Chamber. In the experiment, the Krebs solution was circulated with 95% oxygen and 5% carbon dioxide, the pH was maintained at 7.35-7.45 and temperature was at 37 ℃[15,16].

Two experiments were designed to detect the effect of HSSC on colonic mucosa secretion: *in vivo* drug delivery experiment and *in vitro* drug application experiment.

The *in vivo* drug delivery experiment involved intragastric administration of HSSC to constipation rats. Specifically, the tissues were left to incubate for 60 min and the voltage across the tissues was clamped to 0, then the basal *Isc* was measured (μA/cm^2). To measure the transmembrane resistance, 1 mV electrical stimulation was applied and the resistance was calculated according to Ohm's Law (R = V/I).

The *in vitro* drug application experiment involved application of HSSC on normal isolated colonic mucosa. The final concentration of HSSC (0.1 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 2.5 mg/mL, 5.0 mg /mL, 10.0 mg/mL and 25 mg/mL) was added into the serosal side for 10 min and the *Isc* curve was recorded. The *Isc* at each concentration (%) equaled (*Isc* maximum peak)/(basal *Isc*). In the control group, the same volume of normal saline was added as blank control.

Effect of HSSC on Isc after removal of Cl^- or HCO_3^- and pretreated with different inhibitors

When Cl^- or HCO_3^- was removed from the Krebs solution, or the colonic mucosa was pretreated with a nonselective Cl^- channel blocker (DPC at 1 mmol/L), a cAMP-dependent Cl^- channel blocker (glibenclamide at 1 mmol/L) or a Ca^{2+}-dependent Cl^- channel blocker (DIDS at 500 μmol/L) apically, a Na^+-K^+-$2Cl^-$ cotransporter inhibitor (bumetanide at 100 mol/L), a Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger inhibitor (DIDS at 200 μmol/L), a neural inhibitor (TTX at 1 μmol/L), a muscarinic receptor inhibitor (atropine at 1 μmol/L) or a nicotinic receptor antagonist (hexamethonium at 100 μmol/L) basolaterally, then the HSSC (1.0 mg/mL, 2.5 mg/mL, 5.0 mg/mL and 10.0 mg/mL)

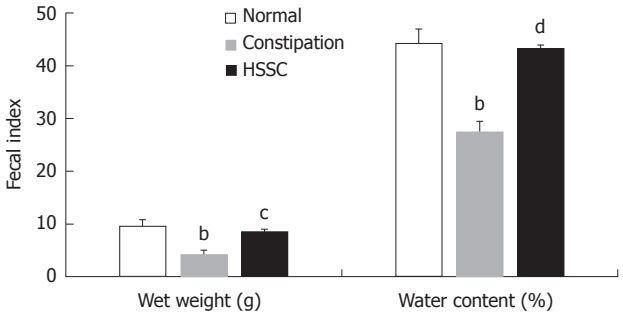

Figure 1 Effect of Hemp seed soft capsule on fecal index in constipation rats. Data are presented as mean ± SEM (n = 8). $^{a}P < 0.01$ *vs* normal group; $^{b}P < 0.01$ *vs* constipation group.

was added consecutively. The *Isc* curve was recorded and its concentration (%) was calculated using the method mentioned above. In the Cl^- or HCO_3^- removal experiment, the normal Krebs solution was used as blank control, and in the inhibitor treatment experiment, the same volume of normal saline was used to replace the inhibitors as blank control. The above experiment was repeated 6 times, each using 6 different rats.

Statistical analysis

The statistical analyses were performed by using SPSS 17.0 software (SPSS, Chicago, IL, United States); data are presented as mean ± SEM. All the original data in the study were distributed normally and conformed to homogeneity of variance. Differences among the three groups were analyzed using one-way analysis of variance (commonly known as ANOVA) followed by the least-significant difference test to compare the differences between two groups. Differences between two groups were analyzed using the *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of HSSC on fecal index in constipation rats

In the constipation group, the fecal wet weight and water content were decreased compared with those in the normal group ($P < 0.01$). After the treatment with HSSC, the fecal wet weight and the water content were increased in the HSSC group, in comparison with the constipation group ($P < 0.01$) (Figure 1).

Effects of HSSC on colonic mucosa secretion

In the constipation group, the basal *Isc* was decreased and the resistance was increased compared to the normal group ($P < 0.01$). After treatment with HSSC, the basal *Isc* was increased ($P < 0.05$) and the resistance was decreased ($P < 0.01$) in the HSSC group, in comparison with the constipation group (Figure 2A).

HSSC at cumulative concentrations from 0.1 mg/mL to 25 mg/mL (experimental group), increased *Isc* dose-dependently, which was not noted in the control group. As shown in Figure 2B, starting from the concentration of 1.0 mg/mL, the difference in *Isc* was found to be significant between the experimental group and the control group. The *Isc* in the experimental group was higher than that in the control group at the same concentration (1.0 mg/mL, $P < 0.05$; 2.5-25 mg/mL, $P < 0.01$).

Effects of Cl^- and HCO_3^- on the secretagogue role of HSSC

When Cl^- or HCO_3^- was removed from the Krebs solution (-Cl^- or -HCO_3^- group), the *Isc* induced by the effective concentration of HSSC (1.0 mg/mL, 2.5 mg/mL, 5.0 mg/mL or 10.0 mg/mL) in the -Cl^- or -HCO_3^- group was lower than that in the +Cl^- or +HCO_3^- group (1.0 mg/mL-2.5 mg/mL, $P < 0.05$; 5.0 mg/mL-10.0 mg/mL, $P < 0.01$) (Figure 3).

Effects of Cl^- channel on the secretagogue role of HSSC

The experimental mucosa was pretreated apically with the nonselective Cl^- channel blocker DPC (+DPC group), cAMP-dependent Cl^- channel blockers glibenclamide (+glibenclamide group) or Ca^{2+}-dependent Cl^- channel blocker DIDS (+apical DIDS group) for 30 min. The control mucosa was pretreated with the same volume of normal saline (-DPC group, -glibenclamide group, -apical DIDS group, respectively). After that, the effective concentration of HSSC (1.0 mg/mL, 2.5 mg/mL, 5.0 mg/mL or 10.0 mg/mL) was added to solicit the *Isc* response. As shown in Figure 4, at the concentrations of 1.0 mg/mL, 2.5 mg/mL, 5.0 mg/mL and 10.0 mg/mL, the *Isc* in the +DPC group was lower than that in the -DPC group (1.0 mg/mL-2.5 mg/mL, $P < 0.05$; 5.0 mg/mL-10.0 mg/mL, $P < 0.01$). At the concentration of 2.5 mg/mL, 5.0 mg/mL and 10.0 mg/mL, the *Isc* in the +glibenclamide group was lower than that in the -glibenclamide group (2.5 mg/mL, $P < 0.05$; 5.0 mg/mL-10.0 mg/mL, $P < 0.01$). At the concentration of 5.0 mg/mL and 10.0 mg/mL, the *Isc* in the +apical DIDS group was lower than that in the -apical DIDS group (5.0 mg/mL, $P < 0.05$; 10.0 mg/mL, $P < 0.01$).

Effects of Na^+-K^+-2Cl^- cotransporter on the secretagogue role of HSSC

The experimental mucosa was pretreated with the Na^+-K^+-2Cl^- cotransporter inhibitor bumetanide (+bumetanide group); the control mucosa was pretreated with the same volume of normal saline (-bumetanide group). After that, the effective concentration of HSSC (1.0 mg/mL, 2.5 mg/mL, 5.0 mg/mL or 10.0 mg/mL) was added to solicit the *Isc* response. As shown in Figure 5, at the concentrations of 2.5 mg/mL, 5.0 mg/mL and

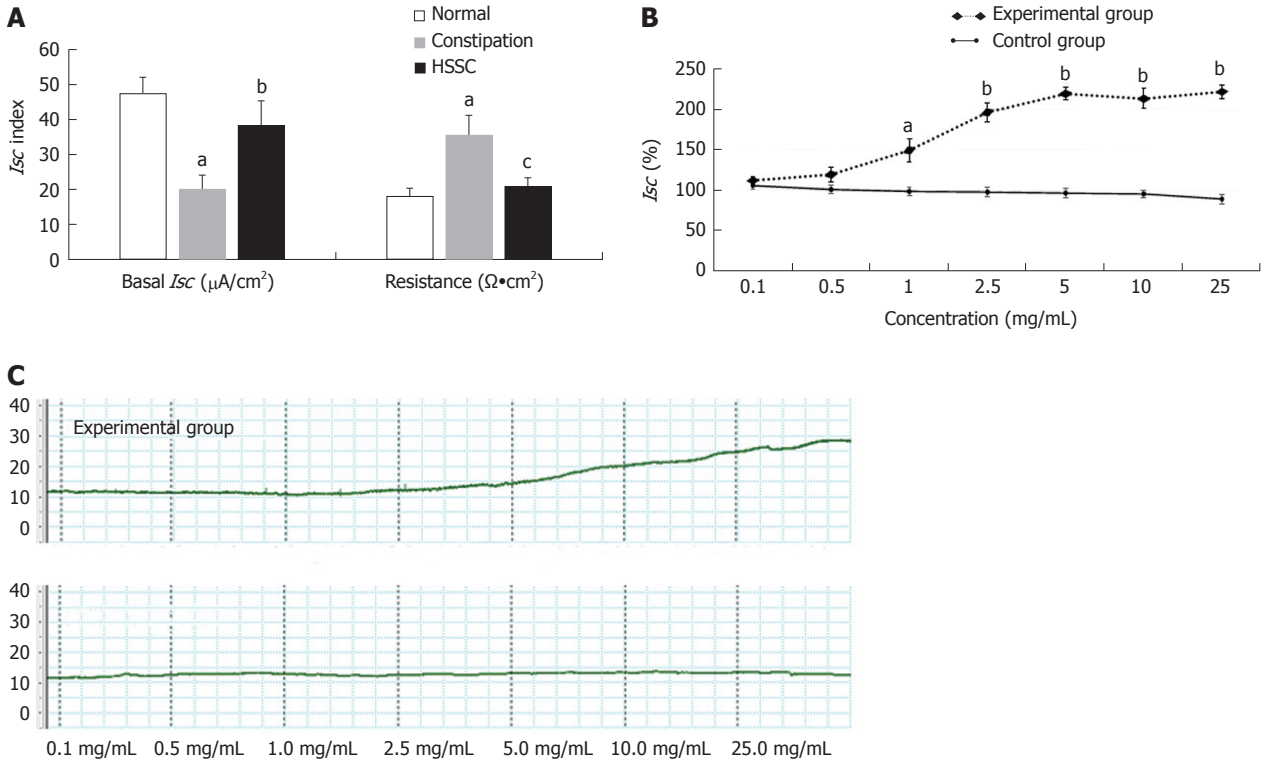

Figure 2 Effect of Hemp seed soft capsule on colonic mucosa secretion. A: In constipation rats (mean ± SEM, n = 8). $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* constipation group; B: In normal isolated colonic mucosa (mean ± SEM, n = 6); C: Representative *Isc* trace in experimental group and control group. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

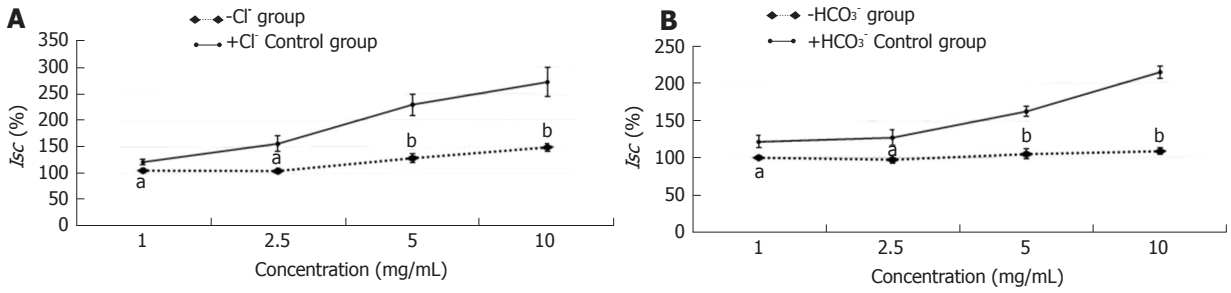

Figure 3 Effect of Cl^- and HCO_3^- on the secretagogue role of Hemp seed soft capsule. Data are presented as mean ± SEM (n = 6). A: When Cl^- was removed from the Krebs solution. The effect of Hemp seed soft capsule on colonic mucosa secretion was determined; B: When HCO_3^- was removed from the Krebs solution. The effect of Hemp seed soft capsule on colonic mucosa secretion was determined. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* +Cl^- group or +HCO_3^- group.

10.0 mg/mL, the *Isc* in the +bumetanide group was lower than that in the -bumetanide group (2.5 mg/mL, $P < 0.05$; 5.0 mg/mL-10.0 mg/mL, $P < 0.01$).

Effects of Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger on the secretagogue role of HSSC

The experimental mucosa was pretreated with the Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger inhibitor (+basolateral DIDS group); the control mucosa was pretreated with the same volume of normal saline (-basolateral DIDS group). After that, the effective concentration of HSSC (1.0 mg/mL, 2.5 mg/mL, 5.0 mg/mL or 10.0 mg/mL) was added to solicit the *Isc* response. As shown in Figure 6, at the concentrations of 5.0 mg/mL and 10.0 mg/mL, the *Isc* in +bumetanide group was lower than that in the -bumetanide group (5.0 mg/mL, $P < 0.05$; 10.0 mg/mL, $P < 0.01$).

Effects of neural pathway on the secretagogue role of HSSC

The experimental mucosa was pretreated with the neural inhibitor TTX (+TTX group), muscarinic receptor inhibitor atropine (+atropine group) or nicotinic receptor antagonist hexamethonium (+hexamethonium group); the control mucosa was pretreated with the same volume of normal saline (-TTX group, -atropine group, -hexamethonium group, respectively). After that, the effective concentration of HSSC (1.0 mg/mL, 2.5 mg/mL, 5.0 mg/mL or 10.0 mg/mL) was added to

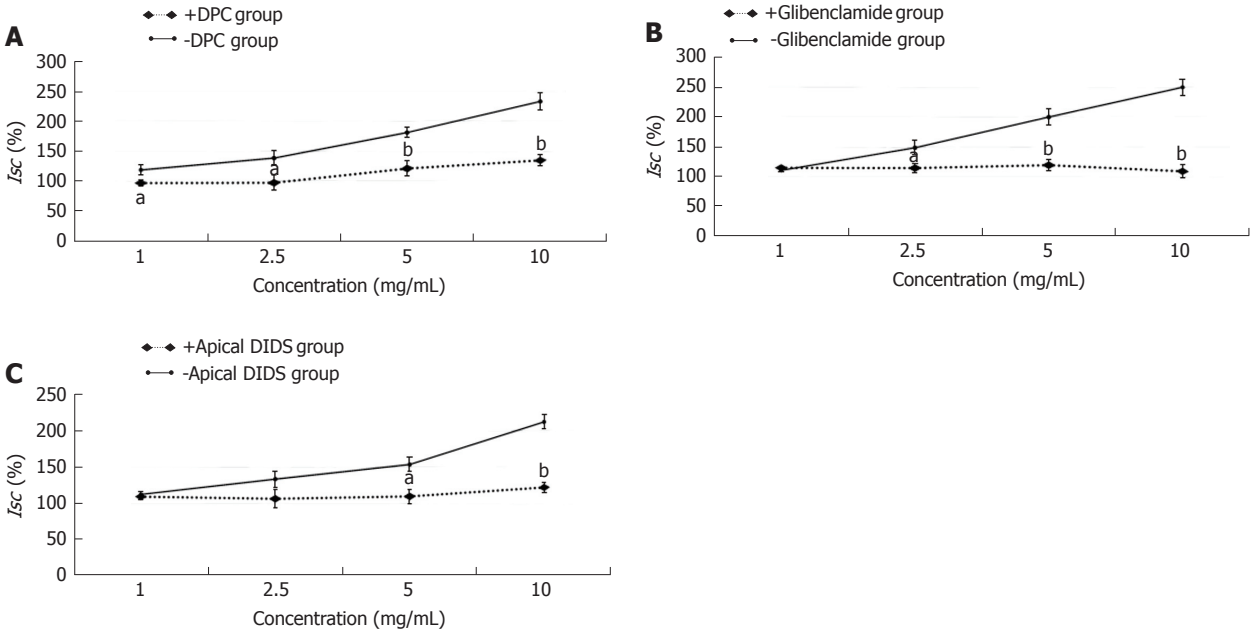

Figure 4 Effect of Cl^- channel on the secretagogue role of Hemp seed soft capsule. Data are presented as mean ± SEM (n = 6). A: Pretreated with nonselective Cl^- channel blocker DPC; B: Pretreated with cAMP-dependent Cl^- channel blocker glibenclamide; C: Pretreated with Ca^{2+}-dependent Cl^- channel blocker DIDS. $^aP < 0.05$, $^bP < 0.01$ *vs* -DPC group, -glibenclamide group or -apical DIDS group.

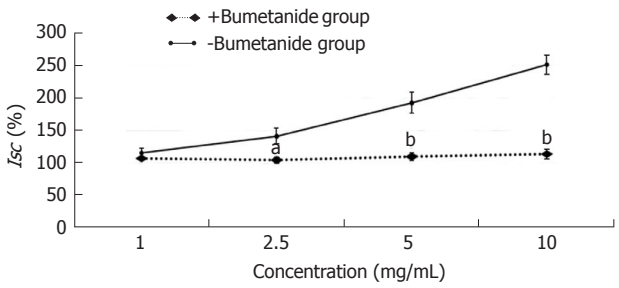

Figure 5 Effect of Na^+-K^+-$2Cl^-$ cotransporter on the secretagogue role of Hemp seed soft capsule. Data are presented as mean ± SEM (n = 6). $^aP < 0.05$, $^bP < 0.01$ *vs* -bumetanide group.

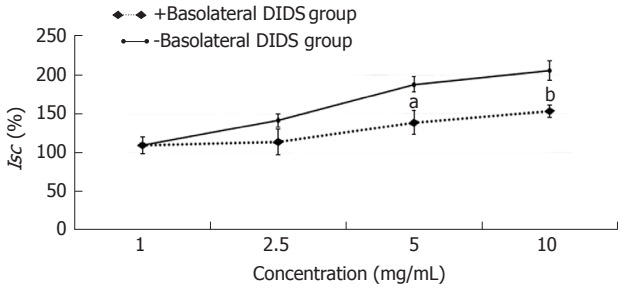

Figure 6 Effect of Na^+-$HCO3^-$ cotransporter or Cl^-/$HCO3^-$ exchanger on the secretagogue role of Hemp seed soft capsule. Data are presented as mean ± SEM (n = 6). $^aP < 0.05$, $^bP < 0.01$ *vs* -basolateral DIDS group.

solicit the *Isc* response. As shown in Figure 7, at each concentration, there was no difference between the experimental group (+TTX group, +atropine group, +hexamethonium group) and the control group (-TTX group, -atropine group, -hexamethonium group) ($P > 0.05$).

DISCUSSION

In our study, HSSC was found to increase the fecal wet weight and water content in constipation rats; meanwhile, colonic secretion was increased, possibly attributable to the combined action of cAMP-dependent and Ca^{2+}-dependent Cl^- channels, NKCC, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger. However, the submucosal neurons, especially the cholinergic neurons, might play little or no role in the secretagogue role of HSSC.

HSSC is developed from the ancient traditional prescription "Hemp seed pills", which consists of *Semen Cannabis*, *Magnolia officinalis*, *Fructus Aurantii* Immaturus, *Radix Paeoniae Alba*, Almond and *Rheum rhabarbarum*. The Hemp seed pill is a representative prescription of TCM in the treatment of constipation, in which *Semen Cannabis* as the monarch drug plays the role of Runchang Tongbian[17]. A series of clinical trials have confirmed the efficiency and safety of HSSC in the treatment of constipation[18,19]. In our study, we established a rodent model of constipation in which the fecal wet weight and water content were decreased and found that HSSC increased the fecal wet weight and water content; these findings confirmed that the HSSC could improve the stool properties in constipation rats.

As research progresses, the pharmacological properties of HSSC have been gradually revealed and the therapeutic mechanisms of HSSC for constipation

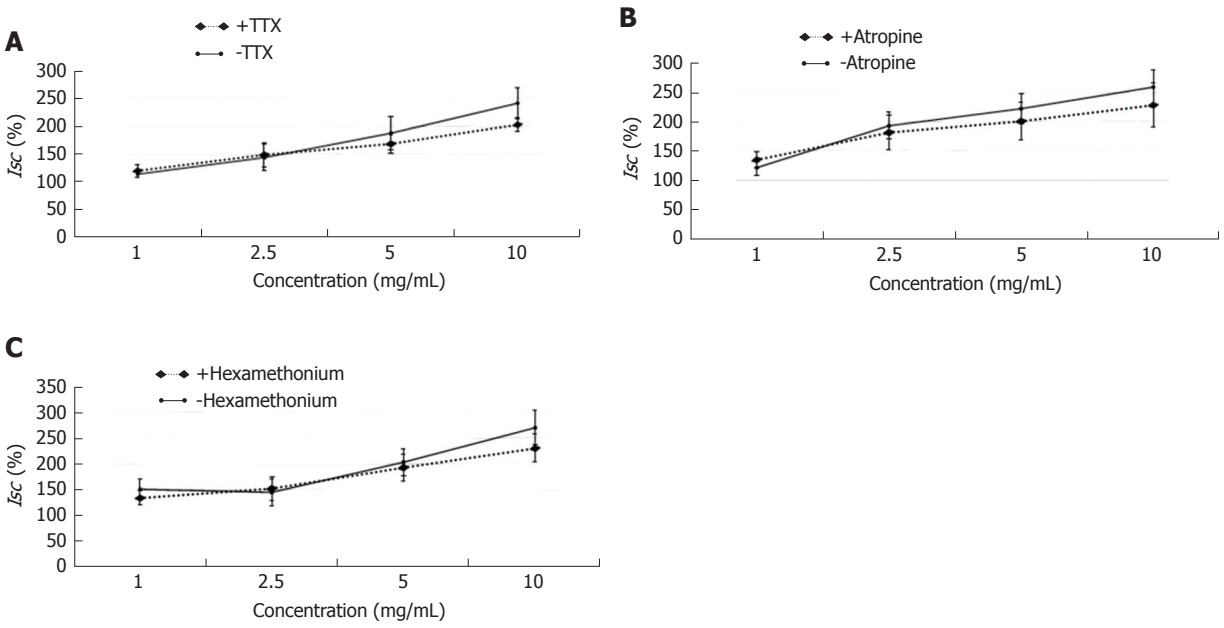

Figure 7 Effects of neural pathway on the secretagogue role of Hemp seed soft capsule. Data are presented as mean ± SEM (n = 6). A: Pretreated with neural inhibitor tetrodotoxin; B: Pretreated with muscarinic receptor inhibitor atropine; C: Pretreated with nicotinic receptor antagonist hexamethonium.

have been partly clarified. Animal experimental studies have confirmed that HSSC could obviously increase the intestinal motility, fecal pellets and weight in constipation rats[20,21]. Further, in rabbits, the intestinal contractile frequency and amplitude were found to be increased after intragastric administration of Hemp seed pills. These previous findings suggested that HSSC might play a laxative effect by regulating the intestinal motility and gastrointestinal hormonal secretions, findings which were consistent with the pathophysiologies of constipation.

Constipation is a common clinical complaint caused by multiple physiological changes. In addition to the above-mentioned intestinal motility and gastrointestinal hormones, the imbalance of intestinal water and electrolytes has also been recognized as one of the most important pathophysiologies[22]. However, it hasn't been reported whether HSSC could regulate the intestinal epithelium electrolyte secretion. In our study, HSSC was found to cause an upward *Isc* response and to have a role of secretagogue.

It is well known that under normal conditions, the Cl^- and HCO_3^- transport in the colon can generate an osmotic driving force for water. The components of Cl^- secretion have been elaborated by Frizzell *et al*[23]: the apical cAMP-dependent and Ca^{2+}-dependent Cl^- channels (CaCC), basolateral NKCC are the main participants for the Cl^- secretion; the HCO_3^- transport is regulated by the Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger. In our experiment, it was found that when the Cl^- and HCO_3^- was removed from the normal Krebs solution, the *Isc* response induced by HSSC was reduced, suggesting that Cl^- and HCO_3^- participated in the ionic regulation of HSSC. Further, the pretreatment of colonic epithelium with the inhibitors of Cl^- and HCO_3^- channels inhibited the *Isc* response induced by HSSC, suggesting that HSSC regulated the anionic transport through the cAMP-dependent and Ca^{2+}-dependent Cl^- channels, NKCC, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger.

As reported, anion secretion can be regulated by neural and non-neural pathways[24]. In the neural pathway, acetylcholine is one of the most important neurotransmitters that mediates intestinal secretion[25]. To determine the role of submucosal neurons, especially the cholinergic neurons, on secretagogue effect of HSSC, the neural inhibitor TTX, muscarinic receptor inhibitor atropine or nicotinic receptor antagonist hexamethonium was used to pretreat the mucosa/submucosa preparation; we found that there was no difference between experimental mucosa and control mucosa, which implied that the secretagogue effect of HSSC seemed not to be dependent on the submucosal nervous system.

In conclusion, the effect of HSSC on constipation was achieved by regulating the colonic secretion, which was mediated *via* cAMP-dependent and Ca^{2+}-dependent Cl^- channels, NKCC, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger.

ARTICLE HIGHLIGHTS

Research background

Constipation is one of the most common gastrointestinal disorders, which maintains a high incidence rate and greatly influences the life quality of patients. As reported, multiple factors and potential pathogenesis contribute to the occurrence and development of constipation. And, a variety of treatment methods have been adopted for constipation, including Traditional Chinese Medicine, in which Hemp seed soft capsule is one of the safe and effective

Chinese patent medicines. However, the treatment mechanisms of Hemp seed soft capsule have been unclear, which restricts its popularization and application. Hence, the exploration of possible mechanisms about Hemp seed soft capsule for treating constipation is necessary.

Research motivation

This study aimed to investigate the effect of Hemp seed soft capsule on colonic ion transport and its related mechanisms in constipation rats, the findings of which will have an important significance for clarifying the pharmacological effect of Traditional Chinese Medicine.

Research objectives

The main objective of the study was to explore whether the regulation of the intestinal secretion was the possible mechanism of Hemp seed soft capsule for treating constipation. This research provided a new thought for us to study Hemp seed soft capsule, and more molecules related to the colonic secretion could be discussed in the future.

Research methods

The constipation rats were induced by oral administration of loperamide (3 mg/kg per day for 12 d). The Hemp seed soft capsule group was given Hemp seed soft capsule by gavage (0.126 g/kg per day for 7 d). The normal and constipation groups were treated with the same volume of distilled water. After treatment, the fecal wet weight and water content were measured. The basal short-circuit current (*Isc*) and resistance were acquired by an Ussing Chamber. Further, after the ion substitution or inhibitor application, the *Isc* induced by Hemp seed soft capsule was also measured.

The statistical analyses were performed by using SPSS 17.0 software and the differences among the three groups were analyzed using one-way analysis of variance followed by least-significant difference test to compare the differences between two groups. The differences among the two groups were analyzed using *t*-test. $P < 0.05$ was considered statistically significant.

Research results

In this study, it was found that Hemp seed soft capsule could increase the fecal wet weight and water content in constipation rats. Meanwhile, Hemp seed soft capsule could increase colonic secretion, and after the application of cAMP-dependent or Ca^{2+}- dependent Cl^- channels inhibitor, NKCC inhibitor, Na^+-HCO_3^- cotransporter inhibitor or Cl^-/HCO_3^- exchanger inhibitor, the colonic secretion induced by HSSC were decreased in experimental group than that in the control group, which demonstrated that cAMP- dependent and Ca^{2+}- dependent Cl^- channels, Na^+-K^+-$2Cl^-$ cotransporter (NKCC), Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger may participate in the HSSC treatment. Meanwhile, after pretreatment with a neural pathway inhibitor (tetrodotoxin, atropine or hexamethonium), there were no differences between experimental mucosa and control mucosa, which implied that the secretagogue effect of HSSC was not dependent on the submucosal nervous system.

Though some results were obtained, the effect of Hemp seed soft capsule on real-time ion current and expression of ion channel protein should be explored in further studies, which will also be important for exploring the specific targets.

Research conclusions

The secretagogue effect of Hemp seed soft capsule for constipation may be achieved *via* the combined action of cAMP-dependent and Ca^{2+}-dependent Cl^- channels, NKCC, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger. However, the submucosal neurons seem to not play a key role in the process. Decreased colonic secretion is found in constipation rats and Hemp seed soft capsule can reverse it. Besides, Cl^- and HCO_3^- participate in the regulative process. The effect of Hemp seed soft capsule for constipation can be achieved by increasing colonic secretion, which is related with the coaction of cAMP-dependent and Ca^{2+}-dependent Cl^- channels, NKCC, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger. The discoveries in this study imply that Cl^-, HCO_3^- and related channels/cotransporters/exchangers participate in the effect of HSSC on constipation.

The new hypotheses proposed in this study is that regulation of the intestinal secretion was the possible mechanism of Hemp seed soft capsule for treating constipation. Ussing Chamber is the acknowledged method, although it is new, to be used in the discovery of the Hemp seed soft capsule therapeutic mechanism. Hemp seed soft capsule can increase the fecal wet weight and water content in constipation rats, while the colonic secretion is increased. Besides, cAMP-dependent and Ca^{2+}-dependent Cl^- channels, NKCC, Na^+-HCO_3^- cotransporter and Cl^-/HCO_3^- exchanger are involved in the effect of Hemp seed soft capsule on colonic secretion. The effect of Hemp seed soft capsule on constipation can be achieved by regulating the colonic secretion, which is related with cAMP-dependent and Ca^{2+}-dependent Cl^- channels, NKCC, Na^+-HCO_3^- cotransporter or Cl^-/HCO_3^- exchanger. Hemp seed soft capsule may increase the fecal water content in constipation patients and can be used as an effective laxative in clinical practice.

Research perspectives

In the study, we found Hemp seed soft capsule could relieve the symptom of constipation and increase the colonic secretion, which implied that the effect of Hemp seed soft capsule on constipation may be achieved by regulating colonic secretion. Further, it was found that Cl^- and HCO_3^- participated in the process, mediated *via* the related channels/cotransporters/exchangers. But, several questions remain unanswered, all of which can be discussed in the future (*e.g.*, what specific pathways, receptors or molecules may be involved in the effect of Hemp seed soft capsule on colonic secretion, such as the 5-HT/5-HTR pathway or the dopamine pathway, or what structural and functional changes can be found in the ion transport protein on the effect of Hemp seed soft capsule, as the expression of cystic fibrosis transmembrane conductance regulator (CFTR, a cAMP- dependent Cl^- channel) protein).

In further studies, we will focus on the effect of Hemp seed soft capsule on Cl^- current and CFTR protein in constipation rats. The methods of patch-clamp and short-circuit current will be used to measure the Cl^- current. Immunofluorescence will be used to investigate the location and expression of the CFTR protein. Finally, western blot and PCR will be conducted to evaluate the expression of CFTR protein and phosphorylated CFTR protein.

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ORIGINAL ARTICLE

Basic Study

Novel D-galactosamine-induced cynomolgus monkey model of acute liver failure

Lei Feng, Lei Cai, Guo-Lin He, Jun Weng, Yang Li, Ming-Xin Pan, Ze-Sheng Jiang, Qing Peng, Yi Gao

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Abstract

AIM

To establish a simplified, reproducible D-galactosamine-induced cynomolgus monkey model of acute liver failure having an appropriate treatment window.

METHODS

Sixteen cynomolgus monkeys were randomly divided

into four groups (A, B, C and D) after intracranial pressure (ICP) sensor implantation. D-galactosamine at 0.3, 0.25, 0.20 + 0.05 (24 h interval), and 0.20 g/kg body weight, respectively, was injected *via* the small saphenous vein. Vital signs, ICP, biochemical indices, and inflammatory factors were recorded at 0, 12, 24, 36, 48, 72, 96, and 120 h after D-galactosamine administration. Progression of clinical manifestations, survival times, and results of H&E staining, TUNEL, and Masson staining were recorded.

RESULTS

Cynomolgus monkeys developed different degrees of debilitation, loss of appetite, and jaundice after D-galactosamine administration. Survival times of groups A, B, and C were 56 ± 8.7 h, 95 ± 5.5 h, and 99 ± 2.2 h, respectively, and in group D all monkeys survived the 144-h observation period except for one, which died at 136 h. Blood levels of ALT, AST, CK, LDH, TBiL, Cr, BUN, and ammonia, prothrombin time, ICP, endotoxin, and inflammatory markers [(tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6)] significantly increased compared with baseline values in different groups ($P < 0.05$). Pathological results showed obvious liver cell necrosis that was positively correlated with the dose of D-galactosamine.

CONCLUSION

We successfully established a simplified, reproducible D-galactosamine-induced cynomolgus monkey model of acute liver failure, and the single or divided dosage of 0.25 g/kg is optimal for creating this model.

Key words: Cynomolgus monkey; D-galactosamine; Acute liver failure; Artificial liver support systems; Intracranial pressure

Core tip: This is an article about a novel D-galactosamine-induced cynomolgus monkey model of acute liver failure (ALF). In this study, we used small saphenous vein puncture instead of jugular vein intubation for different doses of D-gal administration, which not only effectively avoided the trauma caused by intubation, but also significantly reduced the anesthesia time and greatly improved the convenience of operation. This study concluded that a simplified, reproducible D-gal-induced large-animal ALF model with an appropriate treatment window had been established successfully, which is suitable for assessing the safety and efficacy of artificial liver support systems, studying the pathogenesis of ALF, and developing new drugs.

Feng L, Cai L, He GL, Weng J, Li Y, Pan MX, Jiang ZS, Peng Q, Gao Y. Novel D-galactosamine-induced cynomolgus monkey model of acute liver failure. *World J Gastroenterol* 2017; 23(42): 7572-7583 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7572.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7572

INTRODUCTION

Acute liver failure (ALF) results from various causes and is a serious threat to human health[1-3]. Therefore, the establishment of an ALF animal model is of great significance for studying its pathogenesis, developing new drugs, and determining the comprehensive treatment of ALF[4]. In recent years, artificial liver technology has become a topic of great interest to researchers in the ALF field[5-8]. Artificial liver support systems (ALSS) can significantly improve the clinical manifestations and prolong the survival time of patients with liver failure or those awaiting liver transplantation[9]. The safety and efficacy of ALSS must be verified before clinical application because they contain biological substances, such as liver cells; at this point, an ideal animal model of ALF would be an indispensable verification platform[10,11]. Therefore, it is necessary to establish a simplified and reproducible animal model of ALF with an appropriate treatment window.

There are current literature reports of many drugs that have been used to induce animal models of ALF[5,12-14]. D-galactosamine (D-gal) is a disruptor of uridine triphosphate of hepatocytes, causing diffuse hepatic necrosis and an inflammatory response, similar to the pathological changes of clinical viral hepatitis[15,16]. Compared with other drugs, D-gal has many advantages, including better reproducibility and easier dosage control; it is generally accepted as the ideal drug to induce ALF.

At present, large animals used to establish liver failure models are mainly pigs and dogs[15,17,18], but their physiological and biochemical characteristics are dissimilar to those of humans, and results are relatively poor for guiding clinical treatment. As for the methods of drug administration, the main method used is intubation through the jugular vein[8], which is complex and increases the trauma to experimental animals.

In this study, we used different doses of D-gal administered through the small saphenous vein of cynomolgus monkeys, and then observed the clinical manifestations, survival times, changes in biochemical indices, intracranial pressure (ICP) changes, and resulting pathological and histological characteristics, in order to establish a simplified, reproducible D-gal-induced large-animal ALF model with an appropriate treatment window, suitable for assessing the safety and efficacy of ALSS, studying the pathogenesis of ALF, and developing new drugs.

MATERIALS AND METHODS

Animals

Sixteen 6-9-year-old male cynomolgus monkeys, weighing 9.4-11 kg, were purchased from Guangdong Landao Biological Technology Co. Ltd. (Guangzhou, Guangdong Province, China; Certificate of Conformity SCXK [Guangdong] 2014-0010) (Table 1). The

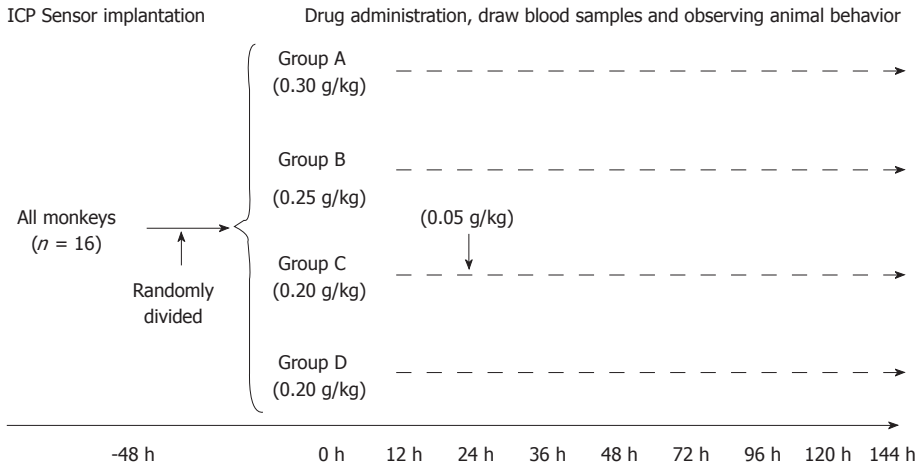


Figure 1 Study design. All monkeys were randomly divided into four groups after ICP sensor implantation; the interval of D-gal administration to group C was 24 h. ICP: Intracranial pressure.

experimental protocol was reviewed and approved by the Institutional Review Board of Zhujiang Hospital, Southern Medical University, China (No. ZJYY-2014-GDEK-003).

Experimental drugs and preparation

D-gal, purchased from Sigma-Aldrich (United States), was dissolved in 5% glucose solution to a concentration of 1.0 g/10 mL, with pH adjusted to 6.8 using 1.0 mol/L NaOH solution. Then, the solution was sterilized by filtration through a membrane with a pore diameter of 0.22 μm and administrated within 2 h after preparation.

Experimental groups

The study design is presented in Figure 1. The 16 monkeys were randomly divided into four groups after an ICP sensor was implanted and then were given different doses of D-gal according to the results of our previous study[19]. The study groups and dosages given were as follows: group A (n = 4), 0.30 g/kg D-gal; group B (n = 4), 0.25 g/kg D-gal; group C (n = 4), 0.20 g/kg D-gal plus 0.05 g/kg D-gal after 24 h; group D (n = 4), 0.20 g/kg D-gal.

Anesthesia and preparation

Basic anesthesia was induced by intramuscular injection of Zoletil (Virbac Laboratory, Carros, France) (15 mg/kg) and atropine (0.5 mg/kg). The experimental monkey was placed on an operating table with a hot blanket after basic anesthesia. After peroral endotracheal intubation, spontaneous breathing was maintained by continuous inhalation of isoflurane (1%-2%) and O_2 (2 L/min) during implantation of the ICP sensor. Animals were placed on the operating table in the prone position; the limbs and head were fixed in place after anesthesia. Skin preparation of the head (for ICP sensor implantation), arms (for collecting blood samples), and hind legs (for drug administration) was performed by using an electric shaver and cleansing with soap and water.

ICP sensor implantation

The detail surgical procedure to implant an ICP sensor in cynomolgus monkeys is shown in Supplementary Meterial 1 and Supplementary Figure 1 .

Establishing the ALF model

Study monkeys were fasted (free access to water) for 12 h before drug administration. Anesthesia was induced by intramuscular injection of Zoletil (15 mg/kg) and atropine (0.5 mg/kg). Blood samples were collected from the forearm and vital signs and ICP were measured as baseline values (0 h). Finally, the prepared D-gal solution was drawn into a 50 mL syringe connected to a disposable needle, air was expelled from the syringe, and then the D-gal solution was administered slowly through the small saphenous vein (Supplementary Figure 2). After D-gal administration, animals were given regular feed and free access to water and fresh fruit.

Parameters

The general condition of study animals was monitored during the experiment and the subsequent observation period, as follows: the ability to stand, to walk, and to eat; the response to sight, sound, and stimulation; and presence of cramps or convulsions. When the animal was conscious, these were recorded every 12 h, whereas the animals were observed every 2 h after unconsciousness occurred. The recorded survival time was defined as the time interval from completion of injection of D-gal to death, and surviving animals were observed for 144 h in total.

ICP, ammonia level, and levels of inflammatory markers [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6] and endotoxin were recorded at 0, 12, 24, 36, 48, 72, 96, and 120 h after D-gal administration. An ammonia determination kit (end-point method) purchased from Sysmex Corporation (Japan) was used to measure whole blood ammonia levels. TNF-α, IL-1β, and IL-6 levels were determined

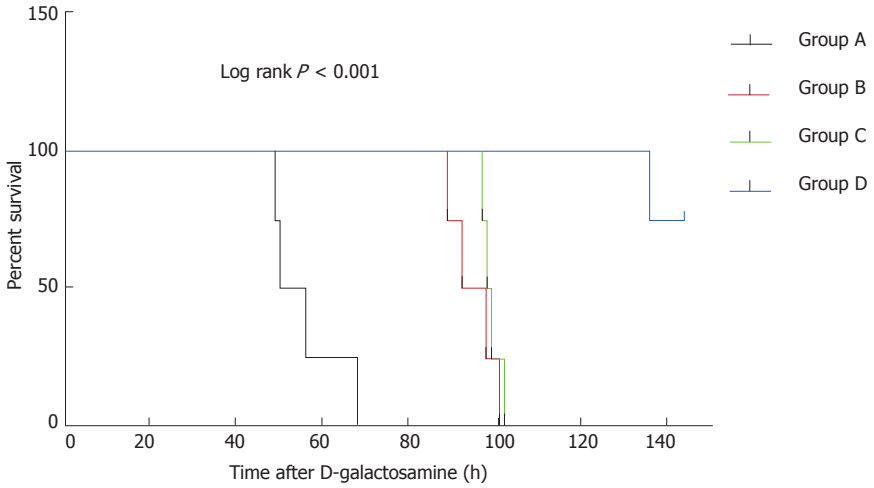


Figure 2 Survival times of monkeys in different study groups. Group A *vs* group B: $P = 0.007$; group A *vs* group C: $P = 0.007$; Group A *vs* group D: $P < 0.001$; Group B *vs* group C: $P = 0.375$.

Table 1 The general condition of cynomolgus monkeys before drug administration

No.	Age (yr)	Weight (kg)	Sexual (F/M)	Dose (g/kg)	BP (mmHg)	T (℃)	Amm (μmol/L)	PT (s)
1	6	9.5	M	0.20	110/68	37.3	37	10.3
2	9	10.2	M	0.25	101/76	37.0	43	10.7
3	7.5	9.4	M	0.20	108/79	36.3	41	11.3
4	8	9.8	M	0.30	100/58	36.4	37	10.5
5	6.5	9.4	M	0.30	121/54	36.5	41	10.3
6	8.5	11	M	0.250	104/70	37.9	41	11.7
7	6.5	9.7	M	0.20 + 0.05	123/67	36.6	49	10.2
8	7	9.6	M	0.25	123/74	36.9	46	10.6
9	8.5	10.4	M	0.30	103/56	36.6	45	9.5
10	9	10.6	M	0.20	102/79	37.4	39	9.6
11	8.5	10.3	M	0.25	116/64	36.6	34	10
12	9	10.7	M	0.20 + 0.05	113/77	36.7	29	9.9
13	7.8	10.3	M	0.30	105/73	37.4	58	10.2
14	6.5	11	M	0.20 + 0.05	118/67	36.6	32	9.8
15	7.5	9.5	M	0.20	112/69	37.3	45	9.8
16	8.5	10.1	M	0.20 + 0.05	113/63	36.8	31	9.9

F: Female; M: Male; BP: Blood pressure; T: Body temperature; Amm: Ammonia; PT: Prothrombin time.

with ELISA kits purchased from Sigma-Aldrich. Endotoxin levels were determined with a Tachypleus Amebocyte Lysate kit purchased from Sigma-Aldrich.

Vital signs were monitored and blood samples to measure liver function indices (AST, ALT, ALB, TBiL, CK, and LDH), renal function indices (BUN and Cr), blood glucose, prothrombin time (PT), and routine blood chemistry tests were collected at 0, 12, 24, 36, 48, 72, 96, and 120 h after drug administration. All tests of blood samples were conducted in the clinical laboratory of Zhujiang Hospital, Southern Medical University, China.

Histopathological examination

From each of the four study groups, one monkey was randomly selected for liver biopsy, which was conducted under ultrasound guidance before D-gal administration (Supplementary Figure 3). Histopathological examination was then performed.

Animals surviving at 144 h were sacrificed with a lethal intravenous injection of pentobarbital and KCl, and a detailed autopsy was performed immediately after animal death. Each animal's liver, heart, kidneys, spleen, lungs, large intestine, small intestine, brain, and pancreas were collected, and all tissue specimens were fixed in 10% formalin solution and cut into 5 mm^3 blocks which were paraffin-embedded and thin-sectioned. Subsequently, slides underwent stepwise alcohol dehydration before hematoxylin-eosin (H&E) staining for observation under a light microscope. In addition, liver specimens were collected from all four groups and TUNEL assays were performed to assess cell apoptosis and necrosis. Finally, liver specimens from all four groups also underwent Masson staining to assess the extent of ALF fibrosis.

Animal care and use statement

The monkeys were cared for in strict accordance with

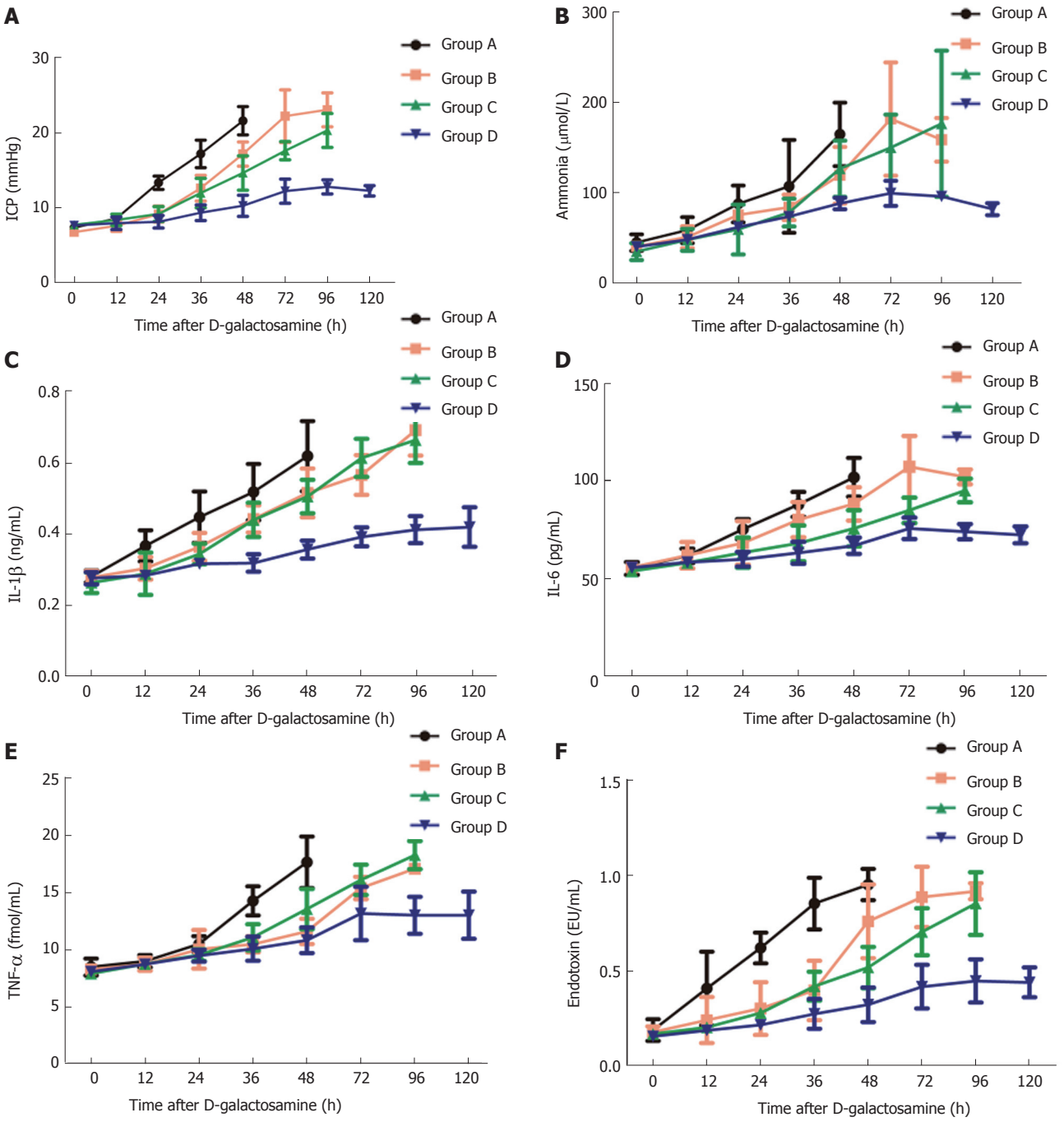


Figure 3 Changes of intracranial pressure, ammonia, inflammation markers and endotoxin at different time points in each group. All data points are mean ± SD, $n = 4$. ICP: Intracranial pressure; Amm: Ammonia; IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α.

the institution's guidelines for experimental animals. Each animal was kept individually in a special iron cage under standard conditions and fed three times a day with free access to water. Animals surviving at 144 h were sacrificed with a lethal intravenous injection of pentobarbital and KCl for tissue collection.

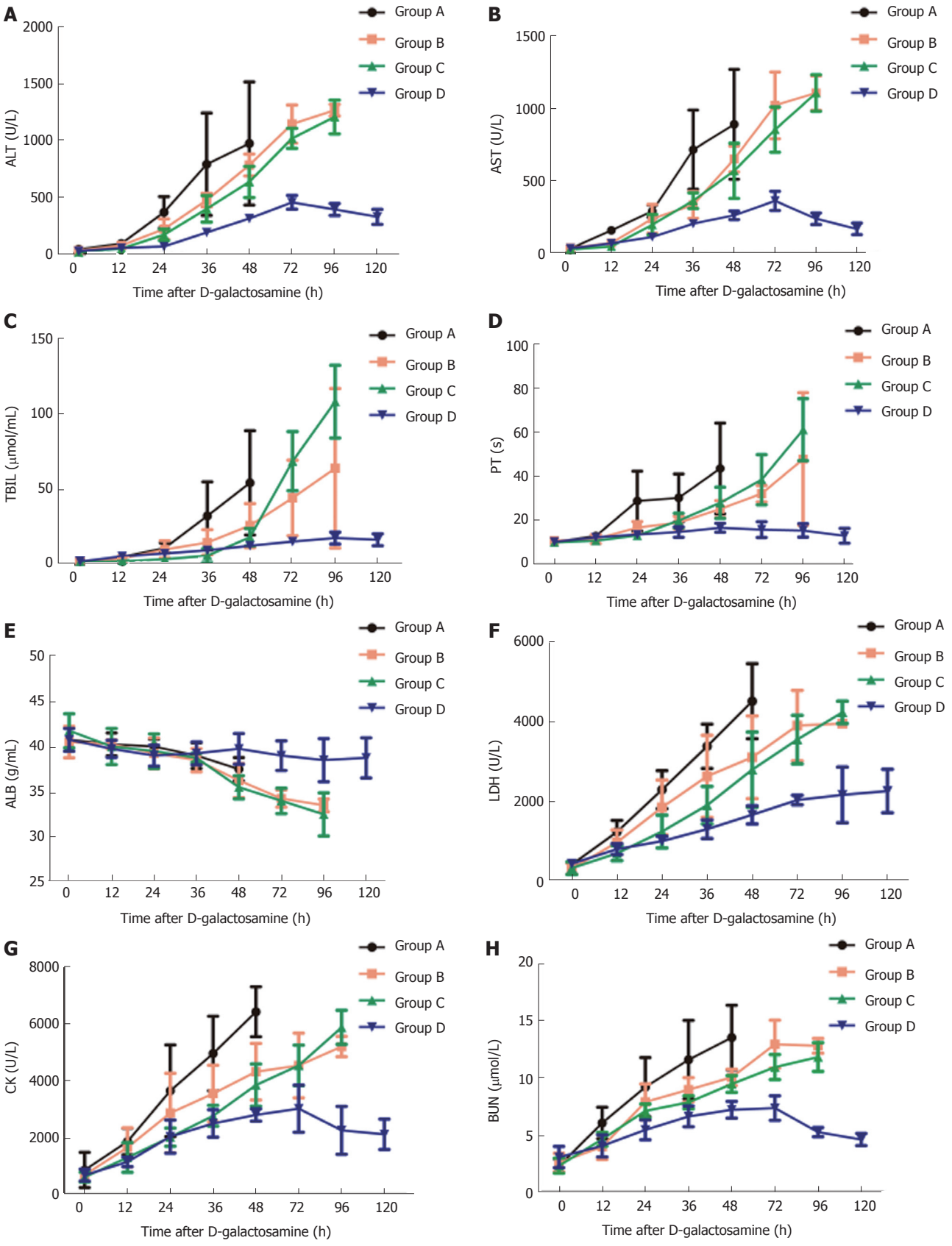
Statistical analysis

Data are expressed as mean ± SD and were analyzed using the SPSS 21.0 statistical package. Differences between baseline values and values at different study time points were analyzed using Student's *t*-test and ANOVA for multiple comparisons. Animal survival was analyzed using the Kaplan-Meier log rank method. *P*-values < 0.05 were considered significant.

RESULTS

General condition

The general condition of the experimental monkeys before D-gal administration is shown in Table 1. After D-gal injection, the experimental monkeys in group A began to eat less at 12 h, responded slowly to the sound stimulus, and two were apparently vomiting at


A
ALT (U/L)
2000
1500
1000
500
0
0 12 24 36 48 72 96 120
Time after D-galactosamine (h)
Group A
Group B
Group C
Group D
B
AST (U/L)
1500
1000
500
0
0 12 24 36 48 72 96 120
Time after D-galactosamine (h)
Group A
Group B
Group C
Group D
C
TBIL (μmol/mL)
150
100
50
0
0 12 24 36 48 72 96 120
Time after D-galactosamine (h)
Group A
Group B
Group C
Group D
D
PT (s)
100
80
60
40
20
0
0 12 24 36 48 72 96 120
Time after D-galactosamine (h)
Group A
Group B
Group C
Group D
E
ALB (g/mL)
50
45
40
35
30
25
0 12 24 36 48 72 96 120
Time after D-galactosamine (h)
Group A
Group B
Group C
Group D
F
LDH (U/L)
6000
4000
2000
0
0 12 24 36 48 72 96 120
Time after D-galactosamine (h)
Group A
Group B
Group C
Group D
G
CK (U/L)
8000
6000
4000
2000
0
0 12 24 36 48 72 96 120
Time after D-galactosamine (h)
Group A
Group B
Group C
Group D
H
BUN (μmol/L)
20
15
10
5
0
0 12 24 36 48 72 96 120
Time after D-galactosamine (h)
Group A
Group B
Group C
Group D

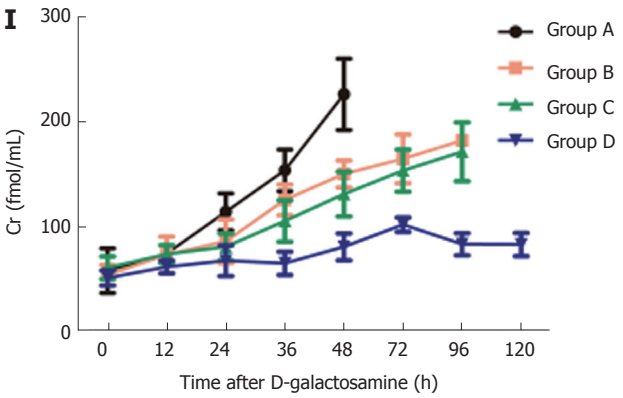


Figure 4 Changes of biochemical indices at different time points in each group. All data points are mean ± SD, n = 4. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; PT: Prothrombin time; ALB: Albumin; LDH: Lactic dehydrogenase; CK: Creatine kinase; BUN: Blood urea nitrogen; Cr: Creaninine.

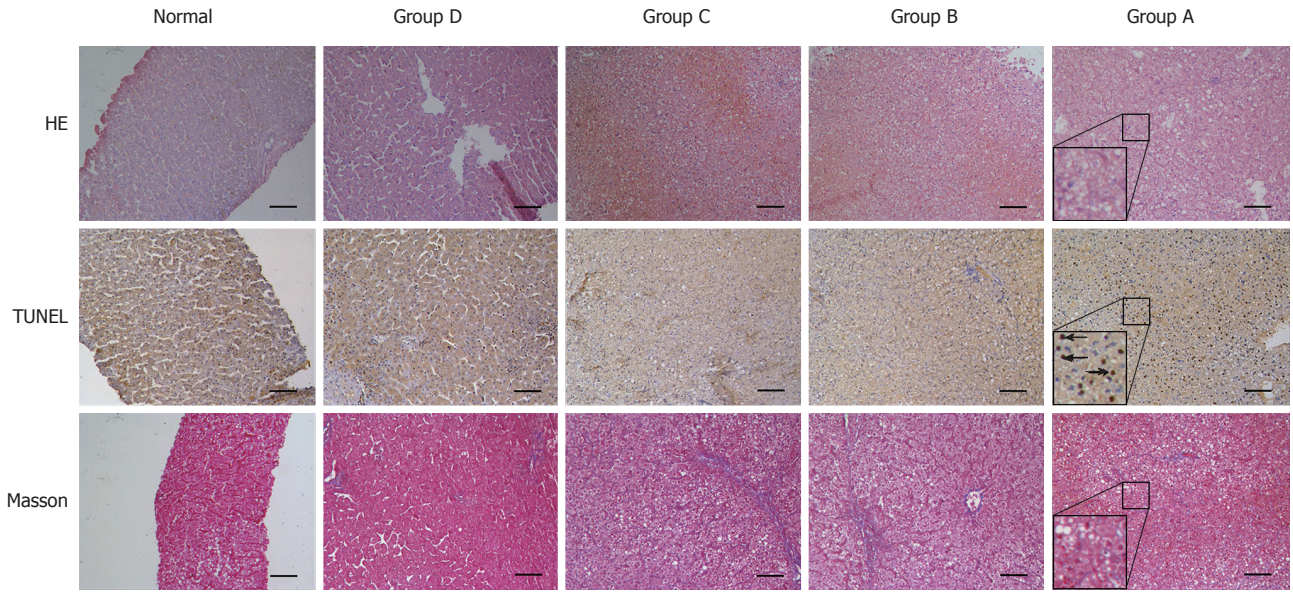


Figure 5 H&E staining, Tunel and Masson assays of post-mortem liver specimens from different groups. H&E: Hematoxylin-eosin staining; TUNEL: Terminal -deoxynucleotidyl transferase mediated nick end labeling; Arrows: Apoptotic bodies. Lower left corner detail: enlarged scale for group A (× 100 magnification, 200 μm scale bars).

16 h and 21 h. All animals in group A had jaundice and very yellow urine after 24 h; their general condition subsequently declined rapidly into a persistent coma, and all animals died within 68 h. In group B, one monkey appeared nauseated and was vomiting at 48 h, while another was discovered to have convulsions and liver coma at 96 h and died a short time later. In group C, two monkeys had nausea, vomiting, and very yellow urine at 72 h after D-gal administration. Group D monkeys eat less and had slower responses at 48 h after D-gal administration, but they were recovering slowly after 96 h.

Survival

All experimental animals in groups A, B, and C died within 5 days. Compared with group D, the survival times of groups A, B, and C animals were significantly shortened (56 ± 8.7 h, 95 ± 5.5 h, and 99 ± 2.2 h, respectively; $P < 0.01$ for all), whereas three monkeys in group D survived until the end of the 144-h observation period, and one died at 136 h. Kaplan-Meier survival analysis suggested that the survival time of each group of monkeys was significantly different ($\chi^2 = 22.42$, $P < 0.001$) (Figure 2).

Changes in ICP and ammonia

Significantly increased levels of ICP and ammonia were observed after D-gal administration in all study groups, compared with baseline values ($P < 0.05$ for all). The ICP and ammonia levels in group A increased to their peaks at 48 h to about 3-fold and 4-fold of baseline, respectively, whereas those in groups B and C had no significant increase ($P > 0.05$) except at 72 h and 96 h, when they all increased to a peak. In group D,

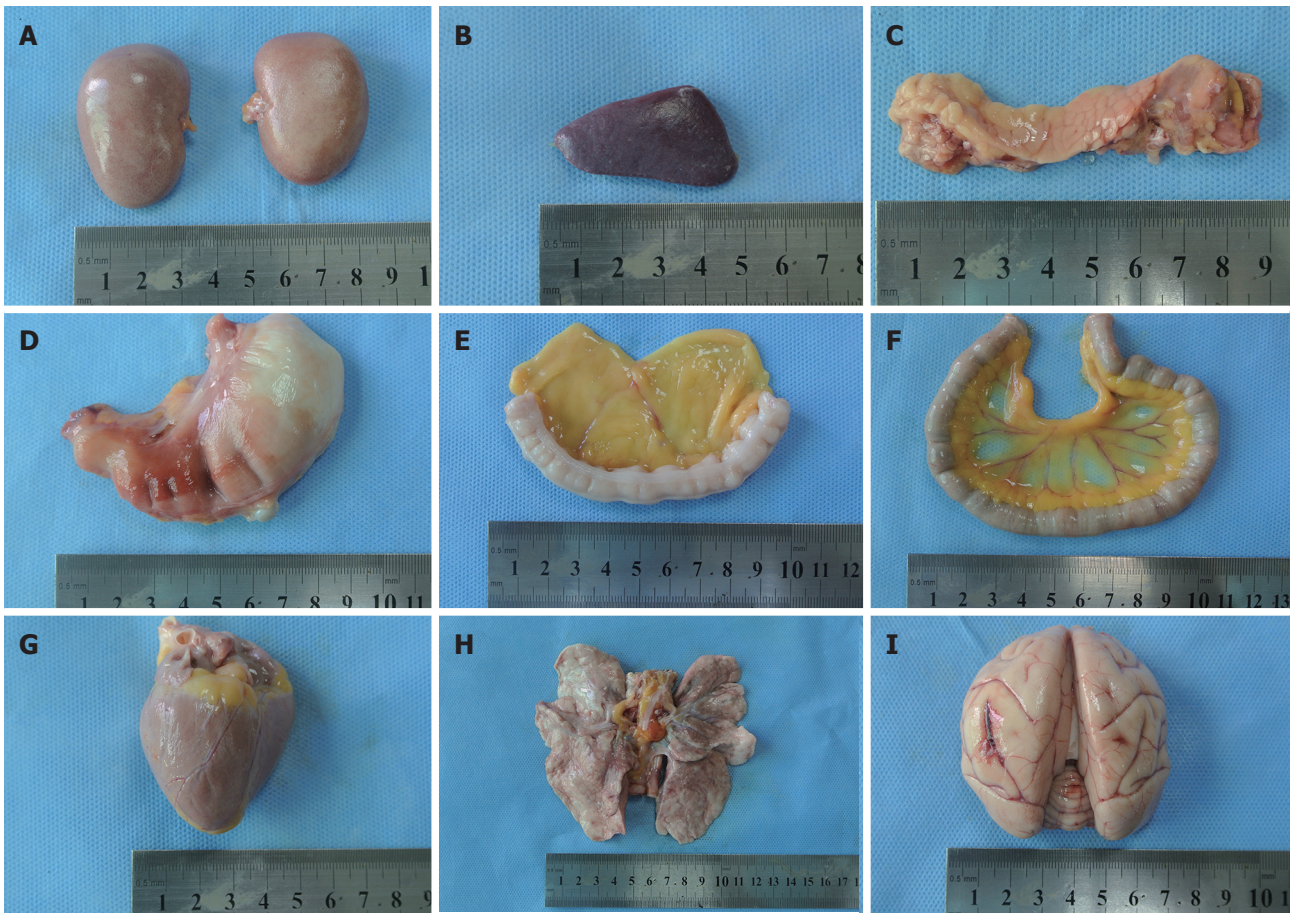


Figure 6 Gross specimens of other organs post-mortem (in group C). A: Renal; B: Spleen; C: Pancreas; D: Stomach; E: Large intestine; F: Small intestine; G: Heart; H: Lung; I: Brain.

ICP and ammonia levels increased slowly and declined after peaking at 96 h (Figure 3 and B).

Changes in inflammatory markers and endotoxin levels

As shown in Figure 3C-F, compared with baseline values, IL-1β, IL-6, TNF-α, and endotoxin all significantly increased in group A at all time points except at 12 h ($P < 0.05$ for all). IL-1β and TNF-α levels were not significantly different between groups B and C at any time point; IL-6 and endotoxin were not significantly different between groups B and C except at 48 h and 72 h. In group D, these values were all lower than those in the other groups.

Biochemical parameters

The progressive increase in the levels of liver enzymes (ALT, AST, LDH, and CK), and TBiL indicated serious liver damage after D-gal administration. The liver enzymes and TBiL in group A significantly increased compared with baseline levels and those of the other groups ($P < 0.05$ for all). However, the liver enzymes in groups B and C were not significantly different ($P > 0.05$), and neither were levels of TBiL except at 72 h and 96 h (Figure 4A-C, F and G).

The PT in all monkeys was prolonged significantly and there were significant differences between different time points after D-gal administration ($P < 0.05$ for all). The PT in group A increased to a peak at 48 h, about 4-fold of baseline; that of groups B and C significantly increased to a peak at 96 h, about 5-fold and 6-fold of baseline, respectively; whereas in group D, the PT increased slowly (Figure 4D).

Significant reductions in the plasma levels of ALB were also observed after D-gal administration in groups A, B, and C, to 37.50 ± 1.29 g/L at 48 h, and 33.50 ± 0.71 g/L and 32.50 ± 2.38 g/L at 96 h, respectively. The plasma level of ALB in group D did not change significantly (Figure 4E).

The BUN and Cr levels of all experimental monkeys significantly increased after D-gal administration. The BUN and Cr in group A increased to a peak at 48 h to about 6-fold and 4-fold compared with baseline levels. In groups B and C, the increase was progressive. In group D, the increase was slow and declined after peaking at 72 h to the baseline level at 120 h (Figure 4H, I).

Histopathology

The histopathology of normal liver clearly showed the expected findings of the central vein, portal area,

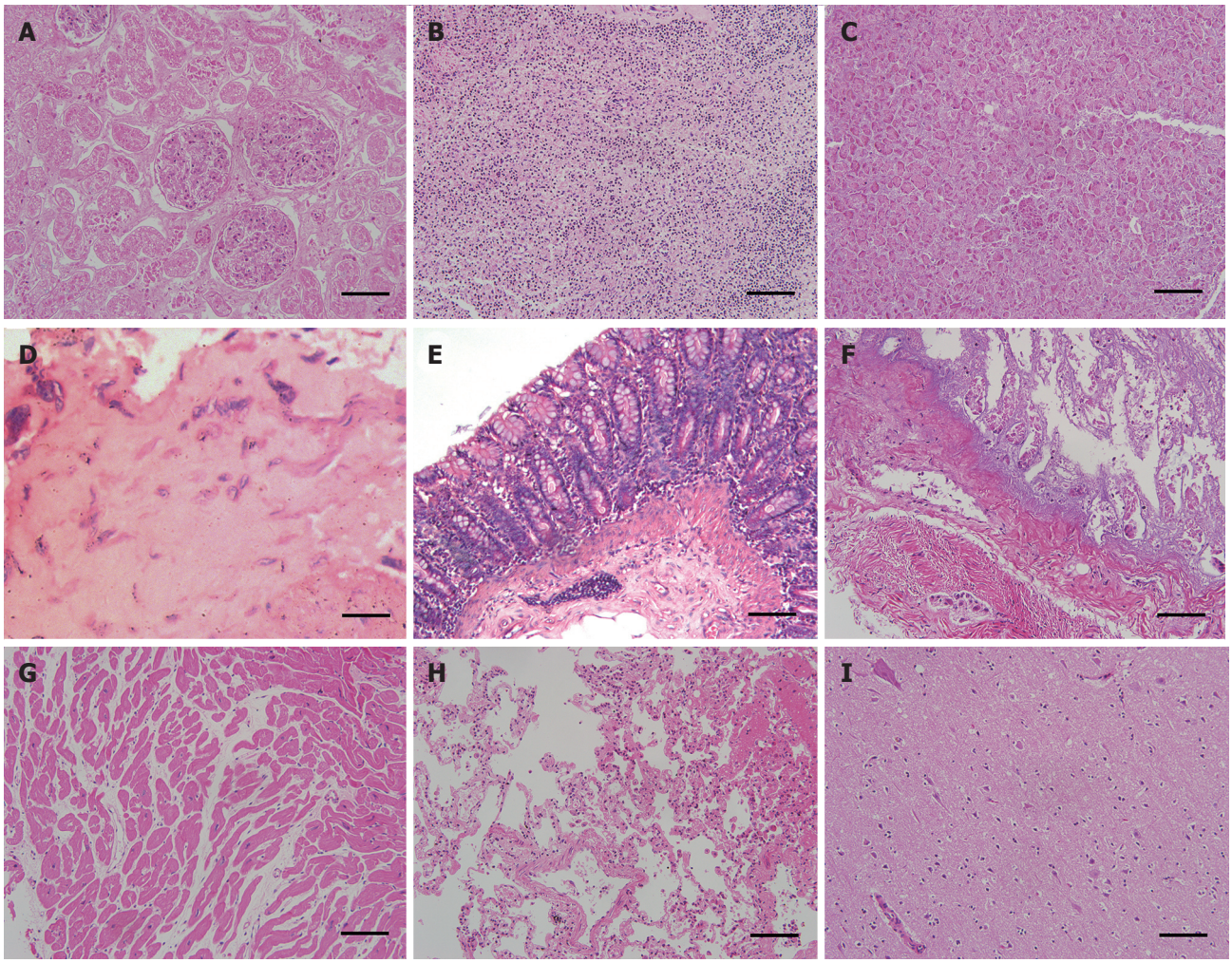


Figure 7 HE staining of other organs post-mortem (in group C). A: The renal tissue profile was clear, and the glomerular capillaries and renal interstitial blood vessels were slightly dilated and congested; B: The splenic sinusoids were mildly to moderately expanded with a large number of red blood cells; C: Pancreas, no abnormalities; D: Stomach, no abnormalities; E: Large intestine, no abnormalities; F: Small intestine, no abnormalities; G: Heart, no abnormalities; H: Lung, the bronchial and alveolar structures of pulmonary tissues were complete, and the interstitial capillaries were diffusely expanded and congested with few red blood cells; I: Brain, the nerve cells were diffusely enlarged with mild degenerative changes (× 100 magnification, 200 μm scale bars).

liver cords, and liver lobules. No swelling, vacuoles, or necrosis of liver cells was observed. In group A after D-gal administration, liver cells presented with extensive necrosis, had visible nuclear fragments, and a large number of vacuolar structures. The situation in groups B and C was similar, with areas of necrotic lesions with diffuse swelling of liver cells having cytoplasmic and vacuolar degeneration. In group D, liver cells had mainly degenerative edema and the liver sinus structure was visible. The TUNEL assay demonstrated obvious positive cells in group A, while in groups B and C positive cells were present in comparatively lower quantities. No positive cells were detected in group D samples. Masson staining revealed mild fibrosis in groups A, B, and C, and no obvious abnormality in group D animals (Figure 5).

Results of examination of gross specimens of the other organs are shown in Figure 6. The results of HE staining of other organs are shown in Figure 7.

DISCUSSION

A simplified, reproducible D-gal-induced cynomolgus monkey model of ALF that is suitable for use to assess the safety and efficacy of ALSS has been successfully established. The ideal criteria for animal models were first proposed by Terblanche *et al*[20], which were promoted and supplemented as a result of subsequent studies[14]. They mainly comprise the following points: reversibility; reproducibility; death from liver failure; suitable treatment window; large animals; causing minimal harm to environment and researchers; consciousness level making hepatic encephalopathy easy to evaluate; similarity to human beings; and ethically acceptable.

At present, large animal models of ALF meeting the above criteria mainly include drug-induced models[5] and surgery-induced models[21]. Drug-induced models are easy to create, having short anesthesia times, and

can be accomplished without use of the highly skilled technical work required to establish surgery-induced models. Although sometimes drug-induced models are unstable because of great individual differences in drug tolerance and metabolic function, these models are of great interest to research scholars because the most common reason for ALF in clinical setting is drug toxicity[4].

Current literature reports of drugs that can induce ALF include those on D-gal, acetaminophen (APAP), and carbon tetrachloride, to name a few[12-14]. Yu *et al*[22] reported on the pharmacokinetics, drug metabolism, and hepatic toxicity of APAP in cynomolgus monkeys and found significant tolerance to APAP; therefore, APAP is not suitable to create a cynomolgus monkey model to study related hepatic injury. Compared with other drugs, D-gal has many advantages for this purpose, including better reproducibility and easier control of the dosage; it is generally accepted as the ideal drug to induce ALF.

For this study, we chose cynomolgus monkeys because their anatomy, physiology, biochemical metabolism, and immune system characteristics are very similar to those of human beings, making them the ideal animal to establish an ALF model. Given the rarity of primate species and the instability of other models, there are few relevant published reports of primate models of ALF. Zhou *et al*[23] induced fulminant hepatic failure (FHF) in the *Macaca mulatta* by intraperitoneal injection of amatoxin and endotoxin, and evaluated the animal model by progressive analysis of clinical features, biochemical indices, and histopathology. However, their study included only two monkeys, so the stability and reproducibility need further verification, and the effective treatment window of this model would make the study of use of ALSS difficult.

Drug dosages and administration methods are important for establishing drug-induced models. The method of drug administration affects the convenience of using a model. Various ALF studies have different requirements for the survival time, which usually means exploring the optimal dosage and induction methods for different purposes. Glorioso *et al*[8] successfully established a pig model of ALF by injecting 0.75 g/kg D-gal through the external jugular vein, which was successfully used in the study of artificial livers. Li *et al*[7,24] established a pig model of FHF by intravenous injection of 1.3 g/kg and 1.5 g/kg[25] D-gal, which was used in studies to verify the safety and efficacy of ALSS. Ding *et al*[26] established a pig model of ALF by injecting 0.45 g/kg D-gal intravenously to study treatment with a novel bio-artificial liver.

Currently, D-gal is usually administered through the external jugular vein or the abdominal cavity[23-25,27]. The abdominal cavity injection is simple and convenient, but resulting models are unstable, while administration through the external jugular vein and portal vein usually requires a long anesthesia time and surgical venous intubation, so the method is more complicated.

In our early study, we administered 0.45, 0.3, and 0.15 g/kg of D-gal through the external jugular vein to establish an ALF model to explore the optimal basic dosage to establish the primate model of ALF[19]. However, venous intubation is not only inconvenient, but also brings certain trauma to the experimental animal. In this study, we used small saphenous vein puncture instead of jugular vein intubation for D-gal administration, which not only effectively avoided the trauma caused by intubation, but also significantly reduced the anesthesia time and greatly improved the convenience of operation. Moreover, we further adjusted and optimized the dosage of D-gal using the previous dose of 0.3 g/kg, as well as a 0.25 g/kg single dose, 0.25 g/kg as a divided dose (0.20 + 0.05 g/kg), and a single 0.20 g/kg dose, and then compared in the different groups for changes of clinical manifestation, survival time, liver function, inflammatory factors, PT, ICP, and histopathology.

The results showed that the experimental monkeys developed different levels of anorexia, anemia, jaundice, and coagulopathy after intravenous injection of different doses of D-gal that were similar to the various degrees of clinical ALF. The animals administered 0.30 g/kg of D-gal had the shortest survival time (56 ± 8.7 h), and there was no significant difference in survival time after 0.25 g/kg given as a single or divided dose (95 ± 5.5 h and 99 ± 2.2 h, respectively). In our study, 81.3% (13/16) of experimental monkeys died, and the survival time of experimental animals was positively correlated with the dose of D-gal.

D-gal can cause liver cell necrosis and lead to ALF, as well as abnormally elevated serum TNF-α, which then triggers the cascade of inflammatory mediators and is closely related to the pathophysiology of ALF[28,29]. In our study, a strong inflammatory response was observed, as evidenced by markedly increased levels of TNF-α, IL-1β, IL-6, and endotoxin, all of which were positively correlated with the dose of D-gal.

Liver enzymes are important indices to assess clinical liver injury. When liver cells are necrotic, inflammation and toxicity can cause damage to the liver cell membrane, leading to serum transaminase elevations; transaminase levels 10-fold higher than the baseline indicate acute liver damage[30]. In this study, ALT, AST, CK, and LDH increased rapidly in a short time after injection of D-gal, with results demonstrating that acute liver injury and the degree of damage were positively correlated with the dose of D-gal.

ALB and PT are important indicators of liver synthesis and reserve function. In our study, serum ALB levels showed a progressive decline after D-gal administration except in group D, and this may explain the anomalous finding of abdominal and pleural effusions on autopsy of the study animals. The PT in the four groups was significantly prolonged, with the peak times 4-, 5-, 6-, and 1.5-fold of the baseline time in groups A, B, C and D, respectively. At autopsy,

the livers in groups A and B had obvious ecchymosis, and four lung specimens had obvious bleeding; these findings are likely associated with the coagulation dysfunction caused by liver failure.

Hepatic encephalopathy is a serious complication of ALF and is closely related to the blood ammonia level, elevations of which cause brain edema, oxidative stress, and inflammation[31,32]. In our study, we measured the progression of ammonia levels and ICP to monitor for hepatic encephalopathy. Ammonia and ICP were significantly increased in groups A and B, and were associated with the clinical manifestations of consciousness changes and hepatic coma before death, as well as histopathological changes, all indicating that the experimental animals developed hepatic encephalopathy before death.

The model established in our study has some limitations. First, we used Zoletil to induce anesthesia before administering D-gal, and although Zoletil has many advantages, including short induction time, minimal side effects, and maximum security compared with ketamine, whether it can affect the effect of D-gal is unknown. In addition, the number of animals used was small, and further studies with larger experimental groups are warranted to verify our results.

In conclusion, we have successfully established a simplified, reproducible D-gal-induced cynomolgus monkey model of ALF that is suitable for assessing the safety and efficacy of ALSS, studying the pathogenesis of ALF, and developing new drugs, and the dosage of 0.25 g/kg as either a single or divided dose is optimal.

ARTICLE HIGHLIGHTS

Research Background

Acute liver failure (ALF) is a serious threat to human health. Artificial liver support system (ALSS) is a novel method to deal with ALF. However, the safety and efficacy of ALSS must be verified before clinical application. Therefore, the establishment of an ALF animal model is of great significance for testing ALSS, studying the pathogenesis of ALF, and determining the comprehensive treatment of ALF. Nowadays, there have been many studies about the acute liver failure in large animals, such as pigs and dogs. However, there have been few previously reported studies of ALF models in cynomolgus monkey. Furthermore, the methods of drug administration are complex and increase the trauma to experimental animals.

Research motivation

In this study, our motivation was to establish an ideal animal model of ALF with an appropriate treatment window which is suitable for assessing the safety and efficacy of ALSS, studying the pathogenesis of ALF, developing new drugs, and determining the comprehensive treatment of ALF.

Research objectives

The primary objective of this study was to establish a simplified, reproducible D-gal-induced large-animal ALF model with an appropriate treatment window. In addition, we wanted to explore the optimal dosage of D-gal to induce ALF in cynomolgus monkey.

Research methods

In this study, we used small saphenous vein puncture instead of jugular vein intubation for different doses of D-gal administration, and then observed the clinical manifestations, survival times, changes in biochemical indices, intracranial pressure changes, and resulting pathological and histological characteristics. This method not only effectively avoided the trauma caused by intubation, but also significantly reduced the anesthesia time and greatly improved the convenience of operation. All experimental data were analyzed using SPSS 21.0 statistical package.

Research results

The results showed that the experimental monkeys developed different levels of anorexia, anemia, jaundice, and coagulopathy after intravenous injection of different doses of D-gal that were similar to the various degrees of clinical ALF. The animals administered 0.30 g/kg of D-gal had the shortest survival time, and there was no significant difference in survival time after 0.25 g/kg was given as a single or divided dose. The degree of acute liver damage and the survival time of experimental animals were positively correlated with the dose of D-gal. The experimental animals given 0.25 g/kg as a single or divided dose had an appropriate treatment window. However, the number of animals used was limited, and further studies with larger experimental groups are warranted to verify our results.

Research conclusions

The authors have successfully established a simplified, reproducible D-gal-induced cynomolgus monkey model of ALF and found that the optimal dosage to induce ALF in cynomolgus monkey is 0.25 g/kg as either a single or divided dose.

Research perspectives

From this study, we found that drug dosages and the administration methods are important for establishing drug-induced models. The method of drug administration affects the convenience of using a model. In addition, we think small saphenous vein puncture for D-gal administration is the best method to induce ALF in cynomolgus monkey and the dosage of 0.25 g/kg as either a single or divided dose is optimal. Furthermore, we can use this method and dosage to induce ALF in cynomolgus monkey to test ALSS or study the pathogenesis of ALF in the future.

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ORIGINAL ARTICLE

Basic Study

Diversity of bacterial lactase genes in intestinal contents of mice with antibiotics-induced diarrhea

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Abstract

AIM

To investigate the diversity of bacterial lactase genes in the intestinal contents of mice with antibiotics-induced diarrhea.

METHODS

Following 2 d of adaptive feeding, 12 specific pathogen-free Kunming mice were randomly divided into the control group and model group. The mouse model of antibiotics-induced diarrhea was established by gastric perfusion with mixed antibiotics (23.33 mL·kg^{-1}·d^{-1}) composed of gentamicin sulfate and cephradine capsules administered for 5 days, and the control group was treated with an equal amount of sterile water. Contents of the jejunum and ileum were then collected and metagenomic DNA was extracted, after which analysis of bacterial lactase genes using operational taxonomic units (OTUs) was carried out

after amplification and sequencing.

RESULTS

OTUs were 871 and 963 in the model group and control group, respectively, and 690 of these were identical. There were significant differences in Chao1 and ACE indices between the two groups ($P < 0.05$). Principal component analysis, principal coordination analysis and nonmetric multidimensional scaling analyses showed that OTUs distribution in the control group was relatively intensive, and differences among individuals were small, while in the model group, they were widely dispersed and more diversified. Bacterial lactase genes from the intestinal contents of the control group were related to Proteobacteria, Actinobacteria, Firmicutes and unclassified bacteria. Of these, Proteobacteria was the most abundant phylum. In contrast, the bacterial population was less diverse and abundant in the model group, as the abundance of *Bradyrhizobium* sp. BTAi1, *Agrobacterium* sp. H13-3, *Acidovorax* sp. KKS102, *Azoarcus* sp. KH32C and *Aeromonas caviae* was lower than that in the control group. In addition, of the known species, the control group and model group had their own unique genera, respectively.

CONCLUSION

Antibiotics reduce the diversity of bacterial lactase genes in the intestinal contents, decrease the abundance of lactase gene, change the lactase gene strains, and transform their structures.

Key words: Antibiotics-induced diarrhea; Lactase genes; Gene diversity; Intestinal bacteria; High-throughput sequencing

Core tip: The mechanism of antibiotics-induced diarrhea has been studied in a wide range of diverse microbes, but less on functional enzymes. The current study aimed to determine the mechanism of lactase activity from genetic diversity and provide a basis for antibiotics-induced diarrhea. Alpha/Beta diversity analysis showed that there were significant differences between the control mice and model mice in types of lactase genes expressed and their activities. Following the antibiotics-induced diarrhea symptoms, the intestinal lactase genes changed, the number of strains was reduced and the abundance decreased, indicating changes in community structure and decreased diversity of lactase genes.

Long CX, He L, Guo YF, Liu YW, Xiao NQ, Tan ZJ. Diversity of bacterial lactase genes in intestinal contents of mice with antibiotics-induced diarrhea. *World J Gastroenterol* 2017; 23(42): 7584-7593 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7584.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7584

INTRODUCTION

Diarrhea is a common complication of antibiotic therapy and any antibiotic may disrupt the intestinal microbiota leading to diarrhea[1]. Antibiotics are usually used to treat diarrhea in children. Most studies on the impact of antibiotics on diarrhea focus on the occurrence of antibiotics-associated diarrhea (AAD)[2]. AAD can be induced by almost all antibacterial agents following the administration of mixed or individual antibiotics for several days. To date, the mechanism of AAD is unknown. However, AAD can cause severe side effects, as it is associated with damage due to intestinal micro-organisms, and disorders in intestinal function and flora[3].

Previous studies have shown that drug- or antibiotics-induced diarrhea is associated with intestinal lactase dysfunction, due to loss of its activity[4]. Antibiotics can destroy or inhibit intestinal lactase activity, leading to diarrhea. Data show that 62.5% of infantile diarrhea is related to intolerance of lactose activity[5]. Similar observations were also found in other drug-induced diarrhea, and treatment with lactase supplements is a good option for most types of diarrhea due to the importance of lactase activity for the control of intestinal function[6].

Lactase, an enzyme also known as β-D-galactosidase (EC3.2.1.23), catalyzes lactose hydrolysis to glucose or galactose[7]. Clinically, various symptoms have been associated with lactose intolerance, such as abdominal pain, abdominal distension, and diarrhea[6,8]. Individuals may be lactose intolerant to varying degrees, depending on the severity of these symptoms. Lactase is mainly produced by bacteria living in the intestinal tract of animals, for example *Lactobacillus* sp., *Bifidobacterium* sp., *Bacillus* sp., *Escherichia coli*, *Streptococcus thermophilus*, and *Enterobacter aerogenes*[9,10].

Lactase activity is tightly regulated by its expression and environment, which can alter and modify the gene expression of lactase isoforms. Various isoforms of the lactase gene have been identified and reported to be widely expressed in the intestinal tract, with diverse enzyme activities. In addition, the expression, protein modification and isoforms can change in different microenvironments[11]. Studies have shown that AAD is not only associated with dysbacteriosis, but also damage to intestinal lactase activity, leading to diarrhea[4].

In the present study, we found that the activity of lactase in intestinal contents was significantly reduced in mice with antibiotics-induced diarrhea. In order to provide a basis for the mechanism of antibiotics-induced diarrhea, we investigated whether the change in lactase activity was caused by altered gene expression. Therefore, we compared the diversity of bacterial lactase genes expressed in control mice and in model mice with antibiotics-induced diarrhea.

MATERIALS AND METHODS

Materials

Animals: Twelve mature specific pathogen-free Kunming mice (six males and six females) weighing 18-22 g were purchased from Hunan Slaccas Jingda Laboratory Animal Co., Ltd (Hunan, China) with license number SCXK (Xiang) 2013-0004.

Reagents: Chemicals were purchased from Yichang Renfu Pharmaceutical Co., Ltd. and Suzhou Zhonghua Pharmaceutical Industry Co., Ltd., including gentamicin sulfate for injection and cephradine capsules. Solutions including protease K, lysozyme, Tris saturated phenol-chloroform-isoamyl alcohol (25:24:1), TE buffer and acetone were purchased from Beijing Ding-Guo Biotechnology Co., Ltd. Other solutions, such as 0.1 mol/L phosphate buffer solution (PBS), hexadecyl trimethyl ammonium bromide (CTAB)/NaCl, 10% sodium dodecyl sulfate (SDS), 5 mol/L NaCl, chloroform-isoamyl alcohol (24:1), 3 mol/L sodium acetate and 70% ethanol, were prepared in the laboratory.

Methods

The mice were randomly allocated to the control and model groups, with six mice in each group. The mice in the model group were administered 0.35 mL antibiotic mixture at a concentration of 62.5 g/L[12], twice a day for 5 d. The mice in the control group were treated in the same way, but received distilled water. The animals were maintained under controlled conditions (23-25 ℃, humidity 50%-70%). Animal surgery followed international regulations and standards.

Collection of intestinal contents

Following the development of antibiotics-induced diarrhea symptoms, the mice were sacrificed using cervical vertebra dislocation, and their intestinal contents from the jejunum and ileum were collected and immediately frozen until analysis[12].

Metagenome extraction

According to a previous report[13], 2.0 g of intestinal contents was collected in a sterile environment, placed in a 50 mL germ-free centrifuge tube and homogenized in 30 mL of 0.1 mol/L PBS, followed by centrifugation at 200 × *g* for 2 min. After washing twice with PBS, the supernatant was transferred into fresh germ-free tubes and centrifuged for 8 min at 10000 × *g*. The sediment was collected, washed once with PBS, twice with acetone, and three times with PBS, then resuspended in 4 mL TE buffer. After sample pretreatment, 500 μL of bacterial suspension was added with 5 μL proteinase K, 20 μL lysozyme and 45 μL TE buffer, and homogenized in 1.5 mL sterile Eppendorf tubes. Samples were incubated at 37 ℃ for 30 min and 30 μL 10% SDS was added and mixed well, followed by incubation at 37 ℃ for 40 min, with vortexing once every 10 min. The mixture was vortexed at 65 ℃ for 10 min after adding 80 μL of CTAB/NaCl and 100 μL of 5 mol/L NaCl. An equal volume of Tris saturated phenol-chloroform-isoamyl alcohol (25:24:1) was then added to the sample, mixed well and centrifuged at 10000 × *g* for 3 min. The supernatant was transferred to new sterile tubes, mixed with an equal volume of chloroform-isoamyl alcohol (24:1), and centrifuged at 10000 × *g* for 3 min. The supernatant was transferred into new sterile tubes and mixed with an equal volume of chloroform-isoamyl alcohol (24:1). The supernatant was transferred into fresh sterile tubes after centrifugation at 10000 × *g* for 3 min, a double volume of absolute ethyl alcohol and 1/10 volume of 3 mol/L sodium acetate were added, and precipitated at -20 ℃ for approximately 12 h. Samples were centrifuged at 10000 × *g* for 3 min. The sediment was collected and washed with 70% ethanol, dried and then dissolved in 50 μL TE buffer for DNA extraction.

PCR amplification and sequencing

To amplify the DNA, universal primers for bacterial lactase genes were designed and purchased from Shanghai Personal Biotechnology Co., Ltd. The sequence for the upstream primer was: 5′-TRRGC AACGAATACGGSTG-3′, and the downstream primer was: 5′-ACCATGAARTTSGTGGTSARCGG-3′. The PCR amplification system (25 μL) contained 11.25 μL sterilized ultrapure water, 0.25 μL Q5 high-fidelity DNA polymerase, 5 μL 5 × reaction buffer, 5 μL 5 × high GC buffer, 0.5 μL dNTP (10 mmol/L), 1 μL template DNA, 1 μL upstream primer (10 μmol/L) and 1 μL downstream primer (10 μmol/L). PCR conditions were as follows: 98 ℃ for 30 s, followed by 32 cycles at 98 ℃ for 15 s, 46 ℃ for 30 s (annealing) and 72 ℃ for 30 s (extension), and then 72 ℃ for 5 min[14]. The PCR products were excised from a 1.5% agarose gel and purified by AxyPred Gel Extraction Kit (Axygen, Scientific Inc., Union City, CA, United States).

Sequence screening and analysis

To identify the population of lactase genes, Qiime (v1.8.0, http://qiime.org/) software[15] was used to align the sequencing results and perform cluster analysis, principal component analysis (PCA) and ACE abundance indexing, and Simpson diversity indexing of the analysis results. The principal coordinates analysis (PCoA), nonmetric multidimensional scaling (NMDS), and heatmap were carried out in R for diversity and similarity. In addition, USEARCH (v5.2.236, http://www.drive5.com/usearch/) was used to exclude chimeric sequences. According to the software, fragments with sequence similarity over 97% were considered as one operational taxonomic unit (OTU)[16]. Evolution and abundance of bacterial lactase genes were analyzed by MEGAN (http://ab.inf.uni-tuebingen.de/software/megan5/) software[17].

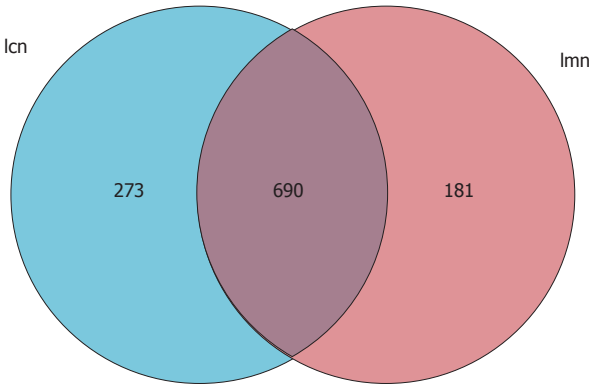

Figure 1 Venn diagram of operational taxonomic units based on the sequences with over 97% similarity under a similar level of clustering. Icn: Control group; Imn: Model group.

Table 1 Alpha diversity index

Group	Chao1	ACE	Simpson	Shannon
Control group	503.00 ± 20.07	585.25 ± 40.84	0.89 ± 0.02	4.81 ± 0.28
Model group	446.67 ± 18.04[a]	500.20 ± 5.10 [a]	0.91 ± 0.02	4.92 ± 0.27

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

Statistical analysis

SPSS21.0 software (IBM Corp, Armonk, NY, United States) was used for statistical analysis. Results are expressed as means ± SE. To compare the significance of differences, the pairwise *t*-test was used with *P* values < 0.05.

RESULTS

Comparison of operational taxonomic units

To investigate the variety and abundance of the expression of lactase genes, the OTUs were measured. We found that there were 871 and 963 OTUs expressed in mice in the model and control groups, respectively. Of these, 690 were identical in the two groups (Figure 1). These findings indicated that antibiotics-induced diarrhea reduced the expression of certain lactase genes and triggered different expression responses to symptoms.

Alpha diversity analysis

Alpha diversity and abundance analysis were estimated by four indices from the Simpson or Shannon diversity analysis, and Chao1 or ACE. According to the definition, a higher value of the Chao1 or ACE index indicated greater abundance of a bacterial population. Higher Shannon and Simpson indices indicated a more diverse bacterial population. Chao1 and ACE indices in the control group were significantly higher than those in the model group ($P < 0.05$; Table 1). This suggested that antibiotics reduced the abundance of bacterial lactase genes in the intestinal contents. As the difference in the Simpson and Shannon indices between the two groups was small, the difference in diversity was insignificant.

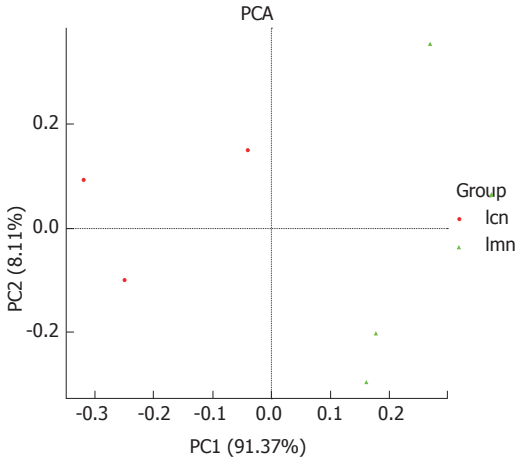

Figure 2 Principal component analysis diagram of bacterial lactase gene similarity at genus level based on DNA sequence data. Each point in the figure represents a sample. Points with the same color belong to the same group. The closer the distance between two points, the smaller the difference in the microbial community. Icn: Control group; Imn: Model group;PCA: principal component analysis.

Beta diversity analysis

PCA analysis was used to measure the variation among individuals within the same group. It can be seen that the distribution was more concentrated in the control group compared with the model group, and the distance between the two groups was relatively great. The percentage contributed to variation of PC1 and PC2 was 91.3% and 8.11%, respectively (Figure 2). These findings suggest that the response to antibiotics-induced diarrhea differed among individuals, and antibiotic modeling changed the structure of the bacterial lactase genes.

To further investigate the homogenous bacterial lactase genes, PCoA was used. In particular, the distances between samples within each group sample were measured. The results showed that the distances between samples from the control group were significantly smaller than those from the model group (Figure 3). NMDS analysis was carried out to compare the similarity of lactase genes expressed within groups. Each point represented one sample, and the points with different colors were the various samples. The closer the distance between two points was, the higher the similarity between two samples, and the smaller the difference was. The distribution of samples from the control group was tightly concentrated compared to the samples from the model group (Figure 4). This indicated that the variations in lactase gene expression in response to antibiotics-induced diarrhea were significantly different from those in the control group.

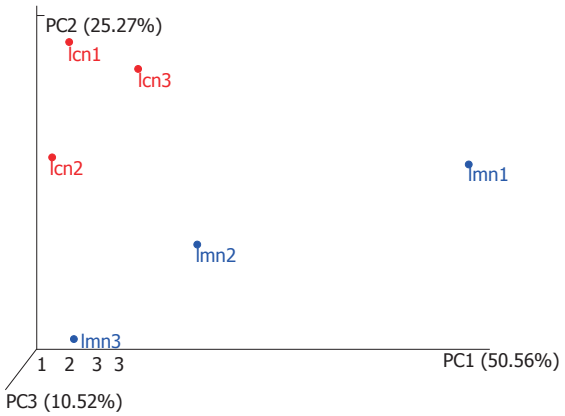

Figure 3 Three-dimensional sorting graph of samples based on weighted UniFrac principal coordinates analysis. Each point in the figure represents a sample. Points with the same color belong to the same group. The closer the distance between two points, the smaller the difference in the microbial community. lcn1-3 were control groups 1-3, and lmn1-3 were model groups 1-3.

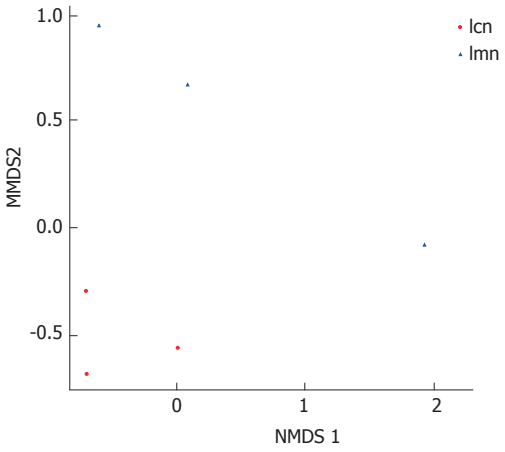

Figure 4 Two-dimensional map of samples based on weighted UniFrac nonmetric multidimensional scaling analysis. Each point in the figure represents a sample. Points with the same color belong to the same group. The closer the distance between two points, the smaller the difference in the microbial community. lcn: Control group; lmn:Model group.

Table 2 Effects of antibiotics-induced diarrhea on species abundance of bacterial lactase genes at genus level in intestinal contents

Genus	Control group	Model group
Corynebacterium	0.000942 ± 0.000365	0.001943 ± 0.000637
Gordonia	0.000114 ± 0.00197	0
Mycobacterium	0.000282 ± 0.000286	0
Frankia	0.000017 ± 0.000029	0
Modestobacter	0.000013 ± 0.000002	0.000098 ± 0.000155
Microbacterium	0.000005 ± 0.000009	0
Arthrobacter	0.000559 ± 0.000233	0.000658 ± 0.000308
Micromonospora	0	0.000016 ± 0.000017
Streptomyces	0.000081 ± 0.000031	0.000886 ± 0.000877
Eggerthella	0.000030 ± 0.000027	0.000074 ± 0.000039
Paenibacillus	0	0.000086 ± 0.000150
Ruminococcus	0.000039 ± 0.000055	0.000455 ± 0.000788
Bradyrhizobium	0.000244 ± 0.000171	0.000202 ± 0.000134
Agrobacterium	0.000140 ± 0.00146	0.000023 ± 0.000026
Ensifer	0	0.000026 ± 0.000045
Rhizobium	0.000100 ± 0.000173	0.000074 ± 0.000128
Novosphingobium	0.000011 ± 0.000020	0
Sphingobium	0.000019 ± 0.000033	0.002638 ± 0.004569
Burkholderia	0.000046 ± 0.000014	0.000079 ± 0.000012
Acidovorax	0.000515 ± 0.000258	0.000251 ± 0.000250
Azoarcus	0.001446 ± 0.002341	0.002971 ± 0.004730
Aeromonas	0.000075 ± 0.000130	0
Citrobacter	0.000051 ± 0.0000089	0.0005557 ± 0.000691
Enterobacter	0.000122 ± 0.000047	0.000380 ± 0.000303
Escherichia	0.000022 ± 0.000007	0.000017 ± 0.000017
Klebsiella	0.000211 ± 0.000040	0.000421 ± 0.000219
Pseudomonas	0.040033 ± 0.008991	0.045158 ± 0.031454
Unclassified	0.729202 ± 0.059025	0.866470 ± 0.048913[a]
No blast hit	0.184757 ± 0.050216	0.024207 ± 0.004006[a]

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

Table 3 Effects of antibiotic-induced diarrhea on species number of bacterial lactase genes at genus level in intestinal contents

	Species number at genus level
Control group	79.33 ± 0.58
Model group	76.00 ± 4.58

Analysis of bacterial lactase gene source and abundance

The pie chart of each branch point of the classification tree shows the abundance of the classification unit in each sample. The larger the fan area, the higher the corresponding abundance of the taxon became. Based on the origins of lactase genes, the phylogenetic tree results showed that the majority of them were members of families, such as Actinobacteria, Firmicutes and Proteobacteria. Interestingly, some of the lactase genes were not from the above species, and there was no clear clue to show which types of bacteria they belonged to (Figure 5).

The data strongly indicated that a mixture of antibiotics induced diarrhea and stimulated the growth of diverse bacteria. Some of the sensitive bacterial lactase genes were inhibited or killed, and insensitive genes were reproduced. In addition, the population sizes, such as *Bradyrhizobium* sp. BTAi1, *Agrobacterium* sp. H13-3, *Acidovorax* sp. KKS102, *Azoarcus* sp. KH32C and *Aeromonas caviae,* were hugely different in the intestinal contents of mice from the model group and control group.

At the genus level, the difference was more apparent. For example, *Gordonia*, *Mycobacterium*, *Frankia*, *Microbacterium*, *Novosphingobium* and *Aeromonas* were commonly seen in the intestinal contents of control mice. However, the lactase genes from these bacteria were not detectable in model mice. In contrast, *Micromonospora*, *Paenibacillus* and *Ensifer* were only found in the model group (Table 2). With regard to quantity, there was no significant difference in bacterial lactase genes in terms of genus in the intestinal contents of mice with antibiotics-induced diarrhea; however, the number of unclassified

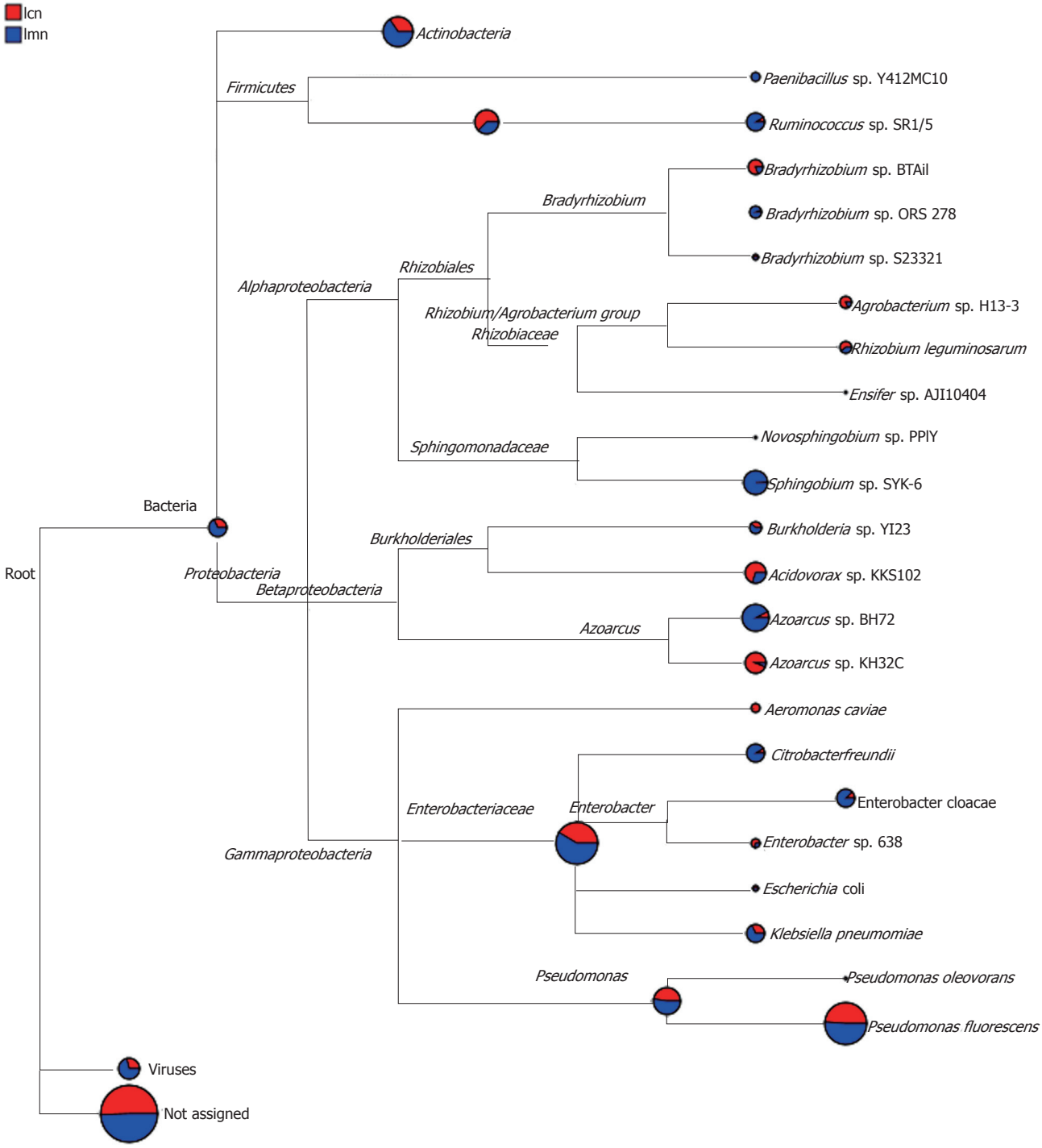

Figure 5 Species evolution and abundance information. The pie chart of each branch point of the classification tree shows the abundance of the classification unit in each sample. The larger the fan area, the higher the corresponding abundance of the taxon became. lcn: Control group; lmn: Model group.

genes was increased ($P < 0.05$) and the abundance of no blast hit genes was reduced ($P < 0.01$; Table 2).

To confirm our findings, heatmap analysis provided by the R package was used, and both the diversity and abundance of bacterial species were clearly shown (Figure 6). The colors in the heatmap images are from high (red) to low abundance (blue). In addition, the images also show the distinction/differences in individuals within the same group. From the image, the lactase genes of *Gordonia*, *Frankia*, *Novosphingobium*, *Mycobacterium*, *Agrobacterium*, *Aeromonas* and *Microbacterium* were highly present in the contents from the model mice compared with those from the control mice. However, the difference in these bacteria was consistent from mouse to mouse in the control group.

DISCUSSION

It is well known that the function or activity of a protein

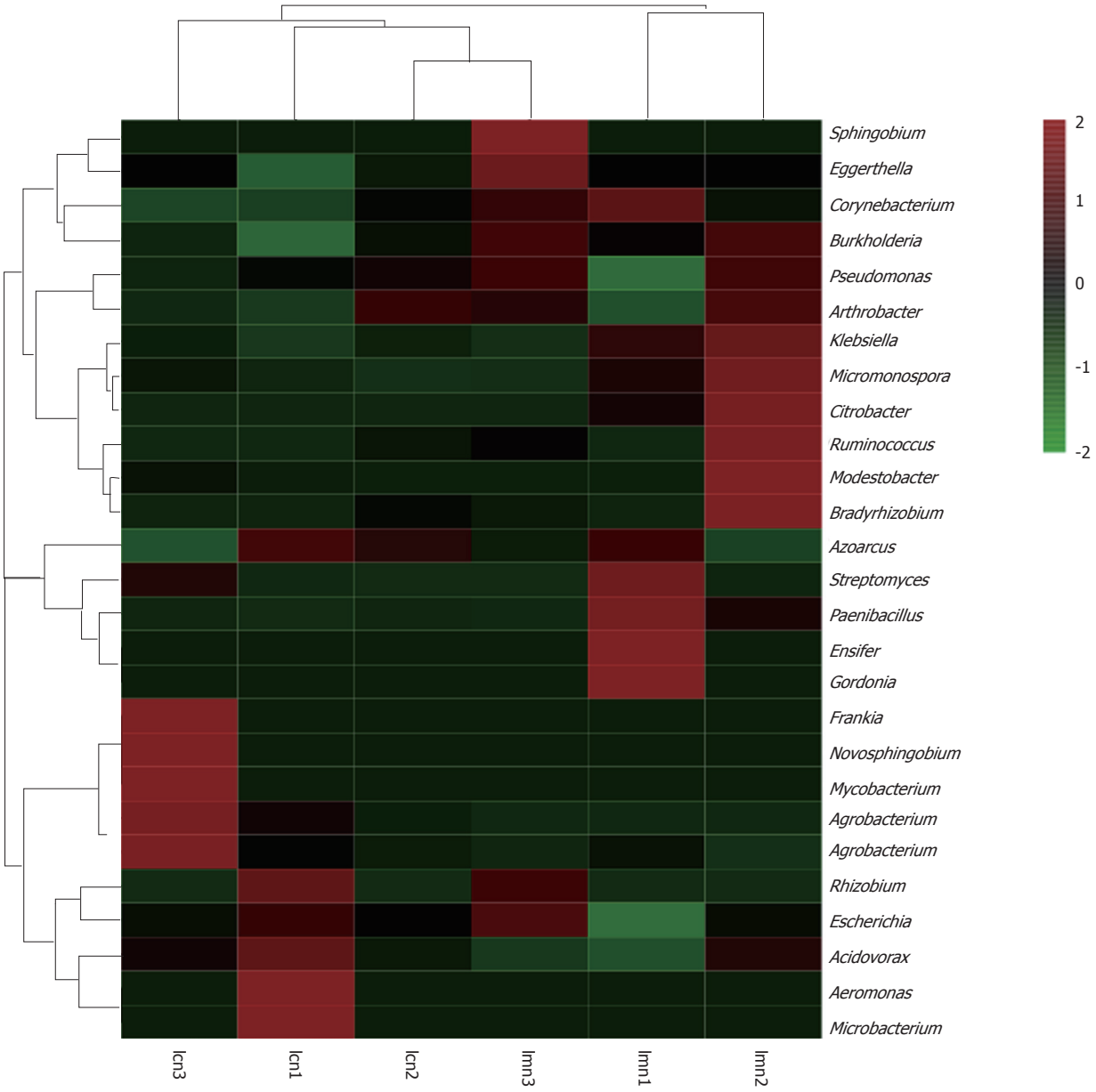

Figure 6 Heatmap analysis at genus level combined with cluster analysis. lcn1-3 were control groups 1-3, and lmn1-3 were model groups 1-3.

is closely related to its structure and modifications. Maintaining routine biological functions and stresses, such as antibiotics-induced diarrhea, requires highly regulated protein expression and its modifications. As with proteins, enzymes require the same[18-21]. In our current research, mice with diarrhea triggered by a mixture of antibiotics showed less diverse bacterial growth than control mice[22-24]. The mechanism of antibiotics-induced diarrhea has been studied in a wide range of diverse microbes[25-27], but there has been less research carried out on functional enzymes.

The current study aimed to determine the mechanism of lactase activity from the viewpoint of genetic diversity and provide a basis for antibiotics-induced diarrhea. The results showed that the number of OTUs, Chao1 index and ACE index of bacterial lactase genes in the intestinal contents were lower in mice with antibiotics-induced diarrhea than in control mice ($P < 0.05$). There were also significant differences between these mice shown by PCA, PCoA and NMDS analysis, which indicated changes in community structure and decreased diversity of lactase genes in antibiotics-induced diarrhea. A possible reason for this could be the diversity of bacterial species, or known species with inhibitory activity. Unknown types of lactase will contribute to the functions triggered by stresses, such as diarrhea.

Besides the unknown species living in the intestinal contents of model mice, bacteria such as Actinobacteria, Proteobacteria and Firmicutes were more abundant in these mice than in control mice. There were 79.33 ± 0.58 and 76.00 ± 4.58 different

genera in the control group and model group (Table 3), respectively, which reflected the diversity of bacterial lactase genes in the intestinal contents. However, bacteria such as *Gordonia*, *Mycobacterium*, *Frankia*, *Microbacterium*, *Novosphingobium* and *Aeromonas* were exclusively detected in the control group. The differences in species and abundance between the two groups indicated that the intestinal environment in model mice treated with antibiotics may be significantly different from that in control mice.

To maintain intestinal health in mice, diverse bacteria may be critical, and our observations also suggested this. For example, the abundance of lactase genes which originated from bacterial species *Bradyrhizobium* sp. BTAi1, *Agrobacterium* sp. H13-3, *Acidovorax* sp. KKS102, *Azoarcus* sp. KH32C and *Aeromonas caviae* was lower in the model group than in the control group. The same pattern was observed at the bacterial genus level. In addition to *Gordonia, Frankia, Novosphingobium, Mycobacterium, Agrobacterium, Aeromonas* and *Microbacterium*, the abundance of other lactase genes was higher in the model group. All these changes in bacteria indicated that antibiotics reduced or increased the number of certain lactase-producing strains, and thereby affected the level of intestinal lactase activity.

In summary, lactase genes are good indicators for determining the diversity of bacteria in intestinal contents, and may be used to monitor the health of mice by comparing the abundance and diversity of bacterial lactase gene expression. The results of this study show that antibiotics-induced diarrhea reduced the diversity of bacterial lactase genes in the intestinal contents and decreased lactase activity by altering the number of lactase-producing strains or reducing the number of key lactase strains, leading to diarrhea. Our current investigation provides strong support for the potential application of this strategy in the clinic.

ARTICLE HIGHLIGHTS

Research background

Studies have shown that drug- or antibiotics-induced diarrhea is associated with intestinal lactase dysfunction due to loss of activity. Thus, treatment with lactase supplements is a good option for most types of diarrhea due to the importance of lactase activity in the control of intestinal function. Various isoforms of the lactase gene have been identified and are widely expressed in the intestinal tract, with diversity enzyme activities. The expression, protein modification and isoforms can change in different micro-environments. Antibiotics-associated diarrhea is not only associated with dysbacteriosis but also intestinal lactase activity damage, leading to diarrhea. In the present study, we found that the activity of lactase in intestinal contents was significantly reduced in mice with antibiotics-induced diarrhea.

Research motivation

The mechanism of antibiotics-induced diarrhea has been studied in a wide range of diverse microbes. However, less research has been carried out on functional enzymes. In our preliminary study, we found that the activity of lactase in intestinal contents was significantly reduced in mice with antibiotics-induced diarrhea. The present study was conducted in order to determine the mechanism of lactase activity from the viewpoint of genetic diversity and provide a basis for antibiotics-induced diarrhea.

Research objectives

This study was carried out in order to provide a basis for the mechanism of antibiotics-induced diarrhea and to determine whether the alterations in activity were caused by its expression. We compared the diversity of bacterial lactase genes expressed in model mice with antibiotics-induced diarrhea and in control mice.

Research methods

Twelve mature specific pathogen-free Kunming mice were randomly allocated to the control and model groups, with six mice in each group. The mouse model of antibiotics-induced diarrhea was created by gastric perfusion with mixed antibiotics (23.33 $mL \cdot kg^{-1} \cdot d^{-1}$) composed of gentamicin sulfate and cephradine capsules administered for 5 days; the control group received an equal amount of sterile water. The contents of the jejunum and the ileum were then collected and metagenomic DNA was extracted, followed by analysis of bacterial lactase genes using operational taxonomic units after amplification and sequencing. Qiime software was used to align the sequencing results and carry out cluster analysis, principal component analysis (PCA), ACE abundance indexing and Simpson diversity indexing analysis. Principal coordinates analysis (PCoA), nonmetric multidimensional scaling (NMDS) and heatmap analysis were carried out in the R for diversity and similarity. SPSS21.0 software was used for statistical analysis and the results are expressed as means ± SE.

Research results

The results showed that there were significant differences in Chao1 and ACE indices between the two groups ($P < 0.05$). As shown by PCA, PCoA and NMDS analysis, sample distribution in the control group was relatively intensive and differences among individuals were small, while in the model group, they were dispersed and more diversified. The bacterial lactase genes in the intestinal contents from the control mice were related to Proteobacteria, Actinobacteria, Firmicutes and unclassified bacteria. Of these, Proteobacteria showed the greatest abundance. In contrast, the bacterial population was less diversified and abundant in model mice, as the abundance of *Bradyrhizobium* sp. BTAi1, *Agrobacterium* sp. H13-3, *Acidovorax* sp. KKS102, *Azoarcus* sp. KH32C and *Aeromonas caviae* was lower than that in the control group. In addition, of the known species, the control group and model group had their own unique genera, respectively. For example, *Gordonia*, *Mycobacterium*, *Frankia*, *Microbacterium*, *Novosphingobium* and *Aeromonas* were only seen in the control group. However, *Micromonospora*, *Paenibacillus* and *Ensifer* were only found in the model group. To confirm our findings, the diversity and abundance of bacterial species were clearly shown using heatmap analysis. The lactase genes of *Gordonia*, *Frankia*, *Novosphingobium*, *Mycobacterium*, *Agrobacterium*, *Aeromonas* and *Microbacterium* were highly present in the intestinal contents from the model group compared with the control group.

Research conclusions

Antibiotics mainly changed the number of the lactase-producing strains or reduced the number of key lactase strains. Antibiotics reduce the diversity of the intestinal bacterial flora, change the lactase gene strains, and transform their structures.

Antibiotics-induced diarrhea reduced the diversity of bacterial lactase genes in the intestinal contents and decreased lactase activity by altering the number of lactase-producing strains or reducing the number of key lactase strains.

The activity of lactase in intestinal contents was significantly reduced in mice with antibiotics-induced diarrhea. The new hypotheses that this study proposed involves how to screen and identify certain key lactase-producing strains in intestinal contents.

The bacterial lactase gene primers were designed to analyze the diversity of bacterial lactase genes in intestinal contents of mice with antibiotics-induced diarrhea by PCR, gene diversity analysis and bioinformatics techniques.

There were significant differences between control group and model group

mice shown by PCR, PCoA and NMDS analysis. Lactase from different bacterial sources has different nature and activity. The diversity of lactase-producing bacteria leads to diversity of lactase genes and their activities.

Antibiotics-induced diarrhea reduced the diversity of bacterial lactase genes in the intestinal contents and decreased lactase activity by altering the number of lactase-producing strains or reducing the number of key lactase strains, leading to diarrhea.

Research perspectives

Lactase genes are good indicators for determining the diversity of bacteria in intestinal contents, and may be used to monitor the health of mice by comparing the abundance and diversity of bacterial lactase gene expression. The lactase genes will help to explore the regulation mechanism of traditional Chinese medicine on intestinal lactase activity based on the relationship between intestinal lactase diversity and antibiotics-induced diarrhea. The best methods for future research are enzyme technology and gene diversity analysis technology.

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ORIGINAL ARTICLE

Basic Study

Potential rat model of anxiety-like gastric hypersensitivity induced by sequential stress

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Author contributions: Jing FC designed and performed the experiments and wrote the manuscript; Zhang J put forward the conceptualization of the experiments; Feng C, Nian YY, Wang JH, Hu H, Yang BD, Sun XM and Zheng JY performed part of the experiments and collected data; Jing FC and Yin XR were responsible for the analysis of data.

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Abstract

AIM

To establish a rat model of anxiety-like gastric hyper-

sensitivity (GHS) of functional dyspepsia (FD) induced by novel sequential stress.

METHODS

Animal pups were divided into two groups from postnatal day 2: controls and the sequential-stress-treated. The sequential-stress-treated group received maternal separation and acute gastric irritation early in life and restraint stress in adulthood; controls were reared undisturbed with their mothers. Rats in both groups were followed to adulthood (8 wk) at which point the anxiety-like behaviors and visceromotor responses to gastric distention (20-100 mmHg) and gastric emptying were tested. Meanwhile, alterations in several anxiety-related brain-stomach modulators including 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), brain-derived neurotrophic factor (BDNF) and nesfatin-1 in the rat hippocampus, plasma and gastric fundus and the 5-HT1A receptor (5-HT1AR) in the hippocampal CA1 subfield and the mucosa of the gastric fundus were examined.

RESULTS

Sequential-stress-treated rats simultaneously demonstrated anxiety-like behaviors and GHS in dose-dependent manner compared with the control group. Although rats in both groups consumed similar amount of solid food, the rate of gastric emptying was lower in the sequential-stress-treated rats than in the control group. Sequential stress significantly decreased the levels of 5-HT (51.91 ± 1.88 *vs* 104.21 ± 2.88, $P < 0.01$), GABA (2.38 ± 0.16 *vs* 5.01 ± 0.13, $P < 0.01$) and BDNF (304.40 ± 10.16 *vs* 698.17 ± 27.91, $P < 0.01$) in the hippocampus but increased the content of nesfatin-1 (1961.38 ± 56.89 *vs* 1007.50 ± 33.05, $P < 0.01$) in the same site; significantly decreased the levels of 5-HT (47.82 ± 2.29 *vs* 89.45 ± 2.61, $P < 0.01$) and BDNF (257.05 ± 12.89 *vs* 536.71 ± 20.73, $P < 0.01$) in the plasma but increased the content of nesfatin-1 in it (1391.75 ± 42.77 *vs* 737.88 ± 33.15, $P < 0.01$); significantly decreased the levels of 5-HT (41.15 ± 1.81 *vs* 89.17 ± 2.31, $P < 0.01$) and BDNF (226.49 ± 12.10 *vs* 551.36 ± 16.47, $P < 0.01$) in the gastric fundus but increased the content of nesfatin-1 in the same site (1534.75 ± 38.52 *vs* 819.63 ± 38.04, $P < 0.01$). The expressions of 5-HT1AR in the hippocampal CA1 subfield and the mucosa of the gastric fundus were down-regulated measured by IHC (Optical Density value: Hippocampus 15253.50 ± 760.35 *vs* 21149.75 ± 834.13; gastric fundus 15865.25 ± 521.24 *vs* 23865.75 ± 1868.60; $P < 0.05$, respectively) and WB (0.38 ± 0.01 *vs* 0.57 ± 0.03, $P < 0.01$) ($n = 8$ in each group).

CONCLUSION

Sequential stress could induce a potential rat model of anxiety-like GHS of FD, which could be used to research the mechanisms of this intractable disease.

Key words: Gastric hypersensitivity; Anxiety; Functional dyspepsia; 5-hydroxytryptamine; γ-aminobutyric acid; Brain-derived neurotrophic factor; Nesfatin-1; Rat model

Core tip: Functional dyspepsia (FD) is a common gastrointestinal disorder in clinic. Gastric hypersensitivity (GHS) and anxiety are important factors triggering or aggravating it, however, the mechanisms by which affect the development of FD are still unknown. In part, this is due to a lack of suitable animal models of FD with anxiety and GHS. Our study provided such a newly developed rat model induced by sequential stress. It demonstrated the complex behavioral characteristics of anxiety and GHS, and the complicated alterations in some anxiety-related neurobiochemical modulators such as 5-hydroxytryptamine, γ-aminobutyric acid, brain-derived neurotrophic factor and nesfatin-1 in the hippocampus, plasma and gastric fundus.

Jing FC, Zhang J, Feng C, Nian YY, Wang JH, Hu H, Yang BD, Sun XM, Zheng JY, Yin XR. Potential rat model of anxiety-like gastric hypersensitivity induced by sequential stress. *World J Gastroenterol* 2017; 23(42): 7594-7608 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7594.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7594

INTRODUCTION

Functional dyspepsia (FD) is a common gastrointestinal disorder that is characterized by persistent or recurrent upper abdominal pain or discomfort in the absence of any structural, morphological or known organic abnormality, often accompanied by psychosocial disturbance. Gastric hypersensitivity (GHS) is one of the characteristic pathogeneses of FD and represents a cardinal pathophysiological change in FD[1-4]. GHS is closely associated with not only postprandial epigastric pain but also some other symptoms such as early satiety, nausea or vomiting[5,6]. Clinical studies have shown that approximately 35%-65% of FD patients suffer from GHS[7], and among them, 10%-25% have been confirmed to have GHS-related postprandial epigastric pain[8,9]. However, although researchers have focused on GHS in the past, its molecular mechanisms and etiology remain largely unclear.

Anxiety is a common psycho-social disturbance[10] and troubles 40%-90% of the FD patients in the clinic[11]. Various studies have suggested that anxiety may influence gastric sensitivity, gastrointestinal movement, gastric emptying and gut neuroendocrine regulation through the hypothalamic-pituitary-adrenal axis (HPA-axis), autonomic nervous system and endogenous pain regulation system[12-14]. Unfortunately, although anxiety has been identified as an important factor triggering or aggravating FD, the mechanisms by which affect the development of FD and the relationship between them are still unknown. In part, this knowledge gap is due to a lack of both available visceral tissue from FD patients and normal human subjects and

suitable animal models of FD with anxiety[15]. Therefore, to elucidate the pathogenesis of FD and to develop new drugs for use FD treatment, the creation of a novel animal model of FD with anxiety-like GHS is of vital importance.

Animal experiments have shown that acute mild gastric irritation or maternal separation (MS) in the neonatal period can induce hypersensitivity to gastric distention in adult rats[16,17]. MS, especially, can have long-lasting influences on emotionality[18], stress responsiveness[19], neurotransmitters in the central nervous system and enteric nervous system[20,21], the HPA-axis[22] and visceral sensitivity[23]. Clinical studies have also demonstrated that adverse physiological or psychological experiences in early life are linked to the development of FD and acute stress in adulthood[24,25]. Meanwhile, some neuromodulators such as 5-hydroxytryptamine (5-HT)[26-28], γ-aminobutyric acid (GABA)[29,30], brain-derived neurotrophic factor (BDNF)[15,31] and nesfatin-1[32,33] are involved in the regulation of anxiety, depression and other psychosocial activities as well as visceral sensations. These anxiety-related brain-gut modulators are neurobiochemical regulatory substances that are shared by the brain and gut of FD patients. Generally, a decrease in 5HT, GABA and BDNF levels or an increase in the nesfatin-1 content in the brain, stomach and plasma may induce or aggravate anxiety-like symptoms and behavior while increasing visceral sensitivity. Based on these findings, the present study was designed to expose animals to novel sequential stress, MS and acute gastric irritation (AGI), early in life followed by exposure to restraint stress (RS) in adulthood, in the hopes of creating a new anxiety-like GHS rat model of FD and exploring the alterations in 5-HT, GABA, BDNF, and nesfatin-1 in the hippocampus, plasma and mucosa of the gastric fundus and the 5-HT1A receptor (5-HT1AR) in the hippocampal CA1 subfield to more deeply understand the molecular mechanisms of these complex clinical disorders from the perspective of brain-blood-stomach axis.

MATERIALS AND METHODS

Animals and reagents

Male Sprague-Dawley rat pups were used in this study and housed with their mothers in cages lined with sterilized bedding materials. The animal protocol was designed to minimize pain or discomfort to the animals. The animals were kept under a constant temperature of 24 ℃ ± 1 ℃ and relative humidity of 55% ± 5% with a 12-h light: 12-h dark cycle (lights on at 7:00 a.m.). The animals had access to regular chow diet and water ad libitum. For the implementation of anesthesia, an intraperitoneal injection of 50 mg/kg of sodium pentobarbital was used. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection. All experiments were performed according to the guidelines established by the European Community for the Care and Use of Laboratory Animals and were approved by the Experimental Animal Care and Use Committee of the Xi'an Jiao Tong University.

Iodoacetamide (IA) was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). The enzyme-linked immunosorbent assay (ELISA) kits for myeloperoxidase (MPO), 5-HT, GABA, BDNF and nesfatin-1 were purchased from Shanghai Hong Ju Biological company (Shanghai, China). The 5-HT1AR antibody was purchased from Abcam company (Cambridge, United Kingdom). They were used following the manufacturer's instructions. All other reagents were of analytical grade and purchased from Guo Yao Group Co., Ltd. (Shanghai, China).

Experimental design

Animals were randomly divided into two groups: Control rats and sequential- stress-treated rats. The rat pups in the sequential-stress-treated group received MS from postnatal day 2 (PND 2) to PND 21, during which AGI was given to them from PND 10 to PND 16. Meanwhile, the pups in the control group were still reared undisturbed with their mothers without undergoing MS or AGI. All animals, including the controls and the sequential-stress-treated ones, were weaned at PND 22, reared up to 8 wk, and then the sequential-stress-treated rats were forced to undergo RS for 7 d, while the control rats were housed freely. From the 9th postnatal week, the control and sequential-stress-treated rats began to undergo the operation for the implantation of the gastric balloon and electrodes for behavioral and electromyographic (EMG) testing. One week after surgery, the rats were first tested on the elevated plus maze (EPM). Then, on the day following the EPM, the open field (OF) experiment was performed on the same rats. Twenty-four hours after the OF test, abdominal withdrawal reflex (AWR) testing, EMG recordings, and blood collection were performed on the rats. According to previously published studies[34,35], the hippocampus is one of the important brain regions closely related to the regulation of mood such as anxiety and depression, while the gastric fundus is modulated mainly by serotonergic mechanisms. Therefore, the present study chose the hippocampus and gastric fundus of the animal as two of its research targets. After blood collection, some of the rats were perfused transcardially and immediately with ice-cold formalin (40 g/L) solution, and the brain and stomach were quickly removed for immunohistochemistry (IHC) measurements. Others of the rats were sacrificed by rapid decapitation and the hippocampus and gastric fundus were isolated carefully and dropped in liquid nitrogen immediately and stored at -80 ℃ for ELISA or Western blot (WB) measurements. Meanwhile, a different group of sequential-stress-treated and control rats was used only for the gastric emptying experiment.

Maternal separation

Maternal separation (MS) was performed as previously described[36]. Briefly, the rat pups that were randomly

assigned to the sequential-stress-treated group were taken away from their maternity cages, placed in a smaller cage and housed individually in a separate room for 3 h daily (9:00 am-12:00 pm) from PND 2 to PND 21. After separation, the pups were then returned to their home cage. Pups in the control group were reared undisturbed with their mothers. At PND 22, all of the pups were weaned and maintained to 8 wk of age.

Acute gastric irritation

The PND 10 rat pups in the sequential-stress-treated group were administered 0.2 mL 1 g/L IA in 20 g/L sucrose daily for 6 consecutive days by oral gavages. Pups in the control group received 0.2 mL 20 g/L sucrose[16].

Restraint stress

The rats in the sequential-stress-treated group were restrained in cylindrical and well-ventilated tubes for 90 min daily for 1 wk beginning at 8 wk of age. They were returned to their rearing cages immediately after restraint[37].

Implantation of the gastric balloon and electrodes

After being fasted overnight prior to surgery, rats were anesthetized with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital[17]. Balloons (2.5 cm long) made from latex gloves were fixed to a long catheter (PE-240). A left lateral epigastric incision was made, and the balloon was placed in the stomach through a small hole made at the tip of the fundus. The pylorus was not obstructed so that there was no blockage of gastric emptying. The hole was then tied tightly to avoid leakage of gastric fluid into the peritoneum. The catheter was exteriorized at the back of the neck along with the EMG electrode leads. The electrodes were implanted into the acromiotrapezius muscle and externalized at the back of the neck. One week later, the behavioral and EMG tests were performed.

AWR and EMG recordings

AWR to graded gastric distention (20-100 mmHg) was tested on day 6 after the implantation of the gastric balloon. Each pressure was achieved by inflating the balloon for 20 s followed by a 5-min interval[15]. The AWR was graded as previously reported[16]: 0, no behavioral response to gastric distention (GD); 1, brief head movement followed by immobility; 2, contraction of abdominal muscles; 3, lifting of abdomen; 4, body arching, lifting of pelvic structures and stretching of body. Visceromotor responses (VMRs) to GD were recorded simultaneously along with measuring the AWR with a BL-420S biological signal collecting and processing system (Techman Software CO., Chengdu, China). EMG activity (including baseline and during every pressure distention of the stomach) was then rectified and quantified by calculating the area under the curve (AUC). VMRs to GD were expressed as a percentage increase over baseline.

EPM

A standard PMT-100 rat EPM (Techman Software CO., Chengdu, China) was used in this experiment. The maze consists of four arms (two open without walls and two enclosed by 30-cm-high walls) 50 cm long and 10 cm wide. Each arm was elevated 50 cm off the floor. The light intensity was set at 300 lux. The rat's movements were automatically recorded by a video-tracking system (Techman Software CO., Chengdu, China) to collect the behavioral data. Every procedure was started by placing the rat at the junction of the open and closed arms, facing the open arm opposite to where the experimenter was standing and lasted 5 min[38]. The maze was cleaned with alcohol between each test animal. The EPM test is based on the rat's avoidance of open spaces. This avoidance is thought to be one of the anxiety-like behavioral characteristics, namely, thigmotaxis, which implies an aversion for open areas by the rats' tendency to remain in enclosed spaces. The increased amount of time spent in the closed arms usually indicates high anxiety-like behavior.

OF testing

The OF consisted of a square arena (100 cm × 100 cm with twenty-five 20 cm × 20 cm virtual grids and four 40-cm-high walls) was divided into a peripheral zone and a central zone (60 cm × 60 cm). Rats were positioned in the center of the OF and allowed to freely explore for 5 min. The light intensity was also set at 300 lux. The time spent in the central area, number of virtual grids climbed and the velocity of the rat's movement were examined[39].

Gastric emptying testing

In another group of sequential-stress-treated and control rats, the gastric emptying of a solid meal was investigated as demonstrated previously[40]. Rats that had been fasted overnight were allowed to freely consume water and a preweighed amount of solid food for 3 h. After that, the water and food were removed, and the rats rested for 3 h. The rats were then killed, and the stomach was removed and emptied thoroughly. The rate of gastric emptying was calculated by the following formula: Gastric emptying (%) = 100 - (gastric content/food intake) × 100.

Histology

Gastric tips cut from along the greater curvature of the killed rats were immersed in 10% formaldehyde for at least 72 h. Then, four-micrometer sections from paraffin-embedded tissue specimens were processed for HE staining for histological measurement.

Assay of MPO activity and levels of 5-HT, GABA, BDNF, nesfatin-1 and 5-HT1AR

Under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally) following the AWR and EMG

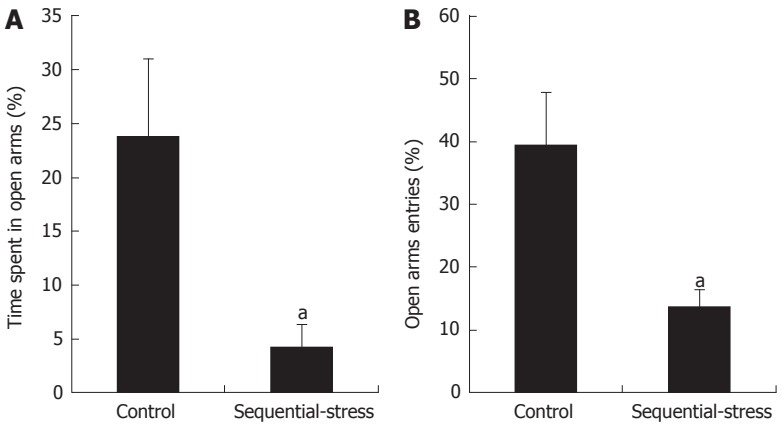

Figure 1 Elevated plus maze examination showed the sequential-stress-treated rats demonstrated anxiety-like behavior. A: They spent less time in the open arms; B: They made fewer entries into the open arms. Data were expressed as the mean ± SEM of the percentage of time spent in open arms and open arms entries (n = 8 in each group). $^{a}P < 0.05$ *vs* control.

testing, 1 mL blood was collected from the rat tail vein using a syringe (1 mL), smoothly injected into a 1.5 mL centrifugal tube and centrifuge 3000 rpm for 15 min. Then, 300 μL supernatant (plasma) was stored in a vial and kept at -80 ℃ until analysis. Once the blood collection was finished, some of the rats were perfused transcardially and immediately with ice-cold formalin (40 g/L) solution, and the brain and stomach were quickly removed and post-fixed by immersing in 40 g/L formalin solution at 4 ℃ overnight for immunohistochemistry (IHC) measurements[45]. Others of the rats were sacrificed by rapid decapitation and the hippocampus and gastric fundus (by another operator) were then isolated carefully, wrapped in foil and dropped in liquid nitrogen immediately and stored at -80 ℃ for ELISA or Western blot (WB) measurements[46]. The ELISA assays of MPO activity and the levels of 5-HT, GABA, BDNF and nesfatin-1 were analyzed as described by the instructions of the ELISA kits and the methods described previously[32,41-44]. The IHC and WB tests of the levels of 5-HT1AR in the hippocampal CA1 subfield or the mucosa of the gastric fundus were also performed using the methods reported previously[45,46].

Statistical analysis

Data were expressed as the mean ± SEM. Student's *t*-test or 2-way repeated measures ANOVA were used for comparisons. *Post hoc* comparisons were made using the Student-Newman-Keuls test. Statistical analysis was performed using SPSS 17.0 software kits. A value of $P < 0.05$ was considered significant.

RESULTS

EPM

The EPM experiment showed that the sequential-stress-treated rats spent less time in the open arms than the control rats (4.26 ± 2.10 s *vs* 23.76 ± 7.24 s, $P < 0.05$, Figure 1A; n = 8 in each group). They also entered into the open arms fewer times than the control group (13.67 ± 2.77 *vs* 39.49 ± 8.31, $P < 0.05$, Figure 1B).

OF testing

In the OF test, animals showing anxiety-like behavior usually demonstrate a decreased amount of time spent in the central area and decreased number of virtual grids climbed. Our results were in accordance with these phenomena (Time spent in the central area: 7.27 ± 1.00 s *vs* 12.53 ± 2.16 s, $P < 0.05$; Figure 2A; Number of virtual grids climbed: 30.86 ± 2.96 *vs* 49.14 ± 6.88, $P < 0.01$; Figure 2B for the sequential-stress-treated and control groups, respectively; n = 8 in each group). The two groups also showed significant differences in the total exploring distance (Locomotor activity: 617.14 ± 59.11 *vs* 982.86 ± 137.56, $P < 0.05$; Figure 2C) and mean velocity in the OF test (Velocity: 123.43 ± 11.82 *vs* 196.57 ± 27.51, $P < 0.05$; Figure 2D).

GHS

At 8 wk, the effects of gastric distention (GD) on the rats were examined. The AWR experiments showed that the behavioral scores in response to GD in the sequential-stress-treated rats were significantly higher than in the control group (20 mmHg: 0.67 ± 0.33 *vs* 0.17 ± 0.17, $P > 0.05$; 40 mmHg: 2.33 ± 0.33 *vs* 1.00 ± 0.26, $P < 0.05$; 60 mmHg: 2.83 ± 0.48 *vs* 1.50 ± 0.22, $P < 0.05$; 80 mmHg: 3.84 ± 0.31 *vs* 2.17 ± 0.17, $P < 0.01$; 100 mmHg: 4.33 ± 0.33 *vs* 2.82 ± 0.30, $P < 0.01$; Figure 3A, n = 8 in each group). Statistical analysis indicated significant differences in the AWR scores at 40, 60, 80 and 100 mmHg.

These results were verified by computing the AUCs of the EMG recordings of the acromiotrapezius, which were used to quantify the VMRs to GD. Rats in the sequential-stress-treated group exhibited significantly greater EMG responses and significantly higher AUCs of the EMG activity than those in the control group at

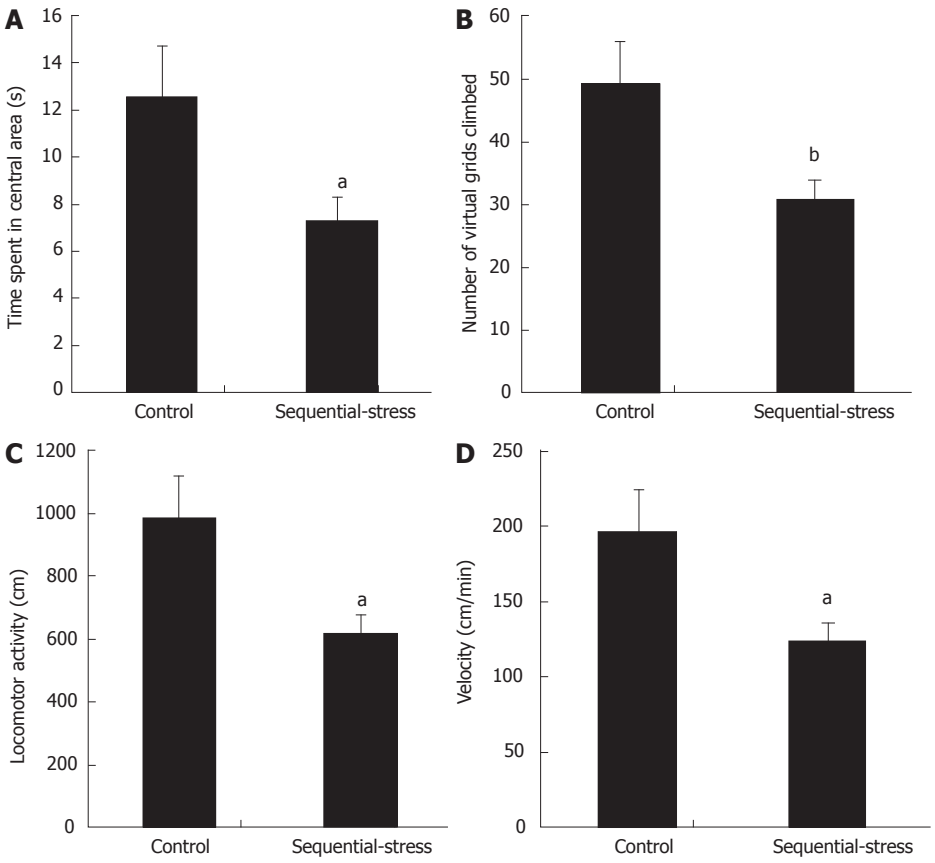

Figure 2 OF test showed the sequential-stress-treated rats demonstrated anxiety-like behavior. A: They spent less time in central area; B: They climbed fewer number of virtual grids in OF box; C: Their total exploring distance was shorter; D: Their mean moving speed was slower. Data were expressed as the mean ± SEM of the time spent in central area, number of virtual grids climbed, total exploring distance and mean moving speed (n = 8 in each group). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

GD pressures 40, 60, 80 and 100 mmHg (146.23 ± 2.95 *vs* 102.97 ± 0.59, 202.39 ± 3.73 *vs* 156.00 ± 2.24, 243.79 ± 3.10 *vs* 193.93 ± 1.22, 293.05 ± 4.75 *vs* 258.17 ± 0.54, respectively, $P < 0.01$; Figure 3B, n = 8 in each group). A representative EMG response from both an adult sequential stress-treated rat and control rat to GD is shown in Figure 3C.

Changes in the levels of 5-HT, GABA, BDNF and nesfatin-1 in the hippocampus

The ELISA results showed that compared with the levels in the control group, sequential stress significantly decreased the levels of 5-HT (51.91 ± 1.88 *vs* 104.21 ± 2.88, $P < 0.01$, Figure 4A), GABA (2.38 ± 0.16 *vs* 5.01 ± 0.13, $P < 0.01$; Figure 4B) and BDNF (304.40 ± 10.16 *vs* 698.17 ± 27.91, $P < 0.01$; Figure 4C) in the hippocampus but obviously increased the content of nesfatin-1 in the same site (1961.38 ± 56.89 *vs* 1007.50 ± 33.05, $P < 0.01$; Figure 4D; n = 8 in each group).

Changes in the levels of 5-HT, BDNF and nesfatin-1 in the plasma

Compared with the levels in the control group, the levels of 5-HT (47.82 ± 2.29 *vs* 89.45 ± 2.61, $P < 0.01$; Figure 5A) and BDNF (257.05 ± 12.89 *vs* 536.71 ± 20.73, $P < 0.01$; Figure 5B) in the plasma of the sequential-stress-treated rats decreased significantly, but the content of nesfatin-1 in the plasma of the sequential-stress-treated rats was obviously increased (1391.75 ± 42.77 *vs* 737.88 ± 33.15, $P < 0.01$, Figure 5C; n = 8 in each group).

Changes in the levels of 5-HT, BDNF and nesfatin-1 in the gastric fundus

Compared with the levels in the control group, the levels of 5-HT (41.15 ± 1.81 *vs* 89.17 ± 2.31, $P < 0.01$; Figure 6A) and BDNF (226.49 ± 12.10 *vs* 551.36 ± 16.47, $P < 0.01$; Figure 6B) in the gastric fundus of the sequential-stress-treated rats decreased significantly, but the content of nesfatin-1 in the gastric fundus of the sequential-stress-treated rats was obviously increased (1534.75 ± 38.52 *vs* 819.63 ± 38.04, $P < 0.01$; Figure 6C; n = 8 in each group).

Changes in the levels of 5-HT1AR in the hippocampal CA1 subfield and mucosa of gastric fundus by IHC

The IHC results revealed that, compared with the levels in the controls, the levels of 5-HT1AR expression in the hippocampal CA1 subfield (Figure 7A) and the mucosa

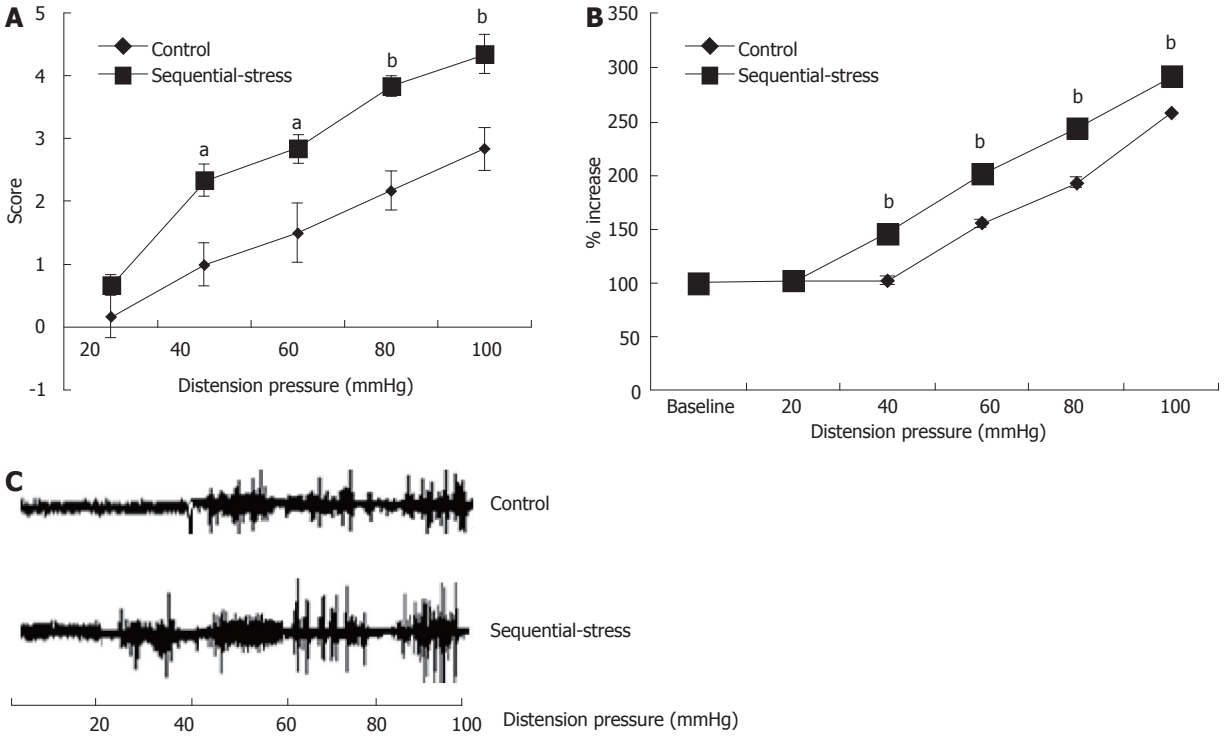

Figure 3 Abdominal withdrawal reflex and electromyographic tests showed the sequential-stress-treated rats exhibited gastric hypersensitivity to gastric distention. A: AWR to GD of the sequential-stress-treated rats was significantly higher at the distention pressure 40, 60, 80 and 100 mmHg; B: EMG responses of the sequential-stress-treated rats were greater at the distention pressure 40, 60, 80 and 100 mmHg; C: The representative EMG response from both an adult sequential-stress-treated rat and control rat to GD. Data were expressed as the mean ± SEM of the AWR score and the percentage of EMG derived AUC increased (n = 8 in each group). [a]P < 0.05, [b]P < 0.01 *vs* control group.

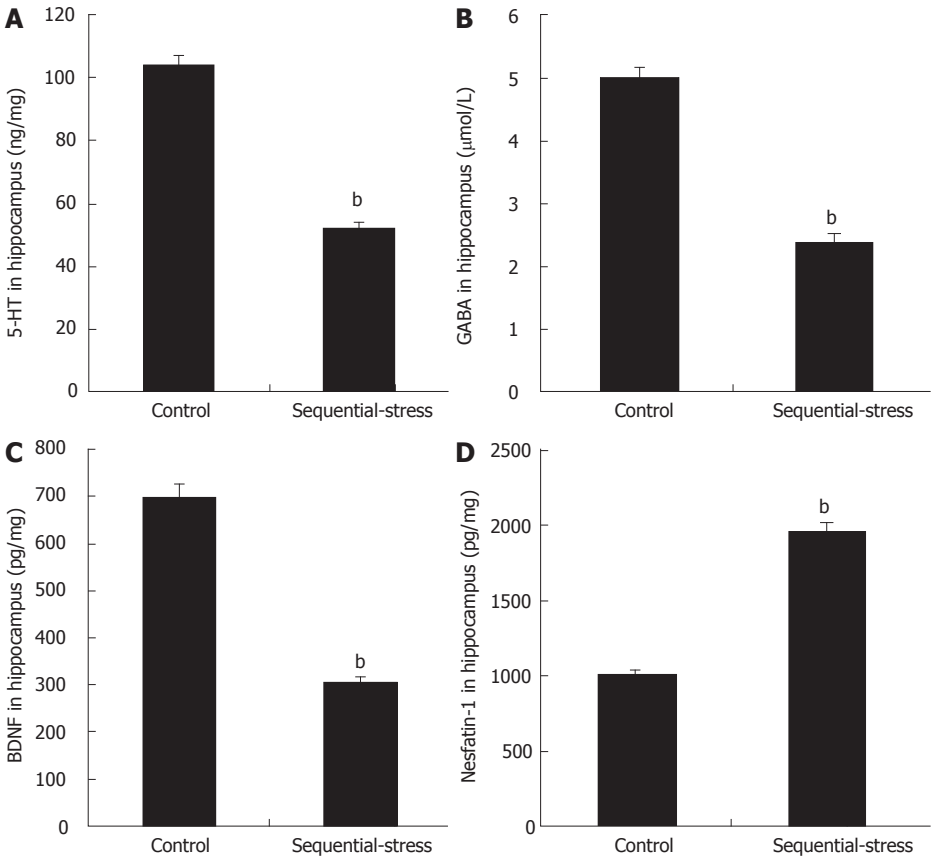

Figure 4 Influence of sequential stress on the levels of 5-hydroxytryptamine, γ-aminobutyric acid, brain-derived neurotrophic factor and nesfatin-1 in hippocampus. A: 5-hydroxytryptamine (5-HT); B: γ-aminobutyric acid (GABA); and C: brain-derived neurotrophic factor (BDNF) were significantly down-regulated; D: Nesfatin-1 was significantly up-regulated. Data were expressed as the mean ± SEM of the levels of 5-HT, GABA, BDNF and Nesfatin-1 in Hippocampus (n = 8 in each group). [b]P < 0.01 *vs* control group.

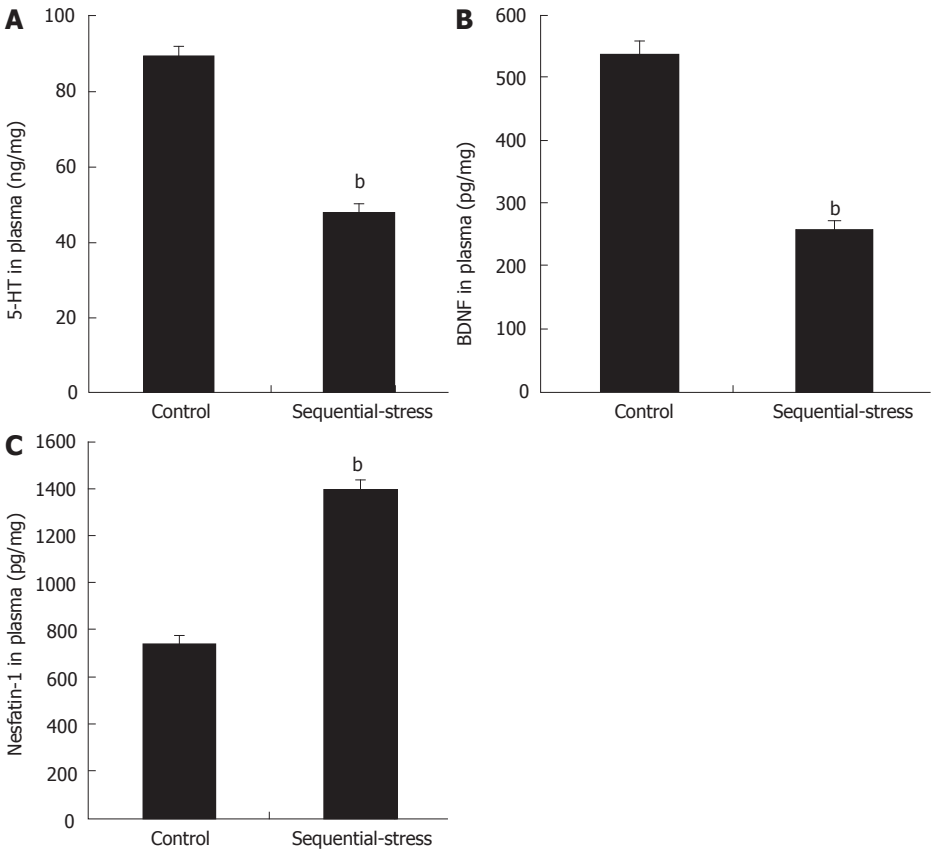

Figure 5 Influence of sequential stress on the levels of 5-hydroxytryptamine, brain-derived neurotrophic factor and nesfatin-1 in plasma. A: 5-hydroxytryptamine (5-HT) and B: Brain-derived neurotrophic factor (BDNF) was significantly down-regulated; C: Nesfatin-1 was significantly up-regulated. Data were expressed as the mean ± SEM of the levels of 5-HT, BDNF and Nesfatin-1 in plasma (n = 8 in each group). $^{b}P < 0.01$ *vs* control group.

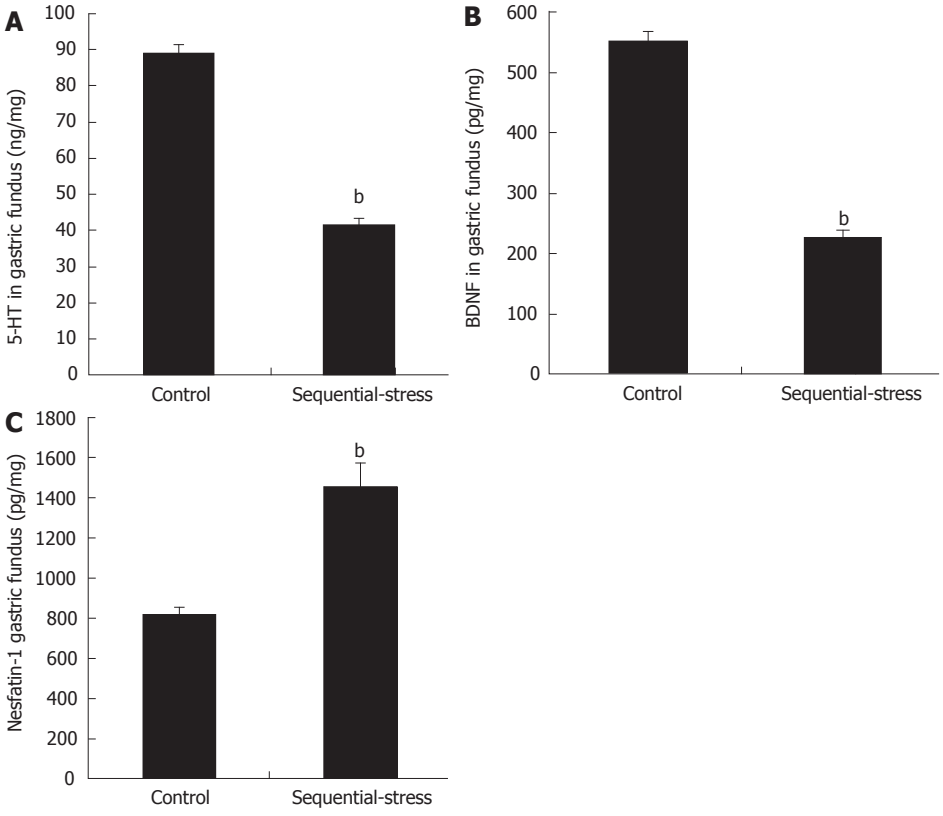

Figure 6 Influence of sequential stress on the levels of 5-hydroxytryptamine, brain-derived neurotrophic factor and nesfatin-1 in gastric fundus. A: 5-hydroxytryptamine (5-HT) and B: Brain-derived neurotrophic factor (BDNF) were significantly down-regulated; C: Nesfatin-1 was significantly up-regulated. Data were expressed as the mean ± SEM of the levels of 5-HT, BDNF and Nesfatin-1 in gastric fundus (n = 8 in each group). $^{b}P < 0.01$ *vs* control group.

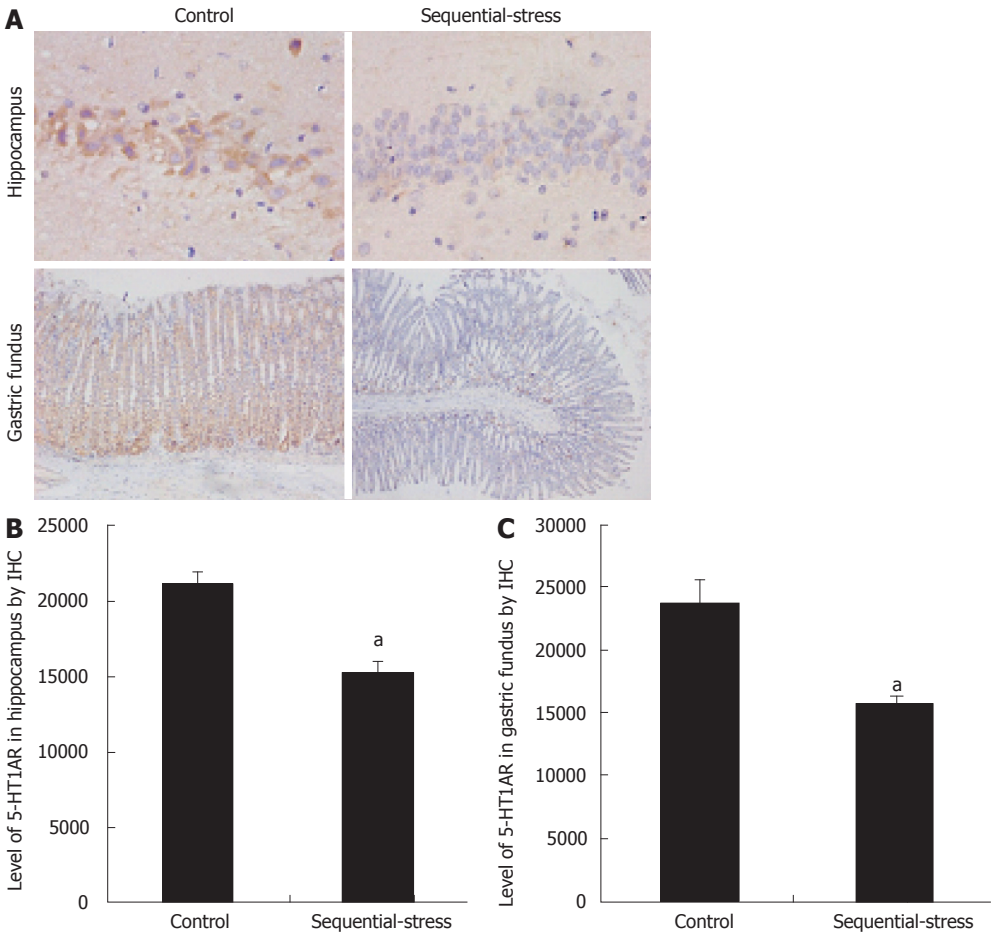

Figure 7 Presentation of 5-hydroxytryptamine 1A receptor expression in the hippocampal CA1 subfield and the mucosa of gastric fundus of each group by immunohistochemistry. A: Magnification, hippocampus, × 400 and gastric fundus, × 100; B: Level of 5-hydroxytryptamine 1A receptor (5-HT1AR) in hippocampus; C: Level of 5-HT1AR in gastric fundus. Data were expressed as the mean ± SEM of the levels of 5HT1AR (n = 8 in each group). $^{a}P < 0.05$ *vs* control group.

of the gastric fundus (Figure 7A). Aof the sequential-stress-treated rats were both down-regulated, while the levels of 5-HT were reduced in these same sites (Optical Density value: Figure 7B hippocampus 15253.50 ± 760.35 *vs* 21149.75 ± 834.13 and Figure 7C gastric fundus 15865.25 ± 521.24 *vs* 23865.75 ± 1868.60; $P < 0.05$, respectively; n = 8 in each group).

Changes in the levels of 5-HT1AR in the hippocampal CA1 subfield by WB

The WB assay of the relative quantitative level of 5-HT1AR in the hippocampal CA1 subfield demonstrated that the expression of 5-HT1AR in this region was significantly lower in the sequential-stress-treated rats than in the control group (0.38 ± 0.01 *vs* 0. 57 ± 0.03, $P < 0.01$; Figure 8; n = 8 in each group).

Lack of an inflammatory response in the gastric wall of FD-like rats

In the sequential-stress-treated group, gastric histology only showed superficial sloughing of the mucosa on day 6, and no deeper injury or inflammation was revealed compared with that in the control group (Figure 9A; n = 8 in each group). At the same time, the myeloperoxidase (MPO) activity assay demonstrated no significant difference between the two groups (505.28 ± 64.10 *vs* 467.00 ± 34.33, $P > 0.05$, Figure 9B; n = 8 in each group). In another set of experiment, sequential-stress-treated rats were followed to adulthood and treated with RS as described before. On day 56 post-treatment, neither histology (Figure 9C) nor the MPO activity test (670.91 ± 79.80 *vs* 462.30 ± 60.07, $P > 0.05$, Figure 9D; n = 8 per group) showed a lesion or abnormality in the gastric mucosa of either group.

Delayed gastric emptying

After 18 h of fasting, the adult rats of the sequential-stress-treated and control groups consumed similar amount of solid food in a 3-h period (6.52 ± 0.34 g *vs* 6.75 ± 0.51 g, $P > 0.05$, respectively; Figure 10A, n = 8 in each group). However, compared with that observed in the controls, the sequential-stress-treated rats exhibited a significant increase in the gastric contents 3 h post food intake (3.50 ± 0.51 *vs* 2.02 ± 0.38, $P < 0.05$; Figure 10A) and decrease in the rate of gastric emptying of the food ingested (49.55 ± 5.11

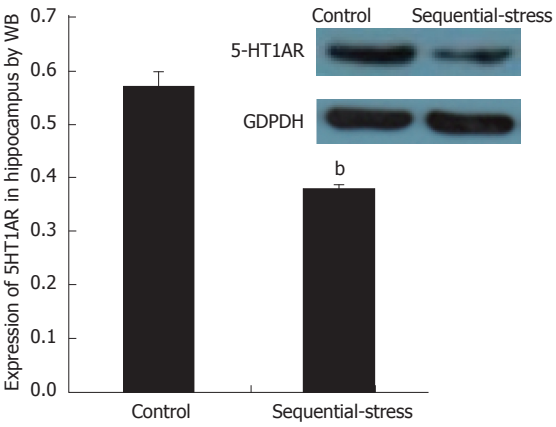

Figure 8 Western blot detection showed a significant decrease of the level of 5-hydroxytryptamine 1A receptor in the hippocampal CA1 subfield of the sequential-stress-treated rats. Data were expressed as the mean ± SEM of the levels of 5-hydroxytryptamine 1A receptor (5-HT1AR) ($n = 8$ in each group). $^{b}P < 0.01$ *vs* control group.

vs 71.37 ± 3.36, $P < 0.05$; Figure 10B).

DISCUSSION

Anxiety is one of the most common symptoms among FD patients and an important cause of refractory FD, with a high incidence of 40%-90% in the clinic[11]. As previously described, anxiety may induce a functional disturbance of the HPA-axis and neuroendocrine regulation system, increase gastric sensitivity, and further promote the development of FD[14,47]. However, the cellular and molecular mechanisms underlying these actions are still unknown. One of the primary reasons for this gap in knowledge is that researchers lack mature animal models mimicking anxiety-like FD with or without GHS.

Previous studies have suggested that neonatal or adolescent adverse physiological or psychological experiences and adult acute stress are all factors associated with the development of FD[24,25]. Acute mild gastric irritation in the neonatal period may be one of the causes of chronic GHS and gut dysfunction[16]. As a negative stimulus in childhood, MS has been proven to induce anxiety-like behavior[48,49], affect the regulation from the hypothalamus to the adrenal gland and the retro-regulation from the adrenal gland to hypothalamus of the HPA-axis, and result in an anxiety-like psychological stress reaction[50]. MS can also have long-lasting influences on emotionality[18], stress responsiveness[19], neurotransmitters in the central nervous system and enteric nervous system[20,21], and visceral sensitivity[23]. Acute stress in adulthood has similar effects on the HPA-axis as MS and has a certain impact on gastric motility and gastric accommodation by influencing the secretion of ghrelin[35,51].

However, although studies published previously have reported some FD-like GHS animal models that have been successfully created using MS, acute stress or neonatal gastric irritation[15-17], few of them focused on the psychological stress responses such as anxiety and depression that the stimuli caused in the experimental animals and the interaction or mechanisms between the psychological stress responses and the GHS in FD from the perspective of brain-blood-stomach axis.

Our study showed that although AGI combined with MS and RS could induce the superficial sloughing of the gastric mucosa in the initiation stage, this change could recover rapidly in a short period, and the histological and MPO tests of the gastric mucosa were completely normal at 8 weeks postnatal, which agrees with the definition of FD. Compared with that in the controls, there was a significant decrease in the rate of gastric emptying in the sequential-stress-treated rats but no significant difference in the food intake amount between the two groups. These findings are in accordance with the clinical observations that up to 40% of patients with FD suffer from a delay in gastric emptying[52-54]. In addition, the AWR and EMG tests of our study also successfully mimicked the GHS in FD, which has been thought to exist in 35%-65% of patients with FD and to be a main cause for refractory FD[1,2,7].

Anxiety is another characteristic of FD. Our study found that the sequential-stress-treated rats spent less time and showed fewer entries into the open arms of EPM than the control rats. Similarly, in the OF test, the sequential-stress-treated rats seemed to avoid staying in the central area, moved a shorter distance, climbed fewer grids, and walked slower than the control animals. The sequential-stress-treated rats demonstrated typical anxiety-like behaviors. These results showed that we have successfully developed a potential anxiety-like GHS rat model of FD.

5-HT is thought to be closely related to both anxiety and the function of the gut system, but the mechanism of its effects is still controversial. In the brain, some reports have found that inhibiting the reuptake of 5-HT by the presynaptic membrane, activating the 5-HT1AR, or up-regulating the expression of the 5-HT1AR protein may produce anxiolytic effects[26,55-57]. In the gut, there are five types of 5-HT receptors, the 5-HT1, 2, 3, 4, and 7 receptor, distributed throughout the gastrointestinal wall. The different types of receptors play different roles in the regulation of gastrointestinal function. The 5-HT1AR and 5-HT7 receptor in the stomach mainly mediate the gastric accommodative relaxation and the delay of gastric emptying[58-60], while the 5-HT2, 3, and 4 receptors mainly participate in the contractive regulation of the gastrointestinal smooth muscle[27]. The present study found that the levels of 5-HT and the expression of 5-HT1AR in the hippocampus and gastric fundus of the sequential-stress-treated rats were synchronously diminished compared with the levels in the control rats, in agreement with the changes observed in the behavioral experiments. These results implied that the 5-HT and 5-HT1AR in the hippocampus and gastric fundus participate in not only the regulation

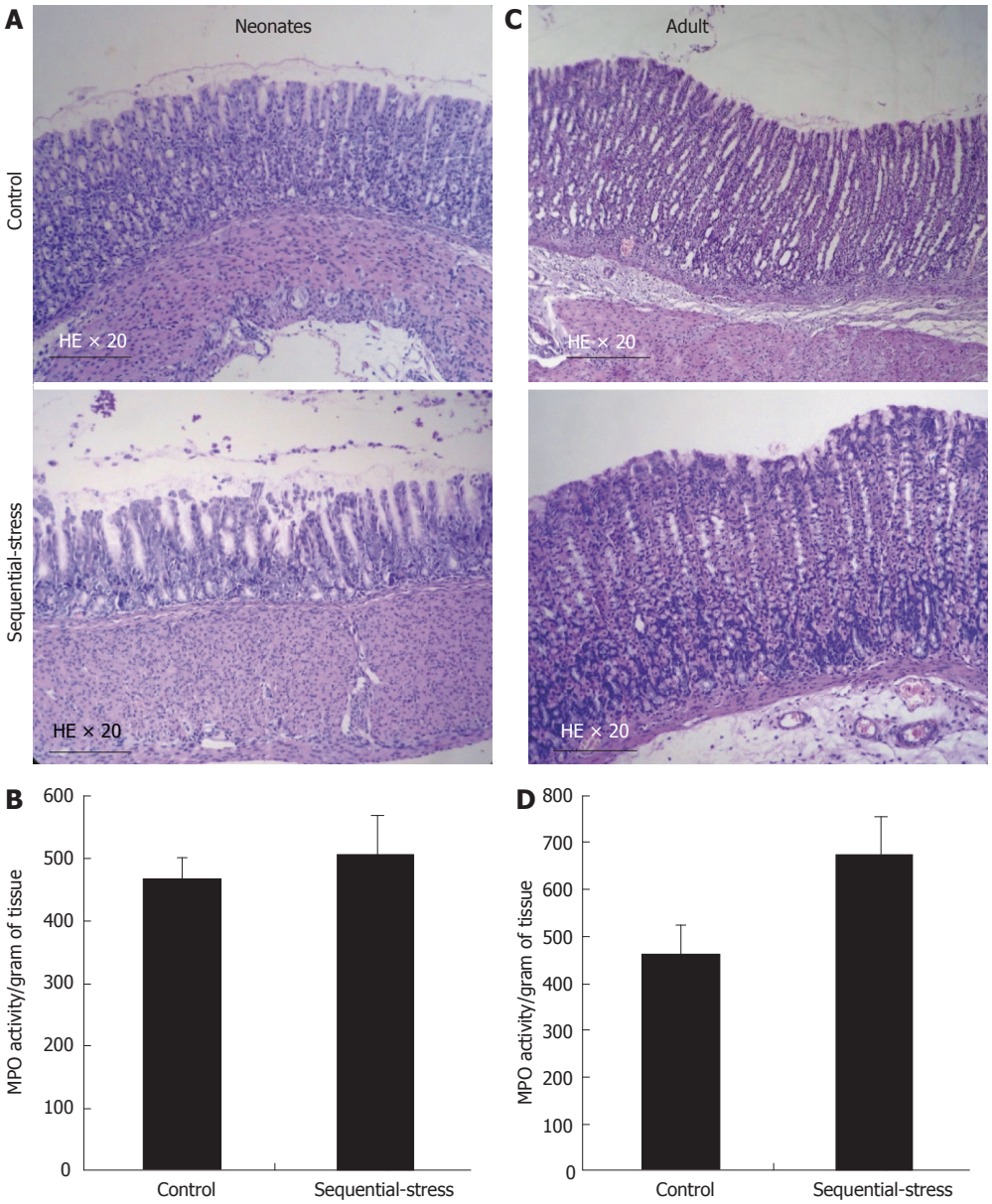

Figure 9 Influence of the sequential stress on inflammatory response of gastric mucosa. A: On day 6 in early life only superficial sloughing of the mucosa was observed in the sequential-stress-treated group; B: Myeloperoxidase (MPO) activity assay at the same time demonstrated no statistical significance between two groups; C and D: after RS treatment, both histology and MPO activity test display no lesion or abnormality in the gastric mucosa of the two groups. MPO activity is represented as activity per unit of dry weight. Data were expressed as the mean ± SEM of the MPO activity (n = 8 in each group).

of anxiety-like responses but also the development of FD symptoms.

GABA is another important inhibitory neurotransmitter in the central nervous system. When it is deficient, animals will manifest obvious psychological responses such as anxiety, insomnia, instable mood and so on[61]. There are known to be two subtypes of the GABA receptor, $GABA_A$ and $GABA_B$, and both of them have anxiolytic effects when activated[29,62]. In addition, a recent study has reported a novel finding that the $GABA_BR$ agonist baclofen produced an analgesic effect on the visceral pain of FD rats. This result indicated that in an anxious state, the deficiency of GABA or $GABA_BR$ in the hippocampus could down-regulate the threshold of visceral pain and elevate the sensitivity of visceral perception[30]. In our current study, the concentration of GABA in the hippocampus of the sequential-stress-treated rats was significantly lower, but the anxiety-like behaviors and the gastric sensitivity were greatly higher than in the control rats. These findings, along with those cited above, suggest that GABA takes part in the formation of anxiety-like GHS in FD.

BDNF is the most prominent neurotrophic factor throughout the mammalian brain including the hippocampus and plays an important role in the regulation of the development, plasticity and survival of dopaminergic, cholinergic, serotonergic and GABAergic neurons[63-64]. BDNF is also related to the formation of anxiety[65]. When the BDNF gene is knocked out or the expression of BDNF is down-regulated in the hippocampus, animals will

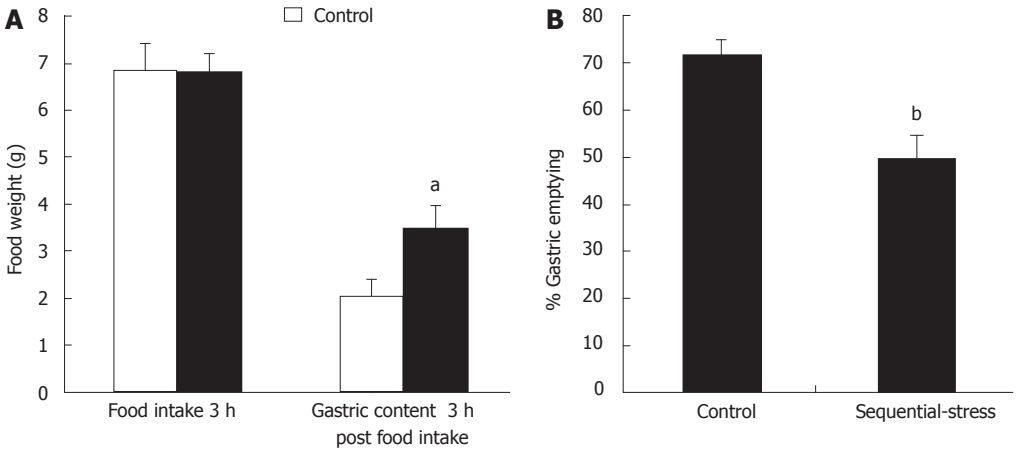

Figure 10 Influence of sequential stress on the rate of gastric emptying. A: Although the food intake in 3 hours after 18 h fast were similar between the two groups, the gastric contents 3 h post food intake in sequential-stress-treated rats increased; B: the rate of gastric emptying of the food ingested in sequential-stress-treated rats decreased. Data were expressed as the mean ± SEM of the food weight and the rate of gastric emptying (n = 8 in each group). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

demonstrate an anxious mood and behavior; however, after BDNF is injected into the cerebral region, these changes are completely alleviated[31,66]. In addition, some research has discovered that changes in the levels of BDNF in certain parts of the body are correlated with gut sensitivity[15,67]. Our study found that the content of BDNF in the hippocampus, plasma and gastric fundus was greatly reduced in the sequential-stress-treated rats, implying that BDNF participated in the regulation of anxiety-like behavior and gastric sensitivity. The changes in BDNF were in line with those of 5-HT, 5-HT1AR and GABA.

Nesfatin-1 is a peptide hormone comprised of 82 amino acids. Nesfatin-1 has been considered to be able to reduce food intake and induce an anxiety-like emotional reaction[68,69]. Evidence has shown that in anorexia nervosa patients with anxiety the plasma levels of nesfatin-1 are elevated, and injection of nesfatin-1 into the cerebral ventricle may delay gastric emptying[70]. Nesfatin-1 could also influence visceral perception and increase visceral sensitivity[33]. The present study found that the levels of nesfatin-1 in the hippocampus, plasma and gastric fundus were significantly elevated in the sequential-stress-treated rats, which was in accordance with the anxiety-like behavior and the delay in gastric emptying. From our study together with previous findings, nesfatin-1 can be inferred to potentially be one of the most important regulatory hormones in anxiety-like GHS in FD.

In brief, sequential using a combination of MS, AGI and RS, the present study provided a potential anxiety-like GHS rat model of FD. This model demonstrated the complex behavioral characteristics of anxiety and GHS. Based on this model, our study initially observed the complicated alterations in some anxiety-related neurobiochemical modulators in brain-blood-stomach axis such as 5-HT, GABA, BDNF and nesfatin-1 in the hippocampus, plasma and gastric fundus of this model. The results indicated that more than one biological regulatory factor is involved in the modulation of FD patients with GHS and anxiety. Furthermore, this study supplied a possible tool for the development of new drugs and the exploration of the mechanisms of FD.

ARTICLE HIGHLIGHTS

Research background

Functional dyspepsia (FD) is a common gastrointestinal disorder that is characterized by persistent or recurrent upper abdominal pain or discomfort in the absence of any structural, morphological or known organic abnormality, often accompanied by psychosocial disturbance. Gastric hypersensitivity (GHS) is one of the characteristic pathogeneses of FD and represents a cardinal pathophysiological change in FD. Clinical studies have shown that approximately 35%-65% of FD patients suffer from GHS, and among them, 10%-25% have been confirmed to have GHS-related postprandial epigastric pain. Anxiety is a common psycho-social disturbance and troubles 40%-90% of the FD patients in clinic. Various studies have suggested that anxiety may influence gastric sensitivity, gastrointestinal movement, gastric emptying and gut neuroendocrine regulation. However, although GHS and anxiety have been identified as important factors triggering or aggravating FD, the mechanisms by which affect the development of FD and the relationship between them are still unknown. In part, this is due to a lack of both available visceral tissue from FD patients and normal human subjects and suitable animal models of FD with anxiety. Therefore, in order to elucidate the pathogenesis of FD and develop new drugs for the treatment of FD, creating a novel animal model of FD with anxiety-like GHS is of vital importance.

Research motivation

The research motivation came from the reality that there are no defined approaches in rat models that simultaneously mimic anxiety-like behaviors and GHS of FD in a standardized way yet. The authors tried to solve this problem by making rats undergo stress early in life and late in adulthood. The authors believed that the establishment of this rat model would be useful to explore the pathogenesis of FD and to develop new drugs for use FD treatment in the future.

Research objectives

The main objective of this study was to develop a new rat model of anxiety-like GHS of FD by the method of novel sequential stress. The results showed that the authors have realized this objective initially. Its significance lies in that researchers will have a new choice in the study of the pathogenesis of FD and in the development of new drugs.

Research methods

In order to create this rat model successfully, the authors took a method of novel sequential stress. It includes maternal separation (MS) and acute gastric irritation (AGI) early in life and restraint stress (RS) in adulthood. Furthermore, the authors used the elevated plus maze, open field experiment, abdominal withdrawal reflex testing and electromyographic recordings to evaluate the rat' s behavioral characteristics. Finally, the alterations of several anxiety-related brain-stomach modulators including 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), brain-derived neurotrophic factor (BDNF) and nesfatin-1 in the rat hippocampus, plasma and gastric fundus and the 5-HT1A receptor (5-HT1AR) in the hippocampal CA1 subfield and the mucosa of the gastric fundus were examined.

Research results

The results showed that the sequential-stress-treated rats simultaneously demonstrated anxiety-like behaviors and GHS compared with the control group. Their rate of gastric emptying was also lower than the control group. Furthermore, sequential stress could significantly decrease the levels of 5-HT, GABA and BDNF in the hippocampus but increase the content of nesfatin-1 in the same site; significantly decrease the levels of 5-HT and BDNF in the plasma but increase the content of nesfatin-1 in it; significantly decrease the levels of 5-HT and BDNF in the gastric fundus but increase the content of nesfatin-1 in the same site. The expressions of 5-HT1AR in the hippocampal CA1 subfield and the mucosa of the gastric fundus were down-regulated. The contribution of this study, as is thought by the authors, is that it provided a novel rat model of FD with anxiety-like GHS to the research in this field and partly explored the molecular mechanisms of this important disease. However, there are still many complicated problems such as the relationship between anxiety-like GHS of FD and hypothalamic-pituitary-adrenal axis to be solved in the future.

Research conclusions

A method of sequential stress, namely, MS and AGI early in life followed by RS in adulthood, were firstly adopted in creating a rat model of anxiety-like GHS of FD, which could mimic the phenomenon observed in clinic that adverse physiological or psychosocial experiences in early life and acute stress in adulthood are linked to the development of FD. The results also showed the alterations of several anxiety-related neurobiochemical modulators in brain-blood-stomach axis such as 5-HT, GABA, BDNF and nesfatin-1, and thus further deepened the understanding of the molecular mechanisms of FD. Besides, this study supplied a possible tool for the development of new drugs in the treatment of FD.

Research perspectives

While doing this study the operation of the implantation of gastric balloon and electrodes is very important in creating this novel rat model of FD with anxiety-like GHS. The direction of the future research in this field lies in the investigation of the molecular mechanisms of the psychological and pathophysiological abnormalities in brain-gut interaction of this important clinical problem and the development of new drugs using this model.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Post-colonoscopy colorectal cancer rate in the era of high-definition colonoscopy

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Abstract

AIM

To investigate the post-colonoscopy colorectal cancer (PCCRC) rate for high-definition (HD) colonoscopy compared with that for standard-definition colonoscopy reported previously.

METHODS

Using medical records at Sano Hospital (SH) and Dokkyo Medical University Koshigaya Hospital (DMUKH), we retrospectively obtained data on consecutive patients diagnosed as having CRC between January 2010 and

December 2015. The definition of PCCRC was diagnosis of CRC between 7 and 36 mo after initial high-definition colonoscopy that had detected no cancer, and patients were divided into a PCCRC group and a non-PCCRC group. The primary outcome was the rate of PCCRC for HD colonoscopy. The secondary outcomes were factors associated with PCCRC and possible reason for occurrence of early and advanced PCCRC.

RESULTS

Among 892 CRC patients, 11 were diagnosed as having PCCRC and 881 had non-PCCRC. The PCCRC rate was 1.7% (8/471) at SH and 0.7% (3/421) at DMUKH. In comparison with the non-PCCRC group, the PCCRC group had a significantly higher preponderance of smaller tumors (39 mm *vs* 19 mm, $P = 0.002$), a shallower invasion depth (T1 rate, 25.4% *vs* 63.6%, $P = 0.01$), a non-polypoid macroscopic appearance (39.0% *vs* 85.7%, $P = 0.02$) and an earlier stage (59.7% *vs* 90.9%, $P = 0.03$). Possible reasons for PCCRC were "missed or new" in 9 patients (82%), "incomplete resection" in 1 (9%), and "inadequate examination'" in 1 (9%). Among 9 "missed or new" PCCRC, the leading cause was non-polypoid shape for early PCCRC and blinded location for advanced PCCRC.

CONCLUSION

The PCCRC rate for HD colonoscopy was 0.7%-1.7%, being lower than that for standard-definition colonoscopy (1.8%-9.0%) reported previously employing the same methodology.

Key words: Post-colonoscopy colorectal cancer; High-definition; Post-colonoscopy colorectal cancer rate; Associated factor; Possible explanation

Core tip: Technological advance from standard-definition to high-definition colonoscopy has the potential to reduce the incidence of post-colonoscopy colorectal cancer (PCCRC). We demonstrated the lower PCCRC rate for high-definition colonoscopy compared for standard-definition colonoscopy reported previously (0.7%-1.7% *vs* 1.8%-9.0%). Our data might help to set a benchmark for the quality of colonoscopy in Asian countries, where data on PCCRC are scarce. We firstly analyzed the possible reasons for both early and advanced "missed or new" PCCRC cases and found differences between the two groups. The leading cause was non-polypoid shape for early PCCRC and blinded location for advanced PCCRC.

Iwatate M, Kitagawa T, Katayama Y, Tokutomi N, Ban S, Hattori S, Hasuike N, Sano W, Sano Y, Tamano M. Post-colonoscopy colorectal cancer rate in the era of high-definition colonoscopy. *World J Gastroenterol* 2017; 23(42): 7609-7617 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7609.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7609

INTRODUCTION

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in females and the third most common in males worldwide[1]. Colonoscopy can reduce the likelihood of CRC-related death by resecting precursor lesions and detecting CRC at an early stage[2-4]. Unfortunately, the quality of colonoscopy is insufficient to prevent all interval CRCs, and some patients still develop CRC before the next recommended surveillance date, an event known as post-colonoscopy CRC (PCCRC).

A better understanding of the factors associated with PCCRC may help to reduce its incidence. Previous reports have suggested that in comparison with non-PCCRC, PCCRC is associated with various clinical factors (*e.g.* older age, female gender, location in the proximal colon, and presence of diverticula) and also endoscopist-related factors (those with less experience at adenoma detection, or non-specialists in gastroenterology)[5-14]. Around 70% of PCCRCs appear to result from lesions that have been missed or incompletely resected at initial colonoscopy, and could theoretically have been avoidable[12]. Therefore, the PCCRC rate has been proposed as a key indicator of the quality of colonoscopy, and a meta-analysis has shown that this varies from 1.8% to 9.0%[13].

High-definition (HD) colonoscopy yields markedly clearer images and has the clinical benefit of increasing the adenoma detection rate in comparison with standard-definition (SD) colonoscopy[15]. Theoretically, HD colonoscopy has the potential to reduce the incidence of PCCRC, but clinical data related to this issue are still insufficient.

We therefore conducted a retrospective observational study at two academic centers to investigate the PCCRC rate for HD colonoscopy in Japan.

MATREIALS AND METHODS

Patients

By reference to the medical records at Sano Hospital (SH) and Dokkyo Medical University Koshigaya Hospital (DMUKH), we included in this study consecutive individuals diagnosed as having CRC between January 2010 and December 2015. Exclusion criteria were as follows: (1) patients with IBD or hereditary disease; (2) those with a previous diagnosis of CRC; (3) those for which data related to CRC (tumor size, shape, site, and histopathology) were insufficient; (4) those with a CRC histopathology other than adenocarcinoma; and (5) those that did not comply with the Japanese clinical guidelines for the management of colorectal polyps at initial colonoscopy[16]. Patients who met the eligibility criteria were divided into a PCCRC group and a non-PCCRC group according to the definition of PCCRC given below. HD colonoscopy with a LUCERA-SPECTRUM or ELITE video processor and HD monitors (Olympus, Japan) had been used for all patients since 2006 at both hospitals. The study protocol was approved by the

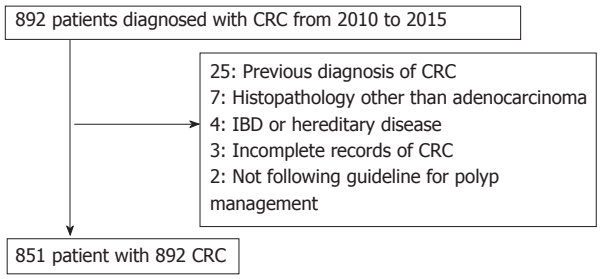

Figure 1 Patient flow chart. CRC: Colorectal cancer.

institutional review boards of both hospitals.

Definition of PCCRC

Based on a previous research method, we defined PCCRC as CRC that had been diagnosed 7 to 36 mo after initial HD colonoscopy, when no cancer had been detected[13]. CRC diagnosed within 6 mo of HD colonoscopy yielding negative findings was considered to have been a cancer confirmed after follow-up of a suspicious lesion, and was classified as non-PCCRC. CRC was defined as tumors that have penetrated through the muscularis mucosae into submucosa according to the classification of the World Health Organization.

Outcome assessment

Primary outcome: The primary outcome of interest was the PCCRC rate for HD colonoscopy, calculated as the number of PCCRC events divided by the total number of CRCs examined during the study period.

Secondary outcome: (1) Factors associated with PCCRC: We collected data on patients (age, sex) and tumors (size, location, shape, depth of invasion, UICC stage) for comparison between the PCCRC and non-PCCRC groups; and (2) possible reason for occurrence of early and advanced PCCRC: We assigned each PCCRC case into one of three categories: "incomplete resection" defined as CRC detected on the scar where an advanced polyp had been incompletely resected at the time of colonoscopy, "inadequate examination" defined as failure to intubate the colon to the cecum or poor bowel preparation, and "missed or new" as "others". Differentiation of "missed" CRC from "new" CRC is challenging. In fact, most CRCs categorized as "missed or new" were thought to have been "missed", in view of the fact that le Clercq had defined "new" CRC as CRC detected > 36 mo after the index colonoscopy[14]. Therefore, we additionally classified the "missed or new" category into four subcategories to determine which factor was most closely associated with "missed" CRC (multiple choice): (1) tumor morphology: Polypoid or non-polypoid; (2) tumor size: Small (< 10 mm) or not; (3) tumor location: In a blind area (*e.g.,* behind a fold or close to the ileocecal valve/junction) or not; and (4) the endoscopist's observational skill: Multiple ($n \geqslant 3$) polyps evident at initial colonoscopy or not. We assumed that if an endoscopist took a long time to examine a patient with multiple polyps, this would prove exhausting and lead to loss of concentration in detecting polyps. We divided 'missed or new' PCCRC into early PCCRC (T1 stage) and advanced PCCRC (T2-4 stage) to clarify how the factors associated with PCCRC differed between the two groups.

Statistical analysis

Categorical variables were compared using the χ^2 test or Mid-P exact test, normally distributed continuous variables were compared using *t*-test, and non-normally distributed continuous variables were compared using the Wilcoxon rank sum test. A two-sided *P* value of < 0.05 was considered statistically significant.

RESULTS

A total of 892 patients with CRC were identified from the records of both hospitals during the period January 2010 to December 2015. On the basis of the exclusion criteria, 41 patients were discarded and 851 patients (444 at SH, and 407 at DMUKH) with 892 CRCs were analyzed retrospectively (Figure 1). All of the CRCs were detected by gastroenterologists with more than 3 years of colonoscopy experience.

PCCRC rate

Among the 892 CRCs (471 at SH, and 421 at DMUKH), 2 (1 at each at SH and DMUKH) were diagnosed within 6 mo after initial colonoscopy and 11 (8 in SH, and 3 in DMUKH) between 7 and 36 mo after initial colonoscopy. The PCCRC rate was 1.7% (8/471) at SH, 0.7% (3/421) at DMUKH, and 1.2 % (11/892) for both hospitals.

Baseline variables in the PCCRC and non-PCCRC groups

Baseline variables in the PCCRC and non-PCCRC groups are listed in Table 1. Among patient-related variables, gender and mean age showed no significant inter-group difference. Among tumor-related variables, there were significant differences in size, depth, morphology and UICC stage between the two groups. In comparison with non-PCCRC patients, those with PCCRC were more likely to have small tumors (mean size, 39 mm *vs* 19 mm respectively, $P = 0.002$), a shallow tumor depth (T1 rate, 25.4% *vs* 63.6%, $P = 0.01$), early CRCs with a non-polypoid macroscopic appearance (39.0% *vs* 85.7%, $P = 0.02$), and an early UICC stage (stage Ⅰ or Ⅱ, 59.7% *vs* 90.9%, $P = 0.03$).

Possible reasons for PCCRC

Details of the 11 patients with PCCRC are shown in Table 2. The possible reasons for PCCRC were "missed or new" in 9 cases (82%), "incomplete resection" in 1 (9%), and "inadequate examination" in 1 (9%). Possible explanations for the 9 "missed or new" cases (6 early and 3 advanced PCCRC) are summarized in Figure 2. The 6 early 'missed or new' PCCRC cases could have

Table 1 Baseline variables in the post-colonoscopy colorectal cancer and non- post-colonoscopy colorectal cancer group *n* (%)

	PCCRC	Non-PCCRC	*P* value
Patients	11	840	
Gender			NS
Male	6 (54.5)	485 (57.7)	
Female	5 (45.5)	355 (42.3)	
Age (yr)			NS
mean ± SD	70 ± 10	68 ± 11	
Range	53-82	29-92	
Tumors Size (mm)	11	881	0.002
Mean ± SD	19 ± 13	39 ± 20	
Range	4-50	4-110	
Location			NS
Proximal	6 (54.5)	283 (32.1)	
Distal	5 (45.5)	598 (67.9)	
Depth			0.010
T1	7 (63.6)	224 (25.4)	
T2-4	4 (36.4)	657 (74.6)	
Shape[1]			0.020
Polypoid	1 (14.3)	136 (61.0)	
Non-polypoid	6 (85.7)	87 (39.0)	
UICC stage			0.033
Stage Ⅰ, Ⅱ	10 (90.9)	526 (59.7)	
Stage Ⅲ, Ⅳ	1 (9.1)	355 (40.3)	

[1]Shape of early CRC (T1 stage). PCCRC: Post-colonoscopy colorectal cancer; NS: Not significant; SD: Standard deviation; UICC: Union for International Cancer Control.

been due to a non-polypoid shape in 5 (83%), presence of synchronous multiple polyps at initial colonoscopy in 4 (67%), a small tumor size (< 10 mm) in 2 (33%), and location at a blind spot in 1 (17%). The 3 advanced 'missed or new' PCCRC cases were likely due to their location at a blind spot (100%). Some representative PCCRC cases are presented in detail in Figures 3-7.

DISCUSSION

In this study, we investigated the PCCRC rate in cases examined by HD colonoscopy. It was anticipated that our data might help to set a benchmark for the quality of colonoscopy in Asian countries, where data on PCCRC are scarce. We analyzed the possible reasons for both early and advanced "missed or new" PCCRC cases and found differences between the two groups.

The PCCRC rate in the present study was 0.7%-1.7%, and lower than that in previous reports from Western countries (1.8%-9.0%) calculated using the same methodology[6-9,13]. There are several possible reasons for this difference. First, as we performed HD colonoscopy in all cases, we might have detected a larger number of pre-malignant polyps or CRC at the time of initial examination. Second, all colonoscopies were performed by experienced gastroenterologists. A population-based study in Manitoba reported that colonoscopy performed by general physicians was associated with a 60% higher risk of missed CRC in comparison with that performed by specialist gastroenterologists[7]. Third, racial differences in the incidence of CRC between Asian and Western countries. Fourth, the rate of recurrence (9.1%

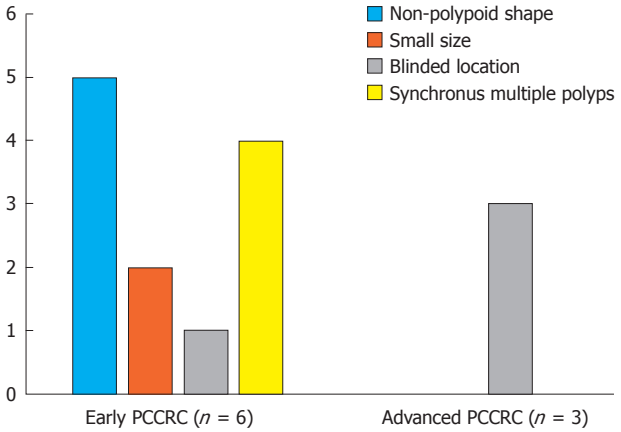

Figure 2 Possible explanations for the 9 "missed or new" post-colonoscopy colorectal cancers. The bar chart shows the number of each possible explanation for the 6 early "missed or new" PCCRCs (left) and the 3 advanced "missed or new" PCCRCs (right). Among the 6 early "missed or new"PCCRCs, possible explanations were a non-polypoid shape in 5 cases (83%), presence of synchronous multiple ($n \geqslant 3$) polyps at initial colonoscopy in 4 (67%), a small size (< 10 mm) in 2 (33%), and a blind location in 1 (17%). For all 3 (100%) of the advanced "missed or new" PCCRCs, a blind location was considered to have been likely. PCCRC: Post-colonoscopy colorectal cancer.

for all incompletely resected lesions including sessile serrated polyps, 0% for adenomas) in this study was low in comparison with previous studies (8.8%-36.8% for adenoma) performed in Western countries[12,14,17]. The difference in the recurrence rate for large colorectal tumors between Asian and Western countries is thought to be attributable to the treatment strategy employed, *i.e.*, whether or not endoscopic submucosal dissection (ESD) is available. The ESD technique, originally developed in Japan for large colorectal ($\geqslant$ 20 mm) tumors, has resulted in higher rates of en bloc resection and lower rates of local recurrence in comparison with conventional endoscopic mucosal resection (EMR) that is generally performed worldwide[18,19]. The ESD technique has not been popular in Western countries because of its technical difficulty, but it is now becoming increasingly available and employed successfully as practitioners gain experience[20,21]. The criteria employed to define PCCRC significantly affects the PCCRC rate[22]. Therefore, we followed the definition of PCCRC adopted in the majority of population-based studies and a recent meta-analysis[6-9,13].

In this study, we were able to identify several tumor-related factors associated with PCCRC. Such cases were significantly associated with a smaller tumor size, a shallower tumor depth, a non-polypoid shape and an earlier UICC stage, which were features characteristic of missed lesions. Our data support previous studies that have investigated tumor-related risk factors for PCCRC, except for tumor location. Although it has been suggested previously that PCCRC is more likely to arise in the proximal colon rather than the distal colon, we did not find any significant difference in the incidence of PCCRC between these two colon regions. This difference in results may have been attributable to the proportion of incomplete examinations, which can potentially lead

Table 2 Data for the 11 patients with post-colonoscopy colorectal cancer

No	Sex	Age(yr)	Interval (mo)	Tumor shape	Size (mm)	Depth	Location	Initial CS	Possible reason
1	M	79	7	Ⅱc	5	T1a	T	Multiple polyps	Missed/new
2	M	76	14	Ⅱa (LST-NG)	15	T1a	S	Multiple polyps	Missed/new
3	M	82	17	Ⅱa (LST-NG)	25	T1a	T	No polyps	Missed/new
4	F	65	22	Ⅱa (LST-NG)	20	T1a	A	Multiple polyps	Missed/new
5	M	59	26	Ⅰs	12	T1a	R	Two polyps	Missed/new
6	F	73	11	Ⅱa	10	T1b	T	Piecemeal EMR	Incomplete resection
7	M	79	15	Ⅰs + Ⅱc	4	T1b	S	Multiple polyps	Missed/new
8	F	70	9	Type 2	30	T3	C	No polyps	Inadequate examination
9	F	53	12	Type 2	17	T3	S (SDJ)	No polyps	Missed/new
10	F	77	12	Type 2	50	T3	RS	One polyp	Missed/new
11	M	66	10	Type 2	20	T4	C	Two polyps	Missed/new

Multiple, $n \geqslant 3$. PCCRC: Post-colonoscopy CRC; CS: Colonoscopy; LST-NG: Laterally spreading tumor non-granular type; SDJ: Sigmoid-descending junction; EMR: Endoscopic mucosal resection.

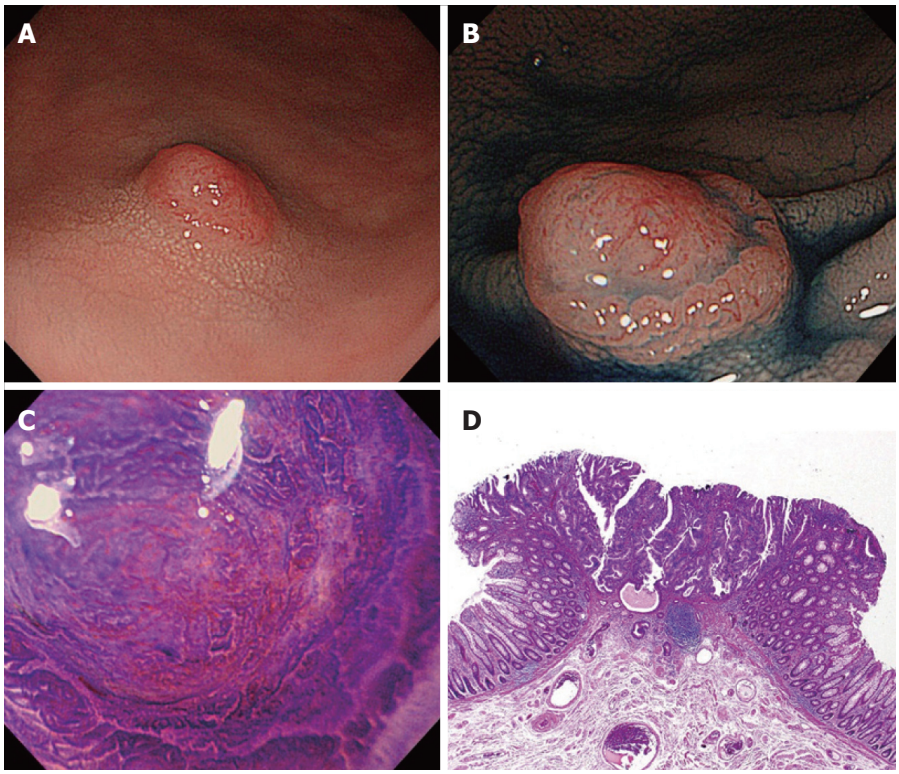

Figure 3 An early "missed or new" post-colonoscopy colorectal cancer case (No. 7 in Table 2). A 79-year-old man underwent initial colonoscopy, and seven small adenomatous polyps in the ascending and sigmoid colon were resected. A: A diminutive lesion 4 mm in size was found in the sigmoid colon during surveillance colonoscopy 15 mo after initial colonoscopy; B: Chromoendoscopy with indigo-carmine dye visualizes the margin of the deep depressed area on the surface of the lesion, and crystal violet stain shows a type-Vi pit with an invasive pattern suggesting submucosal deep invasive cancer (C); D: Histopathological examination of the surgical specimen reveals well to moderately differentiated adenocarcinoma with submucosal deep (3000 μm) invasion and no lymph node metastasis.

to an increase in the rate of proximal colon PCCRC. The rate of complete examination in this study was 99%, as compared with 87%-92% for population-based studies in the United States[11,23]. Although there was a tendency for the PCCRC group to include older patients and a higher proportion of women than the non-PCCRC group, consistent with other reports, the differences between the two groups were not significant[6-11,13]. Other possible explanations may have been an insufficient sample size or the racial composition of the population.

Of the three possible reasons for PCCRC, the majority (82%) of such cases were categorized as 'missed or new', consistent with previous reports[12,14]. We classified 'missed or new' PCCRC into early and advanced cases. The major possible explanations for early 'missed or new' PCCRC were a non-polypoid shape (83%) and the presence of synchronous multiple polyps at initial colonoscopy (67%). Among non-polypoid lesions, the mean size of depressed lesions was 4.5 mm and that of flat lesions including LST-NG (laterally spreading tumor, non-granular type) was 17.5 mm (Figures 3 and 4). As non-polypoid lesions are less conspicuous than polypoid lesions, they

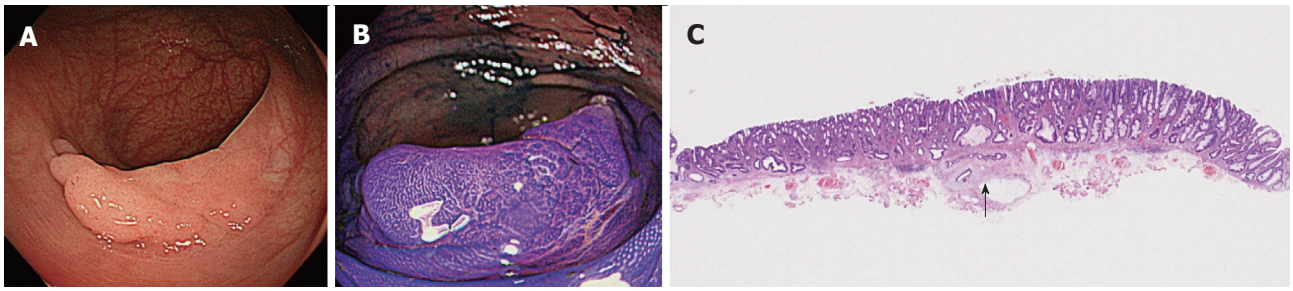

Figure 4 An early "missed or new" post-colonoscopy colorectal cancer case (No. 3 in Table 2). An 82-year-old man underwent initial colonoscopy and was found to have no adenomatous polyps. A: Subsequent colonoscopy 17 mo later revealed a large flat lesion, a laterally spreading non-granular-type tumor (LST-NG), measuring 25 mm in the transverse colon; B: Chromoendoscopy with crystal violet shows a type-Vi pit with a non-invasive pattern suggesting high-grade adenoma or submucosal shallow invasive cancer; C: Histopathological examination of the ESD specimen reveals well differentiated adenocarcinoma with submucosal shallow (200 μm) invasion (arrow) and no lymphovascular involvement. ESD: Endoscopic submucosal dissection.

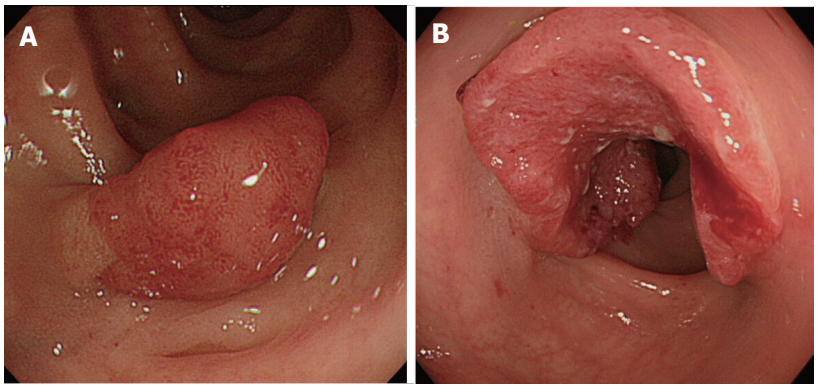

Figure 5 An advanced "missed or new" post-colonoscopy colorectal cancer case (No. 10 in Table 2). A: A 77-year-old woman underwent initial colonoscopy and a pedunculated adenomatous polyp 9 mm in size was resected; B: A large advanced cancer 50 mm in size was found in the recto-sigmoid colon at subsequent colonoscopy for hematochezia 12 mo later. Histopathological examination of the surgical specimen showed well to moderately differentiated adenocarcinoma invading the subserosa, and no lymph node metastasis.

are often missed even if they are large. Endoscopists should pay closer attention to subtle changes in the mucosa, including red areas, loss of vessel visibility, and deformation of the colonic folds, in order to detect flat or depressed lesions[24-26]. We found that the presence of synchronous multiple polyps at initial colonoscopy was a factor associated with around 70% of early "missed or new" PCCRC cases, and was unrelated to advanced cases. We speculated that a long time spent examining a patient with multiple polyps might lead to a decrease in the concentration of the endoscopist, thus increasing the likelihood that small early CRCs (mean size: 13.5 mm), but not large advanced ones (mean size: 39.0 mm), would be overlooked. On the other hand, one possible explanation for advanced "missed or new" PCCRC cases was thought to be the location of lesions at blind spots, such as the junctions of the recto-sigmoid and sigmoid-descending colon and the ileocecal valve (Figure 5). Endoscopists should be aware that even large advanced CRCs can be easily overlooked during colonoscopy. The development of accessory devices and new modalities is expected to improve observation in "blind" areas of the colon[27-29]. One technique for improving the visual field in blind areas where the colon is sharply angled might be to actively push the colonoscope in order to straighten the colon. Among the possible reasons for PCCRC, "incomplete resection" and "inadequate examination" were considered. We experienced a case of PCCRC after piecemeal EMR for a 20-mm sessile serrated adenoma/polyp (SSA/P) in the transverse colon (Figure 6). Although histopathological examination revealed high-grade dysplasia with a negative margin and no lymphovascular involvement, the lesion recurred as a submucosal deeply invasive cancer at 11 mo after the treatment. We speculate that histopathological assessment of the tumor margin for this type of divided specimen may not have been accurate, and that some high-grade dysplasia may have remained *in situ* after initial colonoscopy. Unclear margin of SSA/P may result in incomplete resection. Pohl *et al*[30] reported incomplete resection rate for SSAP was higher than for conventional adenoma (31.0% *vs* 7.2%). Moreover, Zhu *et al*[31] found that for colorectal serrated polyps, a large size (≥ 10 mm) and histologic subtype (SSA/P and conventional serrated adenoma) were significantly associated with synchronous CRC. SSA/P should be resected *en bloc* especially when it exceeds 10 mm in size. Finally, one advanced PCCRC case that arose in the cecum after 9 mo was probably attributable to poor preparation at initial colonoscopy (Figure 7). This case serves to

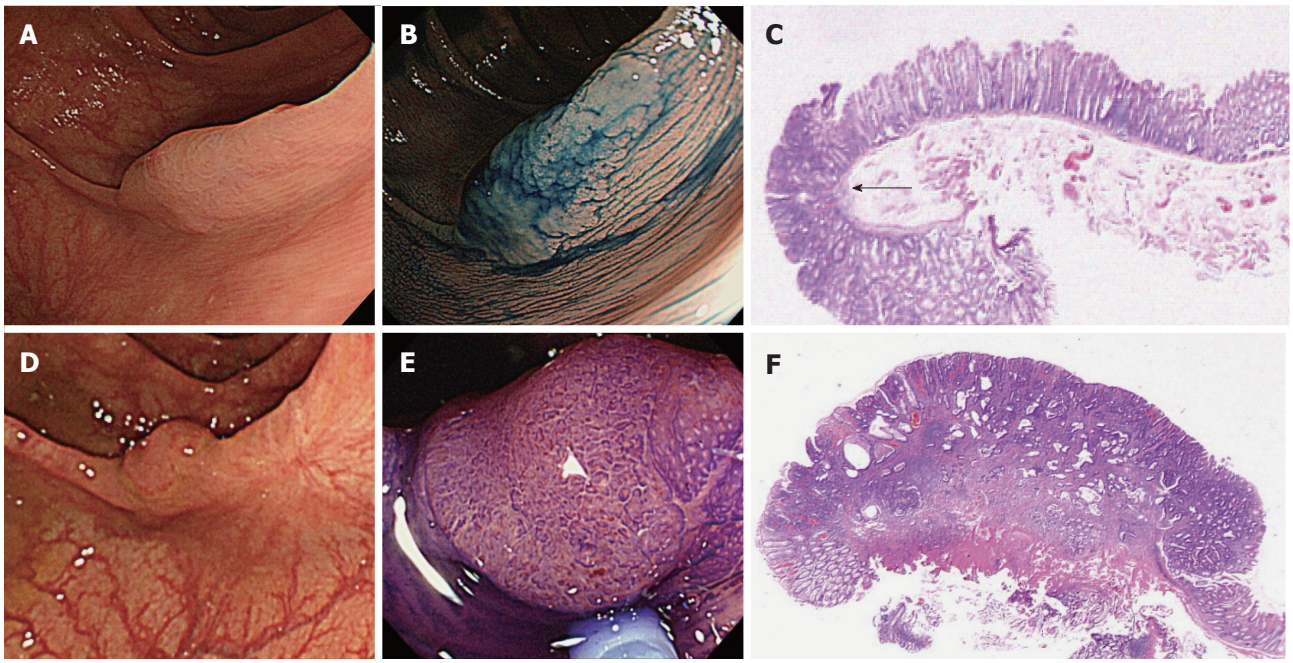

Figure 6 An early post-colonoscopy colorectal cancer case resulting from incomplete resection (No. 6 in Table 2). A 73-year-old woman underwent initial colonoscopy. A large flat lesion 20 mm in size showing type-II and open type-II pits, suggestive of SSA/P, was found by chromoendoscopy in the transverse colon and resected by piecemeal EMR with no macroscopically evident residual lesion (A and B); C: Histopathology of the resected specimen divided into 3 pieces revealed high-grade dysplasia (arrow) in SSA/P with intact vertical and horizontal margins of the dysplasia; D: Surveillance colonoscopy 11 months after initial colonoscopy detected a flat 10-mm lesion on the scar of the initial EMR in the transverse colon; E: Chromoendoscopy with crystal violet revealed unusual type-Vi pits suggesting submucosal invasive cancer; F: Histopathological examination of the EMR specimen revealed moderately differentiated adenocarcinoma invading the deep (2500 μm) submucosa with lymphovascular involvement. Finally, surgery was performed and histopathological examination revealed no residual cancer at the EMR site in the transverse colon and no lymph node metastasis. EMR: Endoscopic mucosal resection; SSA/P: Sessile serrated adenoma/polyp.

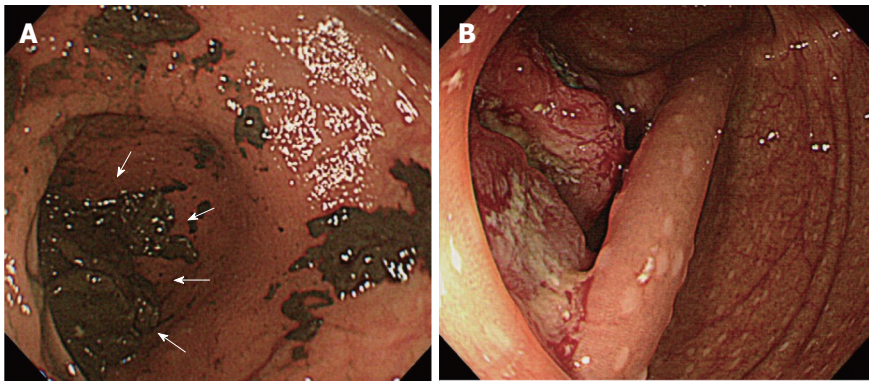

Figure 7 An advanced post-colonoscopy colorectal cancer case resulting from inadequate examination (No. 8 in Table 2). A 70-year-old woman underwent emergency colonoscopy with poor bowel preparation. A: No polyp was found in the colon but a quantity of residual stools covered the lower end of the cecum (arrow); B: Subsequent colonoscopy for hematochezia 9 mo after initial colonoscopy detected a large advanced cancer 30 mm in size at the cecum bottom. Histopathological examination of the surgically resected specimen revealed well differentiated adenocarcinoma invading the subserosa and no lymph node metastasis.

illustrate that residual stools at colonoscopy can hide not only small polyps but also large advanced CRCs. Early repeat colposcopy is therefore recommended for patients who have undergone colonoscopy after low-quality bowel preparation[32].

Our study had several limitations. First, the total number of PCCRC cases at the two hospitals was small (n = 11) during short study period from 2010 to 2015, and insufficient for investigating the factors associated with PCCRC using a multivariate logistic regression model. This is because HD colonoscopy has been available since 2006 at the both hospitals and patients with PCCRC diagnosed within 36 mo after initial HD colonoscopy began to be recruited in 2010. A further study including a larger number of PCCRC cases in an Asian setting will be necessary. Second, we did not have any information about the indications for colonoscopy, use of prophylactic medicines (*e.g.*, aspirin) and family history of CRC, which could potentially affect the incidence of PCCRC. Third, the data on the PCCRC rate with SD colonoscopy in our hospitals were not available before HD colonoscopy was introduced. It would be better to compare the PCCRC rate

using HD colonoscopy with that using SD colonoscopy in the same hospitals. Finally, as all of the examinations were performed by experienced gastroenterologists, our data cannot be generalized to non-gastroenterologists or inexperienced colonoscopists.

In conclusion, we have shown that the PCCRC rate with HD colonoscopy in our present series was 0.7%-1.7%, being lower than that for SD colonoscopy in previous studies using the same methodology. Further advances in technology may help to reduce the PCCRC rate in the future.

ARTICLE HIGHLIGHTS

Research background

Post-colonoscopy colorectal cancers (PCCRC) has been recognized as a key quality indicator for colonoscopy. The data of PCCRC has been reported from Western counties, however that from Asian countries is lacking. Theoretically, HD colonoscopy has the potential to reduce the incidence of PCCRC, but clinical data related to this issue are still insufficient.

Research motivation

The PCCRC rate at two academic centers might help to set a benchmark for the quality of colonoscopy in Asian countries, where data on PCCRC are scarce.

Research objectives

To investigate the PCCRC rate for HD colonoscopy compared with that for standard-definition colonoscopy reported previously.

Research methods

We retrospectively examined the medical records of consecutive adult patients with CRC between 2010 and 2015 at Sano Hospital (SH) and Dokkyo Medical University Koshigaya Hospital (DMUKH) in Japan. Patients with CRC diagnosed within 6 to 36 mo of HD colonoscopy were classified as a PCCCRC group, and the others as a non-PCCRC group. The primary outcome was the PCCRC rate with HD colonoscopy. The secondary outcomes were factors associated with PCCRC and possible reason for occurrence of early and advanced PCCRC.

Research results

We analyzed 851 patients with 892 CRCs including 11 of PCCRC and 881 of non-PCCRC. The PCCRC rate was 1.7% (8/471) at SH and 0.7% (3/421) at DMUKH. Factors significantly associated with PCCRC were smaller size, a shallower invasion depth, a non-polypoid macroscopic appearance, and an earlier stage. The leading possible reason was non-polypoid shape for early PCCRC and blinded location for advanced PCCRC.

Research conclusions

We demonstrated the lower PCCRC rate for high-definition colonoscopy compared for standard-definition colonoscopy reported previously (0.7%-1.7% *vs* 1.8%-9.0%). Technological advance from standard-definition to high-definition colonoscopy has the potential to reduce the incidence of PCCRC.

Research perspectives

Early PCCRC may be missed by inconspicuous macroscopic type, and advanced PCCRC by the position in blinded location. Endoscopists should be aware that even large advanced CRC can be easily missed during colonoscopy. We should learn the reason why we missed CRC during colonoscopy and prevent the PCCRC in the future. The development of accessory devices and new modalities are expected to improve observation in "blind" areas of the colon and decrease the PCCRC.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Right- and left-sided colorectal cancers respond differently to traditional Chinese medicine

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Author contributions: Liu SS and Hou FG designed the research; all authors involved in patients recruitment and data collecting; Liu SS analyzed the data and edited manuscript.

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Abstract

AIM

To explore the differences in the responses of left-sided colorectal cancer (LSCRC) and right-sided colon cancer (RSCC) to traditional Chinese medicine (TCM).

METHODS

Patients with postoperative stage I-III colorectal cancer (CRC) were enrolled and divided into the LSCRC with or without TCM and RSCC with or without TCM groups depending on the primary tumor side and TCM administration. Patients in the TCM group were given TCM for at least 6 mo. Our research adopted disease-free survival (DFS) as the primary endpoint. We applied a Cox proportional hazards regression model for the multivariate factor analysis using Stata 12.0 and SPSS 22.0 software for data analysis.

RESULTS
Of the 817 patients included in our study, 617 had LSCRC (TCM group, *n* = 404; Non-TCM group, *n* = 213), and 200 had RSCC (TCM group, *n* = 132; Non-TCM group, *n* = 68). The 6-year DFS for patients with LSCRC was 56.95% in the TCM group and 41.50% in the Non-TCM group (*P* = 0.000). For patients with RSCC, the 6-year DFS was 52.92% in the TCM group and 37.19% in the Non-TCM group (*P* = 0.003). Differences between LSCRC and RSCC were not statistically significant regardless of TCM ingestion.

CONCLUSION
Patients with either LSCRC or RSCC and who took TCM experienced longer DFS; furthermore, patients with RSCC benefited more from TCM in DFS.

Key words: Colorectal cancer; Left-sided; Right-sided; Traditional Chinese medicine; Disease-free survival

Core tip: In this prospective, observational, multicenter, cohort study, we compared disease-free survival (DFS) of patients with postoperative stage I-III left- and right-sided colorectal cancers who were stratified by ingestion of TCM. The data analysis confirmed that TCM effectively prolonged DFS of patients with stage II-III on both sides, especially individuals with stage III right-sided colon cancer.

Liu SS, Shi Q, Li HJ, Yang W, Han SS, Zong SQ, Li W, Hou FG. Right- and left-sided colorectal cancers respond differently to traditional Chinese medicine. *World J Gastroenterol* 2017; 23(42): 7618-7625 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7618.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7618

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related death in America[1,2]. Resection is the gold-standard treatment for CRC, but 35% of individuals will develop recurrence or metastasis within the first few years after resection[3]. Therefore, preventing postoperative recurrence and metastasis is critical in treating stage I-III CRC. In China, traditional Chinese medicine (TCM) is a common anticancer approach along with chemotherapy and radiotherapy[4,5]. Our preliminary study also proved that chemotherapy coupled with TCM could further reduce the risk of recurrence and metastasis as well as prolong the disease-free survival (DFS) of patients with CRC[6]. Recently, more studies have proposed that location of primary tumor was related to recurrence, metastasis and the therapeutic effect[7-9]. However, there was no evidence regarding whether TCM exerts variable effects on CRC based on the side where the lesion is located. Therefore, we undertook this study to determine whether TCM can prolong the DFS of individuals with either left-sided colorectal cancer (LSCRC) or right-sided colon cancer (RSCC).

MATERIALS AND METHODS

Study design

This was a retrospective, observational, multicenter, cohort study designed to elucidate whether primary tumor location is associated with a differential response to TCM. Eligible postoperative patients with stage I-III disease were screened at affiliated hospitals of Shanghai University of Traditional Chinese Medicine (Shanghai Municipal Hospital of Traditional Chinese Medicine, Shuguang Hospital and Yueyang Hospital) between April 2004 and November 2013. The study protocol was approved by these three individual ethics committees. A total 1020 patients were screened, among which 148 did not present a clear side of colon cancer, 4 presented other primary tumors, 24 were followed up for less than 6 mo, 5 presented an unclear TNM stage, and 22 were treated with non-systemic TCM medication. Notably, systematic TCM medication was defined as at least 6 mo of continuous TCM ingestion before relapse. Finally, 817 patients were included in our research (Figure 1) and divided into the following groups based on tumor location and TCM status: LSCRC with TCM, LSCRC without TCM, RSCC with TCM and RSCC without TCM. All the included patients were followed up by outpatient visits, returning visits or telephone follow-up every 6 mo; clinical data, including age, gender, colon cancer location, surgical pathology, histodifferentiation, TNM stage, lymph nodes examined in surgical specimen, chemotherapy, radiotherapy, comorbidities (including hypertension, diabetes, heart disease, and stroke), period of TCM, time to recurrence and metastasis or cancer-related death, were collected at each follow-up visit. The median follow-up for RSCC was 53.0 mo in the TCM group and 34.3 mo in the Non-TCM group; for LSCRC, the median follow-up was 54.3 mo in the TCM group and 38.5 mo in the Non-TCM group. Follow-up was completed for up to 6 years or until the patients either relapsed or died.

Statistical analysis

DFS, which is defined as the time from surgical resection to relapse, cancer-related death or the last follow-up (whichever occurred first), was evaluated using Kaplan-Meier curves and log-rank tests. Baseline characteristics were analyzed using Pearson's χ^2 test. Propensity score matching was created using logistic regression to model the probability that a patient exhibited a specific characteristic based on

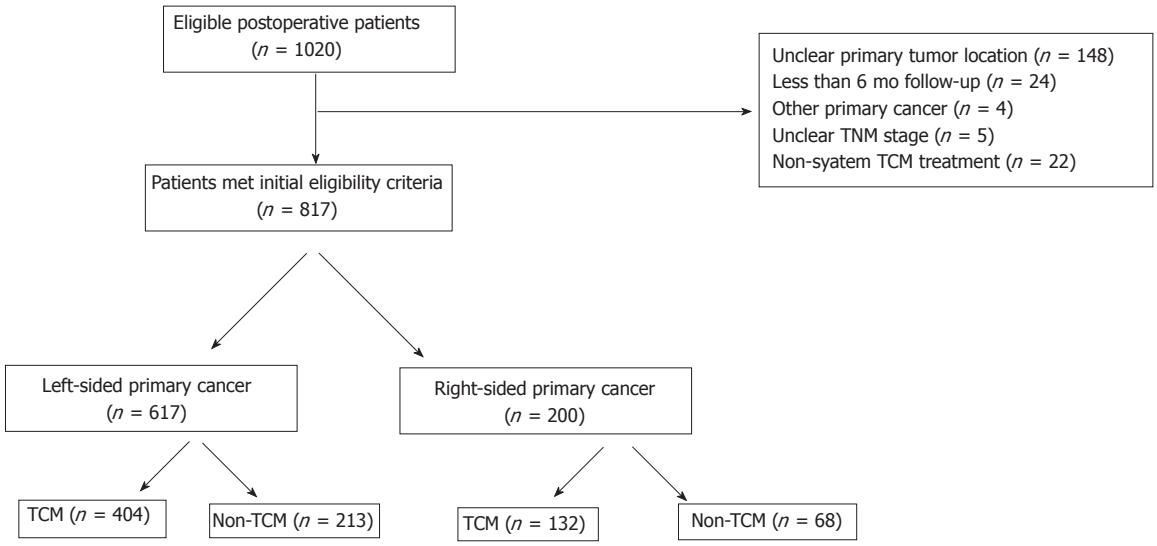


Figure 1 Flowchart of patient selection and grouping.

Table 1 Levels of sIL-2R, ALT, and HBV DNA in the sera of patients with chronic HBV infection (mean ± SD)

	Left-sided		Right-sided		*P* value
	TCM *n* = 404	Non-TCM *n* = 213	TCM *n* = 132	Non-TCM *n* = 68	
Gender					0.035[a]
Man	207	126	63	28	
Woman	197	87	69	40	
Age (yr)					0.027[a]
< 60	163	70	41	17	
≥ 60	241	143	91	51	
Histodifferentiatio, *n*					0.293
Poorly	47	19	18	8	
Moderately	252	123	80	34	
Well	9	7	6	2	
Unknown	96	64	28	24	
Lympho node metastasis					0.125
Yes	176	74	57	32	
No	228	139	75	36	
TNM stage					0.331
I	77	38	19	8	
II	148	95	54	28	
III	179	80	59	32	
Chemotherapy					0.000[a]
Yes	332	149	97	44	
No	72	64	35	24	
Radiotherapy					0.011[a]
Yes	44	19	5	1	
No	360	194	127	67	
Diabetes					0.995
Yes	61	33	21	10	
No	343	180	111	58	
Hypertension					0.872
Yes	131	76	44	22	
No	273	137	88	46	
Heart disease					0.367
Yes	38	30	15	7	
No	366	183	117	61	
Stroke					0.049[a]
Yes	22	13	1	2	
No	382	200	119	66	

[a]*P* < 0.05 statistical difference. LSCRC: Left-sided colorectal cancer; RSCC: Right-sided colon cancer.

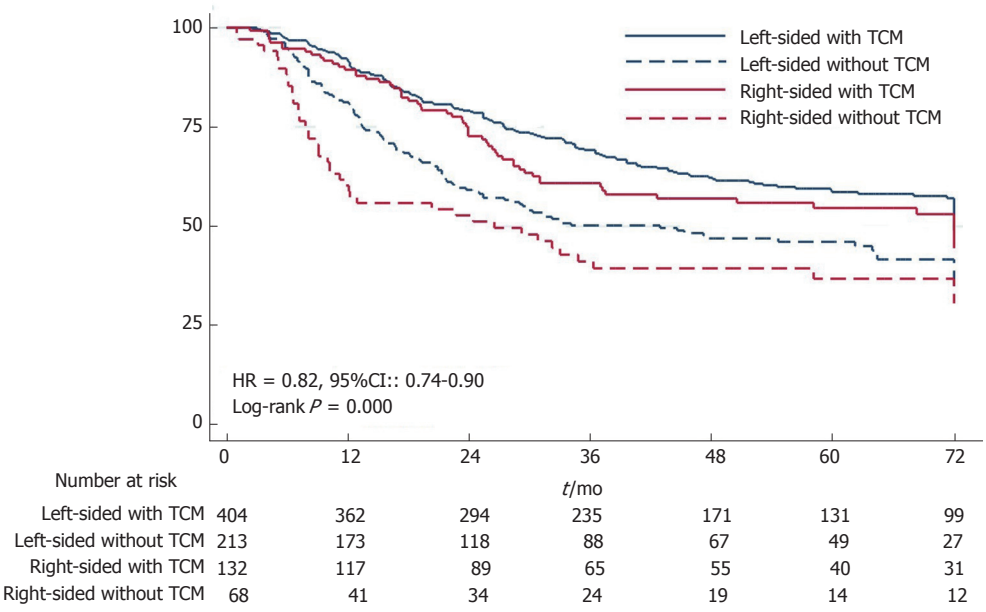


Figure 2 Kaplan-Meier disease-free survival curves for all patients.

gender, age, location, histodifferentiation, TNM stage, lymph node status, chemotherapy, radiotherapy and comorbidities; this matching was conducted to test the stability of our research. A 1:1 match with a random matching order and 0.1 caliper was performed between the TCM and Non-TCM groups in LSCRC and RSCC, respectively, and a 2:1 match was conducted between LSCRC and RSCC within the TCM and Non-TCM groups, respectively; additionally, replacements were not allowed. In addition, we applied multivariate regression analyses for multicollinearity diagnosis and Cox proportional hazards regression model for multivariate factor analysis. Hypothesis testing was conducted using a two-sided alpha set to a 5% level of significance. All analyses were performed using Stata 12.0 and SPSS 22.0 software. The statistical methods used in this study were reviewed by Weibing Wang from the Department of Epidemiology, School of Public Health, Fudan University.

Traditional Chinese medicine treatment

TCM prescriptions were determined by attending physicians on the basis of syndrome differentiation, and their composition, dose were modified every two weeks to tailor them to the distinctive symptom complex. The herbs were processed as a decoction for administration twice per day (200 mL per session). Notably, TCM treatment must have been continually managed for at least six months before relapse for inclusion in the TCM group and was ceased at the patient's request, by the physician or in an instance of relapse.

Definition of left-sided colorectal cancer and right-sided colon cancer

The primary tumor side was identified using post-operative pathology. LSCRC was defined as a primary tumor located between the splenic flexure and the rectosigmoid junction, whereas RSCC was defined as a primary site originating between the cecum and the proximal two-thirds of the transverse colon[10].

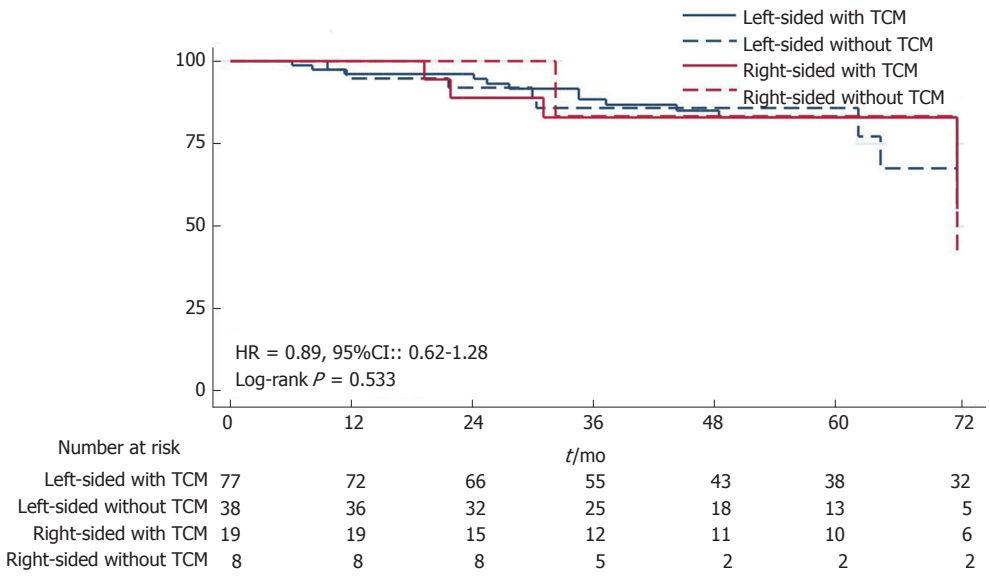
RESULTS

Patients

Among the 817 patients, 617 had LSCRC (TCM group, *n* = 404; Non-TCM group, *n* = 213), and 200 had RSCC (TCM group, *n* = 132; Non-TCM group, *n* = 68). Subjects with LSCRC were more likely to be male than those with RSCC. Patients were commonly older than 60 years old and were less likely to have a stroke. Most patients have received chemotherapy but very few have undergone radiotherapy. Other characteristics such as histodifferentiation, TNM stage, lymph node status, diabetes, hypertension, and heart disease were evenly distributed among the groups and showed no statistically significant differences. The baseline characteristics after propensity score matching were shown in Supplementary Tables 1 and 2.

Effects of traditional Chinese medicine on left-sided colorectal cancer and right-sided colon cancer patients

TCM conferred higher 1-6 year DFS rates on patients with LSCRC (91.98% at 1, 78.95% at 2, 69.15% at 3, 62.04% at 4, 58.45% at 5, and 56.95% at 6 years) than on LSCRC patients who did not receive TCM (81.22%, 59.05%, 50.05%, 46.92%, 46.12%, and 41.50%, respectively). The 1-6 year DFS rates of patients with RSCC in the TCM group were 89.35%, 72.82%, 60.68%, 56.77%, 54.43%, and 52.92%, respectively, whereas in the Non-TCM group, the DFS rates were 60.29%, 52.76%, 41.39%, 39.51%,



Number at risk							
Left-sided with TCM	77	72	66	55	43	38	32
Left-sided without TCM	38	36	32	25	18	13	5
Right-sided with TCM	19	19	15	12	11	10	6
Right-sided without TCM	8	8	8	5	2	2	2

Figure 3 Kaplan-Meier disease-free survival curves for patients with stage I disease.

37.19%, and 37.19%, respectively (Figure 2). DFS was significantly improved by TCM not only in patients with LSCRC (HR = 0.59, *P* = 0.000) but also in those with RSCC (HR = 0.56, *P* = 0.003) (Supplementary Figures 1 and 2). However, the LSCRC and RSCC TCM groups showed similar DFS rates (HR = 0.84, *P* = 0.239) as did the LSCRC and RSCC Non-TCM groups (HR = 0.76, *P* = 0.129) (Supplementary Figures 3 and 4). The above results were relatively unchanged after propensity score matching (Supplementary Figures 5, 6, 7 and 8).

Effect of traditional Chinese medicine on left-sided colorectal cancer and right-sided colon cancer patients in different disease stages

For patients with stage I disease, TCM did not exhibit an obvious advantage in extending DFS in patients with either LSCRC or RSCC (Figure 3; Supplementary Figures 9 and 10). TCM significantly prolonged DFS of patients with stage II LSCRC (HR = 0.60, 95%CI: 0.40-0.89, *P* = 0.011), whereas patients with stage II RSCC in the TCM group showed longer DFS than those in the Non-TCM group; however, this difference was not significant (HR = 0.56, 95%CI: 0.29-1.07, *P* = 0.077) (Figure 4; Supplementary Figures 11 and 12). For stage III disease, TCM was effective for patients with either LSCRC (HR = 0.44, 95%CI: 0.32-0.61, *P* = 0.000) or RSCC (HR = 0.47, 95%CI: 0.28-0.79, *P* = 0.004) (Figure 5; Supplementary Figures 13 and 14).

Cox analysis of baseline characteristics on disease-free survival in left-sided colorectal cancer and right-sided colon cancer

The multivariate regression analyses showed no multicollinearity among variables in this cox analysis (Supplementary Figures 15 and 16). TCM was an independent influencing factor for DFS of patients with either LSCRC (HR = 0.53, 95%CI: 0.41-0.67) or RSCC (HR = 0.47, 95%CI: 0.31-0.71); TNM stage was also an independent factor (HR = 2.39, 95%CI: 1.96-2.90 for LSCRC; HR = 2.63, 95%CI: 1.85-3.72 for RSCC). Histodifferentiation (HR = 1.16, 95%CI: 1.03-1.31) and hypertension (HR = 0.51, 95%CI: 0.31-0.81) were independent influencing factor for DFS of patients with LSCRC and RSCC, respectively (Table 2).

DISCUSSION

Recently, the sidedness of primary colon cancer was demonstrated to be a prognostic factor in survival. Because of their distinct biological characteristics, LSCRC and RSCC tend to be treated separately. To the best of our knowledge, this is the first study discussing the effects of TCM on LSCRC and RSCC separately. Our Cox analysis showed that TCM was an independent influencing factor on DFS for each side. Patients with LSCRC exhibited a relatively longer DFS than those with RSCC regardless of TCM administration, whereas patients with RSCC who took TCM gained a greater benefit regarding DFS-this partially narrowed the disparity in DFS between the different cancer sides.

Our study was somewhat similar to those of previous articles in that TCM coupled with chemotherapy was significantly effective in prolonging DFS of patients with CRC. Tao *et al*[11] L proved that compared to monochemotherapy, chemotherapy integrated with TCM obviously improved the prognosis of patients with stage II-III CRC by reducing recurrence and metastasis. Zhou *et al*[12] LY demonstrated that TCM effectively improved the quality of life, increased body weight and prolonged the survival of patients with CRC. Our preliminary study proposed that individuals with stage

Table 2 Cox analysis for disease-free survival in left- and right-sided colorectal cancer

	Left-sided colorectal cancer			Right-sided colon cancer		
	Univariate	Multivariate		Univariate	Multivariate	
	P value	*P* value	HR (95%CI:)	*P* value	*P* value	HR (95%CI:)
Gender	0.024[a]	0.079	0.81 (0.63-1.02)	0.339	0.023[a]	0.63 (0.42-0.94)
Age	0.955	0.792	1.04 (0.80-1.34)	0.712	0.612	1.12 (0.72-1.76)
Histodifferentiation	0.648	0.016[a]	1.16 (1.03-1.31)	0.685	0.407	1.09 (0.89-1.33)
TNM stage	0.000[a]	0.000[a]	2.39 (1.96-2.90)	0.000[a]	0.000[a]	2.63 (1.85-3.72)
TCM	0.000[a]	0.000[a]	0.53 (0.41-0.67)	0.003[a]	0.000[a]	0.47 (0.31-0.71)
Diabetes	0.948	0.716	0.94 (0.66-1.32)	0.240	0.200	0.67 (0.36-1.24)
Hypertention	0.650	0.120	1.24 (0.94-1.64)	0.019[a]	0.005[a]	0.51 (0.31-0.81)
Heart disease	0.710	0.988	1.00 (0.66-1. 50)	0.461	0.155	1.57 (0.84-2.93)
Stroke	0.171	0.091	0.60 (0.33-1.09)	0.681	0.449	1.34 (0.63-2.83)

[a]$P < 0.05$ statistical difference.

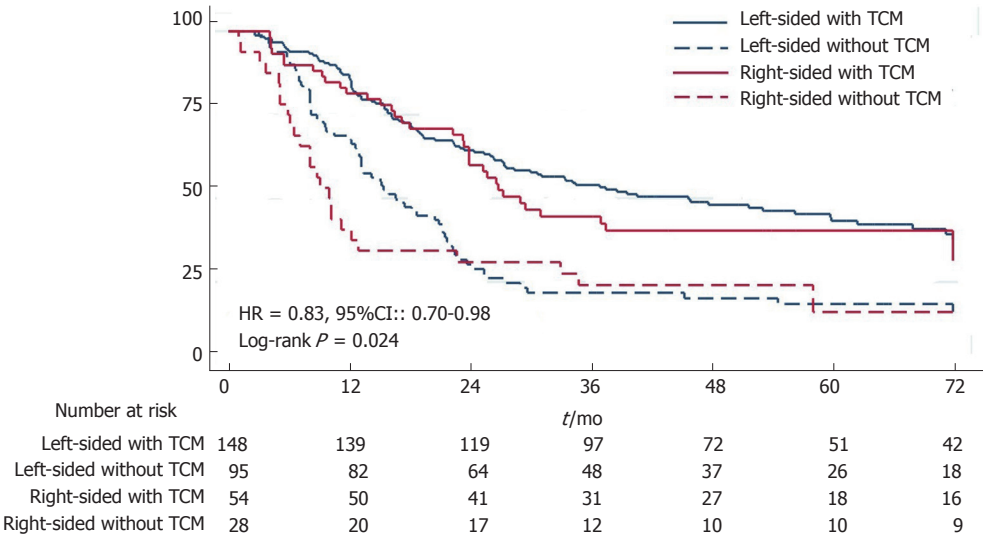


Figure 4 Kaplan-Meier disease-free survival curves for patients with stage II disease.

I CRC do not require TCM[6]. In this study, we found that patients with stage I CRC on either side did not benefit from TCM, whereas subjects with stage II-III LSCRC or RSCC who were administered TCM exhibited a distinct advantage in decreasing the risk of recurrence and metastasis; however, this advantage was not statistically significant in subjects with stage II RSCC. We thought that the primary reasons why TCM improved DFS of CRC on both sides lie in the advantages of TCM in attenuating toxicity, improving immunity and quality of life and enhancing medication sensitivity in patients with tumors, which can prevent metastasis and extend survival[13-17].

The different responses of LSCRC and RSCC to treatment have been previously reported. The GALGB/SWOG 80405 study, which investigated the effect of cetuximab and bevacizumab on cancer located on different sides, found that patients with LSCRC benefited more from cetuximab, whereas those with RSCC responded better to bevacizumab in terms of survival[18]. Moreover, Elsaleh *et al*[19] found that men with RSCC obtained increased benefits from adjuvant chemotherapy whereas men with LSCRC did not; one possible explanation may be partially due to the higher frequency of microsatellite instability (MSI) in RSCC which may be a prognostic factor for a more favorable response[19].

Whether due to differences in biological characteristics between the two sides or TCM producing a relatively better effect on RSCC, RSCC exhibited a greater benefit from TCM than LSCRC in our study; this finding is worth further study. Thus, differences between LSCRC and RSCC regarding embryological origin, blood supply, morphology, carbohydrate antigens and other factors should be considered[20]. In addition, RSCC was more commonly associated with RAS and BRAF mutations, a high CpG island methylator phenotype, mutagenic metabolites of cytochrome p450, MAPK signaling and MSI, whereas LSCRC was associated with APC, K-ras, DCC, p53 mutant EGFR signaling, Wnt signaling and HER1 and HER2 amplification which played a vital role in cancer generation and progression[21-24]. Interestingly, previous studies suggested that the prolonged survival of CRC patients by TCM was mediated by demethylating DNA, antagonizing gene mutations, targeting the MSI

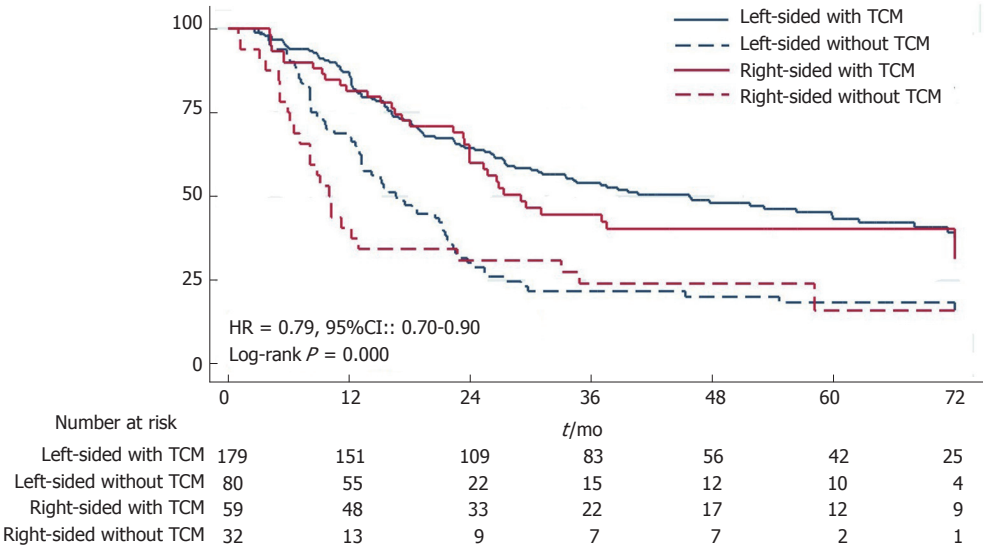


Figure 5 Kaplan-Meier disease-free survival curves for patients with stage III disease.

pathway and promoting apoptosis, which are more characteristics of RSCC[25-27].

There were some limitations in our study. Administration of TCM was tailored to each patient's symptoms, signs and constitution. Although these highly individualized TCM prescriptions were adapted to the patients' conditions to the greatest extent possible, the many different kinds and doses of herbs were difficult to be stratified in this paper[28]. In the future research, we are going to extract some core herbs used in treating CRC based on the primary tumor sides. In addition, TCM in our study was administered as a decoction, the quality of TCM herbs may differ based on the source region, season, and processing factories inevitably. We recommend granular or powder TCM formulations for future treatments. Another limitation lies in a lack of knowledge regarding the optimal period of TCM medication for patients with different TNM stages of CRC, which will be studied in our future research.

In conclusion, our study showed that TCM conferred longer DFS on patients with stage II-III CRC on both sides. Patients with LSCRC and RSCC responded differently to TCM; those with RSCC benefited more from TCM than those with LSCRC. Thus, TCM was recommended to postoperative patients with CRC of both sides, especially the right side. The mechanism of different responses of primary tumor location to TCM is worthy of further study.

ARTICLE HIGHLIGHTS

Research background

The background, present status and significance of the study have been described detailedly in the section "Introduction" of the text.

Research motivation

The background, present status and significance of the study have been described detailedly in the section "Introduction" of the text.

Research objectives

The main objectives, the objectives that were realized, and the significance of realizing these objectives for future research in this field were described in the last sentence of "Introduction", "Discussion".

Research methods

"Statistical analysis" has introduced the methods used in realizing the objectives of our manuscript in detail.

Research results

Research results have been detailedly described in the first and last paragraphs of "Discussion".

Research conclusions

The authors have addressed the above questions mainly in "Statistical analysis" and "Discussion". Recently, the sidedness of primary colon cancer was demonstrated to be a prognostic factor in survival. Because of their distinct biological characteristics, LSCRC and RSCC tend to be treated separately. However, there was no evidence regarding whether TCM exerts variable effects on CRC based on the side where the lesion is located. This is the first study discussing the effects of TCM on LSCRC and RSCC separately. Our results showed that patients with LSCRC exhibited a relatively longer DFS than those with RSCC regardless of TCM administration, whereas patients with RSCC who took TCM gained a greater benefit regarding DFS. Because of their distinct biological characteristics and the therapeutic effect, LSCRC and RSCC should be treated separately in future. The DFS was evaluated using Kaplan-Meier curves and log-rank tests. The authors adopted propensity score matching to model the probability that a patient exhibited a specific characteristic based on gender, age, location, histodifferentiation, TNM stage, lymph node status, chemotherapy, radiotherapy and comorbidities to test the stability of the research. In addition, the authors applied multivariate regression analyses for multicollinearity diagnosis and Cox proportional hazards regression model for multivariate factor analysis.

Research perspectives

Recent studies have proposed that location of primary tumor was related to recurrence, metastasis and the therapeutic effect. The author's results indicated that patients with LSCRC and RSCC responded differently to TCM; those with RSCC benefited more from TCM than those with LSCRC. Thus, TCM was recommended to postoperative patients with CRC of both sides, especially the right side. In the future research, CRC should be treated separately based on the primary tumor sides.

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ORIGINAL ARTICLE

Observational Study

Hepatitis B virus outreach to immigrant population in Greater Boston Area: Key to improving hepatitis B knowledge

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Abstract

AIM

To characterize the understanding of hepatitis B virus (HBV) and determine if outreach improves HBV understanding among Greater Boston Area immigrants.

METHODS

Six outreach sessions were held in various community venues in the Greater Boston Area. Verbal consent was obtained from participants prior to starting each session. Each session included a pre-session questionnaire, followed by a teaching session, and then a post-session questionnaire. In person interpreters were present for translation during the teaching session and assistance for questionnaire completion when needed. The questions were developed based on the HBV clinical experience of physicians who serve largely immigrant populations. Questionnaires included Likert-type scale, open-ended, and true-false questions. All results were anonymous.

RESULTS

One hundred and one people participated in this study. Participants were 30% male with ages ranging from 19 to 87 years. The study population included immigrants from 21 countries, as well as seven United States-born participants. The greatest numbers of participants were from Somalia (44%), Morocco (10%), and Cameroon (8%). Pre session questionnaires revealed that 42% of participants were unaware that HBV can cause cancer, and 50% were unaware that therapies for HBV exist. Our brief teaching intervention led to improved scores on post session questionnaires. For example, at baseline, 58% of participants responded correctly to the question "HBV infection can cause scarring of the liver and liver cancer", whereas 79% of participants responded correctly after the teaching session (P = 0.01). Furthermore, the mean of total correct answers in the true or false portion of the questionnaire increased from 5.5 to 7.6 (P < 0.001).

CONCLUSION

A teaching session targeting Boston Immigrants at-risk for HBV helped improve scores on HBV knowledge questionnaires. Outreach may empower at-risk patients to pro-actively seek HBV care.

Key words: Hepatitis B virus; Outreach; Linkage to care; Immigrant; Boston

Core tip: Awareness is the key to the mitigation of transmittable diseases such as hepatitis B virus (HBV). Therefore, characterizing the baseline understanding of HBV, and improving that baseline, are the first steps toward improving HBV linkage to care among at-risk persons. To characterize and improve the baseline understanding of HBV we performed HBV teaching sessions with pre and post session questionnaires in multiple community venues in and around Boston. These sessions revealed that (1) baseline understanding of risks related to HBV are limited; and (2) a brief teaching session can significantly improve understanding of HBV risks.

Djoufack R, Cheon SSY, Mohamed A, Faye F, Diouf K, Colvin R, Morrill J, Duffy-Keane AM, Perumalswami P, Jourdain G, Fusco DN. Hepatitis B virus outreach to immigrant population in Greater Boston Area: Key to improving hepatitis B knowledge. *World J Gastroenterol* 2017; 23(42): 7626-7634 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7626.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7626

INTRODUCTION

Roughly 240 million people worldwide are chronically infected with hepatitis B virus (HBV), defined as hepatitis B surface antigen (HBsAg) positive for over 6 mo[1]. Over 686000 people are estimated to die every year due to complications of HBV infection, including cirrhosis and liver cancer[1]. HBV prevalence varies worldwide, with highest prevalence in sub-Saharan Africa and East Asia, where between 5%-10% of the adult population is chronically infected[1,2,13]. While precise data on some countries in sub-Saharan Africa are limited, prevalence estimates have been generated from studies of migrant populations, and estimate high prevalence[5,17]. Roughly half of the 800000 to 1.4 million United States residents chronically infected with HBV were born in other countries. The Center for Disease Control and Prevention current recommendations for HBV testing and evaluation have evolved during the last two decades to include more and more persons born abroad (currently countries with HBsAg prevalence of greater than 2%). Appropriate

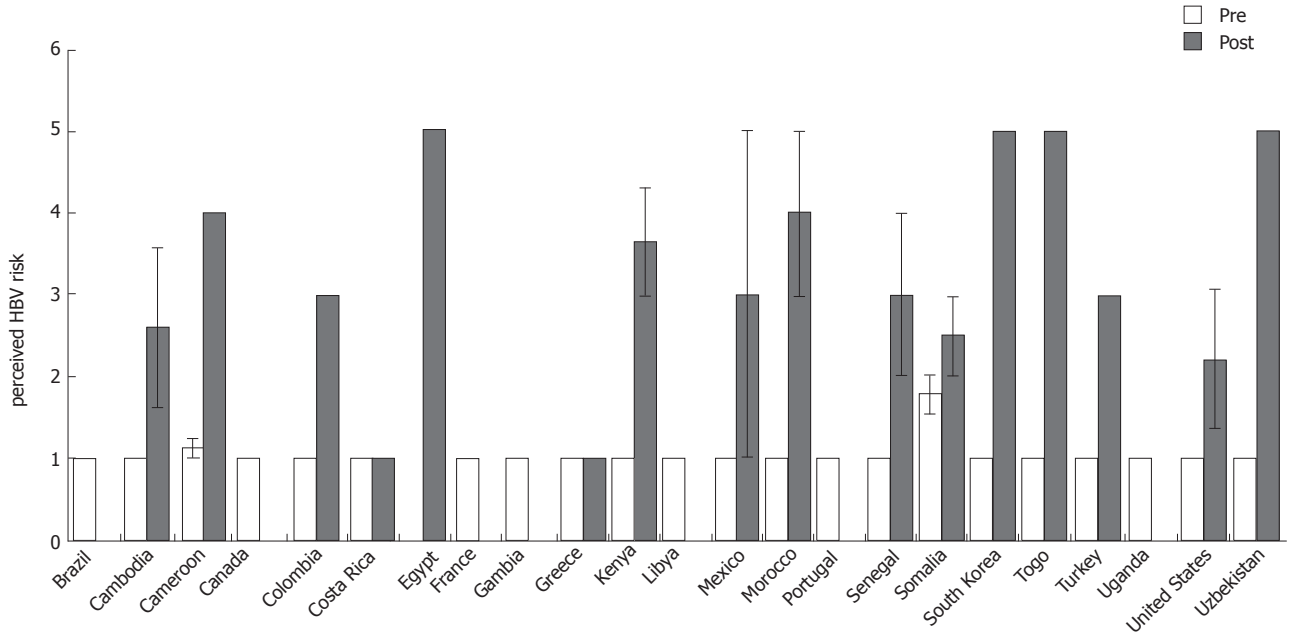

Figure 1 Perceived hepatitis B virus risk by country: responses to "How high is your risk for having hepatitis B infection, on a scale of 1 to 5?" In the pre session, 98 participants answered this question with a mean response score of 1.33. In the post session, 53 participants answered and a mean response score of 3.13 was obtained. There is a statistically significant increase in the perceived risk of hepatitis B with [a]*P* value of < 0.05.

HBV care can both prevent progression of HBV related disease in infected persons and may decrease the likelihood of spread to others[1].

A recent study in the United States found that of 277 health care providers, only 42% reported performing HBV screening in over 50% of patients at risk for HBV[12]. This study identified language as a major barrier to care. Providers speaking an Asian language or caring for > 25% Asian patients were more likely to screen for HBV. Fewer studies have focused on barriers to HBV care among African immigrants in the United States, though they are at high risk, similar to Asian immigrants[3,15]. Between 1980 and 2009, there was a seven-fold increase in the number of African-born United States residents, underscoring a need for HBV outreach targeting these at-risk populations[3,9,10]. While the United States Department of Health and Human Services have initiated efforts to increase HBV services among minority populations, there is an ongoing need for population-specific HBV linkage-to-care strategies[3,6,8].

Prior studies among immigrants in the New York City area have revealed that HBV outreach to immigrants from countries where HBV is endemic, including immigrants from Sub Saharan Africa, leads to improved HBV diagnosis and engagement in follow up care[3,7,14,16,18]., including HCC screening in those who are infected Awareness and knowledge of the disease are prerequisites to screening for HBV infection, then monitoring and treatment. We undertook a study in Boston, which has a large Sub-Saharan African immigrant population[4] to determine (1) baseline HBV knowledge among Greater Boston Area immigrants from HBV endemic regions; and (2) whether a brief teaching intervention could improve HBV knowledge among these at-risk persons.

MATERIALS AND METHODS

Ethical approval

Approval by the Partners/Massachusetts General Hospital Institutional Review Board (Protocol 2014P000921, DNF) was obtained prior to initiating this study. Verbal consent was provided by all participants.

Outreach sessions

After obtaining IRB approval, six "HBV Linkage to Care" community outreach sessions were conducted in the Greater Boston Area targeting foreign-born communities. Although the study targeted foreign born persons, we did not exclude any United States born persons who attended from participating. Event one was held at a Somali restaurant. Event two was held at a Community Health Center. Events three, four, and five were held at private residences/recreation rooms in apartment complexes throughout Boston. Event six was held at a Turkish community center. Food was provided for hospitality at each event. Participants for each event were solicited by word of mouth by a local community outreach liaison, including several members of the Somali community. Each event included the following structure: participants were greeted and informed that the session was part of a study, and verbal consent obtained in native language or in English. In person interpreters were present for Somali events (Somali speaking), Cambodian event (Khmer speaking), and the Turkish Center event (Arabic and Spanish speaking). Interpreters translated the teaching session

Table 1 Demographics of the study population

Characteristics	*n* (%) or mean ± SD
Age (yr)	47 (18)
19-30	20 (22)
31-40	16 (18)
41-50	17 (19)
51-60	15 (17)
> 60	22 (24)
Gender	
F	62 (70)
M	26 (30)
Years in the United States	13 (12)
0-5	24 (28)
6-10	14 (16)
11-15	12 (14)
16-20	21 (24)
> 20	16 (18)
Country of origin	
Somalia	44 (44)
Morocco	10 (10)
Cameroon	8 (8)
Others[1]	39 (39)

[1]See Figure S1. Study population demographics provided by participants as number (No) and percent (%) or mean and standard deviation values (for age and years in the United States).

verbally and assisted participants in questionnaire completion. Participants were then asked to complete a pre-session (baseline) questionnaire within about 30 min. The questionnaire was developed by physicians based on HBV clinical experience in Community Health Centers serving largely immigrant populations. Participants were asked three types of questions (1) six questions answered with a Likert-type scale where responses were scored along a range from 1 to 5); (2) seven open ended questions; and (3) ten true/false questions (Figure S1). The complete teaching session was developed by the study team and then presented to community-based clinicians who manage patients with viral hepatitis and outreach workers serving both immigrant and non-immigrant health center populations for input and editing prior to IRB submission and approval. During outreach events, the teaching session was presented by a member of the study team and took approximately 45 min including time for questions after the session. Participants were then asked to complete a post-session questionnaire (identical to the pre-session questionnaire), though a significant number of participants left without completing the post-session questionnaire. Of note, participants were not instructed to complete their questionnaires alone, and many participants worked in small subgroups, completing questionnaires together. At the end of each event, participants were given (1) a navigator card, in English, which stated a request for HBV testing (Figure S2); and (2) a list of local HBV providers verified to be accepting new patients and their contact information, with contents of each document translated by interpreters. The list of local providers was developed by members of the study team who identified five major medical centers and two community health centers in the Greater Boston area with whom linkage to care agreements were established by contacting outpatient Gastroenterology and/or Infectious Disease clinics at each site, and verification of each site contact information and ongoing acceptance of new patients. All participation was anonymous with no study identifiers being collected.

Data analysis and statistics

All questionnaires were stored in a secure file cabinet accessed only by study staff for entry into a secure database. We used the Kruskal-Wallis test or Fisher's exact test for comparisons. A P value < 0.05 was considered significant, with no correction for multiple comparisons.

RESULTS

There were 101 participants in the pre-session questionnaire, 56 of whom also completed the postsession questionnaires. All 101 pre-session questionnaires were included in data. Participants were male and female, ranging from 19 to 87 years of age (Figure 1). Several female-only teaching sessions were requested by the Somali community leader, leading to greater number of female (62) than male (26) participants overall (Table 1). The study population included immigrants born in 21 countries outside the United States, as well as seven participants born in the United States (Table 1, Figure S3). Countries with the greatest number of participants were Somalia (44%), Morocco (10%), and Cameroon (8%) (Table 1, Figure S3). The mean number of years the participants had spent in the United States was 13 (SD = 12), with 24% participants having been in the United States for less than 5 years (Table 1).

Likert-type scale responses

Responses to the "How high is your risk for having HBV infection?" question were scaled from 1 (lowest risk) to 5 (highest risk) (Figure 1, Figure S1). For this question of perceived HBV risk, mean response scores from pre-teaching session participants from all countries scored at or near 1 except Somalia (1.78). Following the teaching session, mean perceived risk scores increased for participants from the following countries (pre, post mean score): Cambodia (1, 2.6), Cameroon (1.1, 4), Colombia 1, 3), Kenya (1, 3.7), Mexico (1,3), Morocco (1, 4), Senegal (1, 3), Somalia (1.8, 2.5), South Korea (1, 5), Togo (1, 5), Turkey (1, 3), United States (1, 2.2), Uzbekistan (1, 5), though the statistical significance of many of these values is limited by small number of participants (Figure 1). Overall, the mean score for perceived HBV risk was 1.34 prior to the teaching session and increased to 3.26 following the teaching session (P < 0.001; Table 2), independent of gender, age, or the number of years since immigration to the

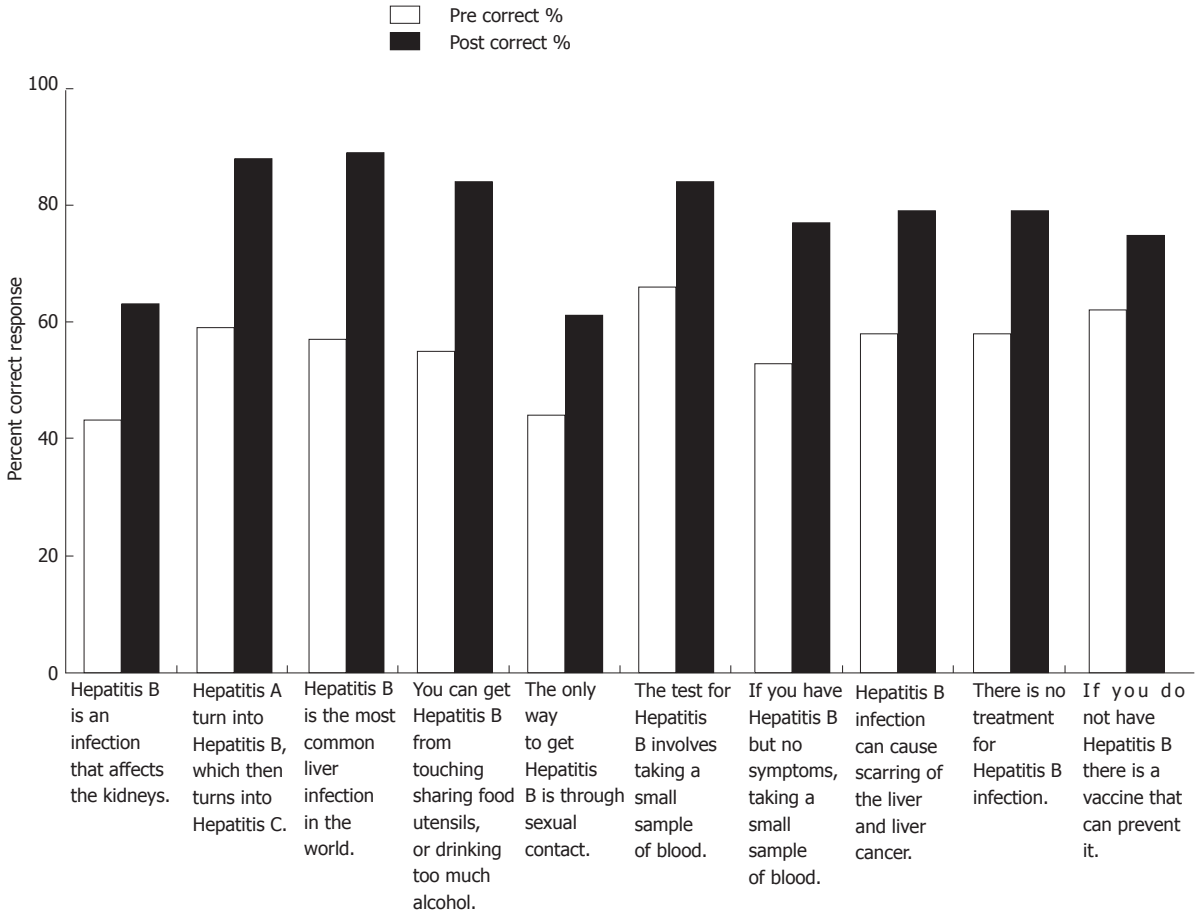

Figure 2 Responses to true/false questions. Participants were asked to answer a series of true/false questions prior to (Pre) and following (Post) the teaching session. The percentage of correct responses to true/false questions increased, following the teaching session, for all questions.

United States (logistic regression, data not shown). Among reasons for possible increased risk of infection, foreign origin was the most cited (40%). Interestingly, the major cause of infection in high prevalence countries, *i.e.,* mother-to-child transmission, was cited only once, post teaching.

Open ended questions

Participants were asked 5 open-ended questions regarding HBV testing including barriers, associated stigma and treatment knowledge (Figure S1). For the open-ended question "What makes your risk for HBV infection higher?" the percent of participants responding "Country of origin" increased slightly from 40% (pre) to 44% (post), following the teaching session (Table 2). The percent of participants answering "I am not at risk" decreased from 20 % (pre) to 12% (post) following the teaching session (Table 2). Responses to additional open ended questions revealed that 73% (pre) -81% (post) of participants reported that HBV testing is important, and 31%-40% of participants cited concerns regarding insurance coverage as a barrier to access to HBV care (Table 3).

True/False questions related to HBV knowledge

Participants were asked to answer ten true/false questions prior to (pre) and after (post) the teaching session (Figure 2, Table 4). The percentage of participants answering each true/false HBV question correctly increased following the teaching session (Figure 2, Table 4), from a mean of 5.5 (report SD) out of 10 correct answers, to a mean of 7.6 (report SD) out of 10 correct answers ($P < 0.001$). For the question "The only way to get HBV is through sexual contact" (false), the percentage of correct answers increased from 45 (pre) to 61 (post) ($P = 0.04$). For the question "If you have HBV but no symptoms, no need to worry about your liver" (false), the percentage of accurate answers increased from 53 (pre) to 77 (post) ($P = 0.006$). For the question "HBV can cause scarring of the liver and liver cancer" (true), the percentage of correct answers increased from 58 (pre) to 79 (post) ($P = 0.01$). For the question "There is no treatment for HBV" (false), the percentage increased from 50 (pre) to 66 (post), but was not statistically significant ($P = 0.06$). For the question "If you do not have HBV, there is a vaccine that can prevent HBV" (true), the percentage increased from 62 (pre) to 75

Table 2 Perceived hepatitis b risk before and after teaching session *n* (%)

Perceived at least some risk of being HBV infected	Pre session (*n* = 101)	Post session (*n* = 56)	*P* value
Mean	1.34	3.26	< 0.001
At least some risk	11 (11)	35 (70)	
Gender			
Female	7 (12)	24 (71)	
Male	3 (12)	5 (63)	
Age (yr)			
< 45	2 (5)	23 (70)	
> 45	8 (18)	9 (75)	
Years in the United States			
< 5	2 (9)	11 (79)	
> 5	9 (13)	19 (63)	
Continent of Origin[1]			
Africa	11 (16)	19 (73)	
Asia	0	6 (67)	
Europe	0	4 (57)	
America	0	1 (50)	
Reasons why your risk is high			
Country of origin	30 (40)	22 (44)	
Needle exposure	15 (20)	9 (18)	
Sexual exposure	14 (19)	9 (18)	
I don't know	11 (15)	9 (18)	
I am not at risk	15 (20)	6 (12)	
Other reasons[2]	8 (10)	9 (18)	
Mother to child transmission	0	1 (0.02)	

[1]Africa (Cameroon, Gambia, Kenya, Libya, Morocco, Senegal, Somalia, Togo, Uganda), Asia (Cambodia, South Korea, Uzbekistan), Europe (France, Greece, Turkey), America (Brazil, Canada, Colombia, Costa Rica, Mexico, United States); [2]Other reasons: Pre (genetic, food, public transportation, wound, job, utensils, contact with blood, sharing blade), Post (lack of information); Participant answers to perceived HBV risk were categorized as either Low risk, if answered 1, or Some risk, for answers of more than 1 in a Likert Scale (1= low; 5 = high risk); HBV: Hepatitis B virus.

(post), but, again, was not statistically significant (P = 0.2).

Other points raised during discussion

Several participants from Somalia clarified that, although they were originally from Somalia, they had spent multiple years in Kenya in refugee camps prior to their arrival to the United States, disrupting both formal education and access to health care for many. Several Somali participants stated that access to vaccines prior to immigration, even when they were available at some sites in their native country, were not accessible due to political instability.

DISCUSSION

The goals of this study were to determine the baseline HBV knowledge state of at-risk immigrants in the Greater Boston Area, as well as the ability to improve HBV understanding through a brief teaching intervention. Of all immigrant populations, the highest number of participants by far was among Somali immigrants, suggesting a particular openness to seek health education and highly effective community networking (recruiting participants) within this community in Boston, likely due to the pre-existing community network established by our Somali liaison.

There were several limitations to our study. One limitation was the lack of retention of participants for the entire event, with many (45%) participants leaving before answering the post-session questionnaire. Furthermore, the sample size of this study was relatively small as this was exploratory research, and there had been no a priori calculation of a target sample size based on a specific hypothesis to be tested. The data collected in our study may help determine the design and sample size of future studies. Another limitation was language barrier, despite presence of in-person interpreters, due to the high ratio of participants per interpreter, and failure to obtain education information, which may have influenced questionnaire responses, from participants. Another limitation of this study was that the questionnaire was not validated. There was some difficulty interpreting changes in answers to open ended questions between pre- and post- teaching sessions. For example, for the open-ended question "Why is it important to test those at risk for HBV", in the pre-session questionnaire, 24% of participants answered that HBV testing could prevent the spread of infection, whereas in the post-session questionnaire, 16% of participants answered that HBV testing could prevent spread of infection. In contrast, post session responses to this question increased for "initiate treatment early" (from 16% to 20%), and "prevent long term complications" (from 10% to 16%), indicating that participants likely shifted the focus of their answers, without any clear evidence of loss of knowledge. These results highlight the complexity of interpreting open-ended questions in a quantitative manner, an area for improvement in future studies. Another limitation is that the impact of this education intervention on longer term knowledge retention as well as accessing HBV screening is not known and should be included in a future study.

While knowledge overall improved after the teaching intervention (report the overall change score with P value), there were two areas that demonstrated more room for improvement including vaccination and treatment. During teaching sessions, it was explained that treatment for HBV exists, but is not curative, and must be taken for the long-term, rather than a discrete time course. It is possible that this description of a suppressive but not curative treatment led to confusion. Regarding vaccines, it was explained that they are effective if given prior to infection, but not if given once someone is already infected. This point was repeated several times in an attempt to clearly communicate that the simple receipt of an HBV vaccine does not eradicate HBV if a person from an endemic area was already infected. In fact, the authors note that communication of this message was one of the

Table 3 Responses to additional open ended questions *n* (%)

Variable	Pre	Post
Why is it important to test those at risk for HBV?		
Prevent the spread of infection	21 (24)	8 (16)
Initiate the treatment early	14 (16)	10 (20)
To be aware of our status	12 (14)	8 (16)
Prevent long term complications	9 (10)	8 (16)
Because no symptoms are present until late stage	2 (2)	
I don't know	27 (31)	11 (22)
To vaccinate those unaffected		2 (4)
2 of the above	2 (2)	2 (4)
Why you do not think it is important to test		
It is important	36 (73)	22 (81)
It is not important	3 (6)	1 (3)
I don't know	6 (12)	2 (7)
Because it is not very common	1 (2)	
I don't have enough information to recommend the test	2 (4)	
Patient has the right to decide if he wants it	1 (2)	1 (3)
Because everybody has Hepatitis B		1 (3)
Barriers of access to medical care		
Insurance coverage	18 (31)	17 (40)
It is easy for me	28 (48)	16 (38)
I don't know	3 (5)	2 (5)
Absence of good doctors	1 (2)	
Lack of available doctors	2 (3)	1 (2)
Lack of time	2 (3)	
Language barrier	1 (2)	1 (2)
Others reasons	2 (3)	3 (7)
2 factors above	1 (2)	2 (5)
Uncomfortable asking for Hepatitis B testing		
I don't find it uncomfortable	45 (90)	26 (76)
I am afraid of the result/consequences of the infection	2 (4)	2 (6)
Shyness/fear of what he might think	2 (4)	2 (6)
Because it is sexually transmitted	1 (2)	
Because I feel well		1 (3)
Because it is taboo		3 (9)
Why it will be difficult to get tested for HBV		
It would not be difficult for me	38 (64)	24 (61)
Insurance coverage	6 (10)	5 (13)
Fear of the results	4 (7)	5 (13)
I don't know	5 (8)	1 (2.5)
Cost of the test	4 (7)	1 (2.5)
Concerns about confidentiality	2 (3)	1 (2.5)
Lack of time		1 (2.5)
2 or 3 factors above	2 (3)	1 (2.5)
Why it could be difficult to get treated for HBV		
It would not be difficult for me	37 (65)	19 (59)
Insurance coverage	7 (12)	2 (7)
Cost of treatment	4 (7)	3 (9)
Treatment side effects	2 (3.5)	
I don't know	3 ((5))	
Lifelong treatment		2 (7)
Lack of treatment providers	1 (2)	
Other reasons	1 (2)	
2 0r 3 factors above	2 (3.5)	6 (19)

Pre and Post indicate answers provided prior to and following teaching session, respectively; HBV: hepatitis B virus.

reasons for the study: multiple patients interactions reveal that patients often perceive that they cannot be HBV infected because they have been vaccinated, although some may have been infected before vaccination. Results of this study reveal that, while knowledge related to HBV vaccines and treatments did improve following teaching intervention, additional refinement in these areas to the teaching intervention could enhance HBV knowledge. Specifically, additional slides illustrating the populations in whom vaccine will and will not work should be included.

This study revealed that outreach events, in accessible and hospitable community venues, can provide an effective forum for HBV health teaching sessions to raise HBV awareness for at-risk immigrants in the Greater Boston Area. Events held in Health

Table 4 True or false questions prior to (Pre) and following (Post) the teaching session *n* (%)

Variable	Pre Correct	Post correct	*P* value[1]
Mean of total correct answers (Std.dev) (mean ± SD)	5.5 ± 3.3	7.6 ± 2.9	< 0.001
1 Hepatitis B is an infection that affects the kidneys	43(43)	35 (63)	0.02
2 Hepatitis A turns into Hepatitis B, which then turns into Hepatitis C	60 (59)	49 (88)	< 0.0001
3 Hepatitis B is the most common liver infection in the world	58 (57)	50 (89)	< 0.0001
4 You can get hepatitis B from touching, sharing food utensils, or drinking too much alcohol	56 (55)	47 (84)	< 0.0001
5 The only way to get hepatitis B is through sexual contact	44 (45)	34 (61)	0.04
6 The test for hepatitis B involves taking a small sample of blood	67 (66)	47 (84)	0.02
7 If you have Hep B but no symptoms, no need to worry about your liver	54 (53)	43 (77)	0.006
8 Hepatitis B infection can cause scarring of the liver and liver cancer	59 (58)	44 (79)	0.01
9 There is no treatment for hepatitis B	50 (50)	37 (66)	0.06
10 If you do not have hepatitis B, there is a vaccine that can prevent it	65 (64)	42 (75)	0.20

[1]Indicates *P* value calculated using Fisher's exact test. Non-significant *P* values are indicated in bold.

Centers may be more poorly attended, though this requires confirmation as our health center event targeted a Cambodian population, whereas our other events targeted populations of African origin. Therefore multiple factors may have influenced attendance. Baseline knowledge of HBV relation to liver disease and hepatocellular carcinoma among Greater Boston Immigrants ranged among countries of origin. Although HBV status of participants is unknown, most participants came from countries of high HBV endemicity, thus improved HBV knowledge is desirable. Our simple teaching intervention revealed a significant improvement in HBV knowledge, specifically including the information that (1) country of origin can be a factor of HBV risk; (2) HBV causes cancer; and (3) HBV infection may cause pathology long before any symptoms. As noted, a limitation of our teaching sessions was the ability to communicate efficacy of vaccines and treatments. We only observed a modest increase in participants aware that HBV treatment exists (50% to 66%). This may be related to our limitations to communicate that antiviral treatment to prevent or delay complications exists but no cure for HBV highlighting the need for additional outreach on this topic. While outreach into the community does cost money and time, the benefit of added HBV knowledge may outweigh these costs. Additional studies determining the percent of participants that present for HBV testing and, if positive, receive appropriate care would be required to confirm this. This study highlights the finding that (1) persons at-risk for HBV are living in the Greater Boston area; (2) there are important gaps in HBV baseline knowledge in the community; (3) teaching sessions in the community are greeted with warm reception and high participation numbers, particularly among Somalis; and (4) significant improvement in HBV understanding can be made with teaching sessions.

COMMENTS

Background

Hepatitis B virus (HBV) infection and HBV related liver disease are a major public health problem worldwide. In 2016 the World Health Organization announced the initiative "Combating hepatitis B and C to reach elimination by 2030". However, many people at risk for HBV infection and HBV related liver complications are unaware that they are infected or unaware of HBV-related risks.

Research frontiers

They propose that reaching the final frontier of HBV elimination requires vigorous efforts to step up HBV awareness, at the global level. Awareness serves as the first step empowering at-risk persons to seek appropriate testing and care. Awareness in a given individual may also catalyze dissemination of information throughout at-risk communities. This pilot study represents a local first step in an effort to trigger rapid and accurate dissemination of HBV knowledge among persons at risk.

Innovations and breakthroughs

This study is innovative in that we worked to disseminate HBV directly to at-risk persons working through social networks who were brought together by community liaisons. While similar work has been initiated in the greater New York City metropolitan area, by co-author Ponni Perumalswami MD, we are not aware of similar initiatives elsewhere in the United States. This study was unique in the engagement of multiple immigrant populations (Somali, Turkish, Moroccan, *etc.*,) simultaneously, rather than focus on any one particular group.

Significance of the applications

Practical applications of this study are the following: (1) the authors have demonstrated that a small group of health care workers can effectively increase HBV knowledge among relatively large groups of at-risk persons in community based, brief teaching sessions; and (2) they have further demonstrated that there is a significant need for these teaching sessions, as many participants from extremely high risk regions for HBV are unaware of their HBV risk. They hope that this model for HBV outreach will be directly applied by others around the globe to quickly and effectively prompt at-risk persons to seek HBV care. To underscore that intention, we have provided a "toolkit" with copies of our pre/post-session questionnaire (Figure S1) and navigator card (Figure S2) for immediate use by others toward the concerted effort of 2030 HBV Eradication.

Peer-review

The manuscript described a teaching session to improve understanding of hepatitis B virus infection by people in a local community. The questionnaire is well designed and the procedure properly conducted.

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ORIGINAL ARTICLE

Observational Study

Predictors of healthcare-seeking behavior among Chinese patients with irritable bowel syndrome

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Abstract

AIM

To analyze predictors of healthcare-seeking behavior among Chinese patients with irritable bowel syndrome (IBS) and their satisfaction with medical care.

METHODS

Participating patients met IBS Rome Ⅲ criteria (excluding those with organic diseases) and were enrolled in an IBS database in a tertiary university hospital. Participants completed IBS questionnaires in face-to-face interviews. The questionnaires covered intestinal and extra-intestinal symptoms, medical consultations, colonoscopy,

medications, and self-reported response to medications during the whole disease course and in the past year. Univariate associations and multivariate logistic regression were used to identify predictors for frequent healthcare-seeking behavior (≥ 3 times/year), frequent colonoscopies (≥ 2 times/year), long-term medications, and poor satisfaction with medical care.

RESULTS

In total, 516 patients (293 males, 223 females) were included. Participants' average age was 43.2 ± 11.8 years. Before study enrollment, 55.2% had received medical consultations for IBS symptoms. Ordinary abdominal pain/discomfort (non-defecation) was an independent predictor for healthcare-seeking behavior (OR = 2.07, 95%CI: 1.31-3.27). Frequent colonoscopies were reported by 14.7% of patients (3.1 ± 1.4 times per year). Sensation of incomplete evacuation was an independent predictor for frequent colonoscopies (OR = 2.76, 95%CI: 1.35-5.67). During the whole disease course, 89% of patients took medications for IBS symptoms, and 14.7% reported they were satisfied with medical care. Patients with anxiety were more likely to report dissatisfaction with medical care (OR = 2.08, 95%CI: 1.20-3.59). In the past year, patients with severe (OR = 1.74, 95%CI: 1.06-2.82) and persistent (OR = 1.66, 95%CI: 1.01-2.72) IBS symptoms sought medical care more frequently.

CONCLUSION

Chinese patients with IBS present high rates of frequent healthcare-seeking behavior, colonoscopies, and medications, and low satisfaction with medical care. Intestinal symptoms are major predictors for healthcare-seeking behavior.

Key words: Irritable bowel syndrome; Colonoscopy; Healthcare seeking; Treatment; Outcomes

Core tip: The prevalence of irritable bowel syndrome (IBS) in the general Chinese population is about 6.5%. Many patients are dissatisfied with the efficacy of traditional IBS treatments. Data about healthcare-seeking behavior among these patients in China are lacking. We analyzed a database of patients with IBS from Peking Union Medical College Hospital to identify predictors for healthcare-seeking behavior and satisfaction with medical care among this population. We found high rates of frequent healthcare-seeking behavior, colonoscopies, and medications, and low satisfaction with medical care. Intestinal symptoms were major predictors for healthcare-seeking behavior. Anxiety influenced satisfaction with medical care.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional bowel disorder with a global prevalence of 11%[1]. A meta-analysis found the pooled prevalence of IBS in a Chinese community was 6.5%[2]. Rome Ⅲ criteria indicate IBS is characterized by persistent or recurrent abdominal pain or discomfort associated with altered bowel habits, and patients with IBS report lower quality of life[3]. In the United States, IBS is associated with an annual economic burden of more than 20 billion dollars (direct and indirect healthcare costs)[4]. Data from Korea in 2008 showed the annual average National Health Insurance costs for IBS per person were USD64.1, the cost for outpatient care was USD43.7, and that for inpatient care was USD1087.9[5]. A Chinese study focused on medical costs showed that IBS accounted for 3.3% of the total healthcare budget for the entire Chinese nation[6]. Data from Western countries indicated intestinal symptoms (including increasing pain severity and duration) were independently associated with seeking healthcare for IBS[7], and frequent consulters were more likely to have coexisting anxiety or depression[8]. In France, 71.9% of patients consulted their general physicians, 45.9% consulted gastroenterologists, and 8% had been hospitalized for IBS[9]. An epidemiological study in China demonstrated that 22.4%[10] of patients with IBS symptoms sought healthcare, but there were no detailed data revealing the predictors for healthcare-seeking behavior among patients with IBS in China.

The pathogenesis of IBS is unclear, and its diagnosis depends on Rome diagnostic criteria. However, in France, 67% of patients who met Rome Ⅱ criteria underwent additional investigations to determine etiologies[9]. The therapeutic goals of IBS are to alleviate intestinal symptoms, reduce episodes, and improve quality of life. Nevertheless, many patients with IBS are dissatisfied with the efficacy of traditional treatment options and undergo frequent consultations, referrals, multiple medications, and even unnecessary abdominal or pelvic surgeries[11]. The present study aimed to provide evidence for IBS management strategies through a database analysis of patients with IBS from Peking Union Medical College Hospital (PUMCH).

MATERIALS AND METHODS

Participants

Participants were consecutive patients with IBS enrolled in a gastroenterology clinic at PUMCH (a tertiary university hospital) from June 2009 to February 2016. Eligible patients were aged 18-65 years. All patients met Rome Ⅲ diagnostic and subtype criteria[12], including recurrent

abdominal pain or discomfort at least 3 d/mo in the last 3 mo associated with two or more of these features: (1) improvement with defecation; (2) onset associated with a change in the frequency of stools; and (3) onset associated with a change in the form of stools. Criteria were fulfilled in the last 3 mo with symptom onset at least 6 months before diagnosis. Patients with organic gastrointestinal diseases and metabolic diseases were excluded based on the results of routine tests for blood, urine, stool; liver, kidney, and thyroid function; measurements of carcinoembryonic antigen, erythrocyte sedimentation rate and C-reactive protein; and abdominal ultrasound and colonoscopy in the past year. Eligible patients needed to be able to complete the questionnaires. After being informed about the study, some participating patients provided informed written consent and others provided oral consent to participate before study enrollment. This study was approved by the PUMCH Ethics Committee (S-234).

Methods

IBS symptom questionnaires were administered by well-trained investigators in face-to-face interviews. Information collected included demographic data, IBS disease course, frequency and severity of IBS symptoms, defecation-related symptoms, extra-intestinal symptoms, examination results in the past year, and psychological and sleeping status and management. Symptom score for IBS with diarrhea (IBS-D) was calculated according to Zhu *et al*[3], with a total possible score of 15 that reflected the frequency and severity of abdominal pain/discomfort, frequency of bowel movements during symptom onset, and improvement of abdominal pain/discomfort with defecation. We defined mild symptoms as a symptom score $\leqslant$ 8, moderate symptoms as 9-10, and severe symptoms as > 10, based on symptom score percentiles and the severity and frequency of abdominal pain, number of other symptoms, health-related quality of life, and healthcare use[13]. In this questionnaire, ordinary abdominal pain/discomfort referred to abdominal pain/discomfort during non-defecation, whereas persistent symptoms referred to having IBS symptom onset every day.

Patients with difficulty falling asleep, light sleep/dreaminess, sleeping time < 6 h, or early awakening in the past 3 mo were defined as having sleeping disorders. The Hamilton anxiety (HAMA) and Hamilton depression (HAMD) scales were used to evaluate patients' psychological status by specially trained professionals through conversation and observation[14].

The validated simplified Chinese version of the IBS-Quality of Life (IBS-QOL) instrument was completed by patients and transformed to scores according to the instructions provided[3,15]. Healthcare-seeking conditions consisted of healthcare-seeking behavior throughout the whole disease course and the past year, medical costs, treatment efficacy evaluation, and satisfaction with medical care as reported by patients. Medical costs were converted and presented as USD, based on the average exchange rate during 2009-2015 from the National Bureau of Statistics of China (USD1 = CNY6.4195).

Statistical analysis

All analyses were performed using SPSS version 19.0 (IBM Corporation, Somers, NY, United States). Parametric data were presented as mean ± SD. Nonparametric data were presented as median (interquartile range). Comparisons among the two groups were made by Student's *t*-tests for parametric data. The Mann–Whitney *U* test was used to compare nonparametric data between the two groups. Chi-square tests were used for categorical variables. Spearman's test was performed to assess nonparametric correlations between two quantitative variables. Univariate associations were identified by χ^2 tests. Variables that were significant in the chi-square tests were included in a multivariate logistic regression model to identify independent predictors for healthcare-seeking behavior among patients with IBS. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic data

Data for 516 patients with IBS were included in the final analysis. Patients' average age was 43.2 ± 11.8 years, and the sample included 56.8% males and 43.2% females. The median IBS disease course was 6.5 (8) years; 30.8% of patients had a disease course $\geqslant$ 10 years, and 12.0% $\geqslant$ 20 years.

IBS-D, IBS with constipation (IBS-C) and mixed IBS (IBS-M) accounted for 94.4%, 3.5%, and 2.1% of patients, respectively. We did not include patients with unsubtyped IBS. The average symptom score for IBS-D was 9.4 ± 1.6; 26.2% had mild symptoms, 51.7% moderate symptoms, and 22.1% severe symptoms. In addition, 58.1% of patients had coexisting sleeping disorders, with a median duration of 3.5 (9) years. A total of 362 patients (70.2%) completed HAMA and HAMD assessment. The average HAMA score was 16.2 ± 7.3 and the average HAMD score was 13.2 ± 6.1. We found that 62.1% of patients had coexisting anxiety, of which 49.6% were moderate to severe. In addition, 29% of patients had coexisting depression, with 14.2% being moderate to severe. The average IBS-QOL score was 71.7 ± 17.9, and there was no significant difference between males and females (73.0 ± 17.7 *vs* 71.0 ± 19.1, $P = 0.22$).

Healthcare-seeking behavior among patients with IBS

During the whole disease course, 285 patients (55.2%) had sought healthcare at least once for IBS symptoms (current consultation not included). These patients were defined as the consulter group. In the past year this figure increased to 79.3%, with an average number of visits of 4.5 ± 6.2. The majority of patients

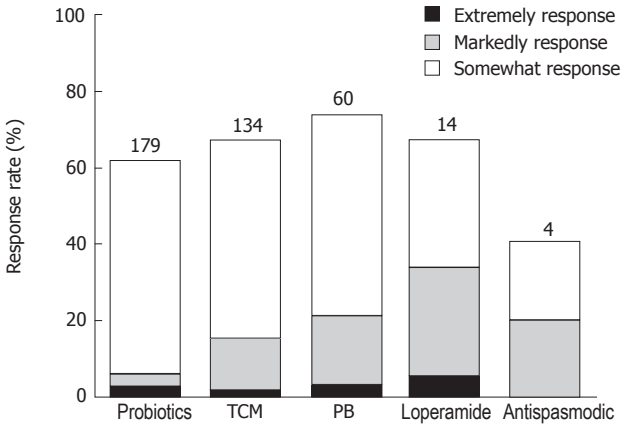

Figure 1 Patients with irritable bowel syndrome with diarrhea reported effective rate in the past year. Number on top of the column referred to number of patients who used that kind of drug. TCM: Traditional Chinese medicine; PB: Pinaverium bromide.

(79.3%) consulted with tertiary hospitals; primary/secondary care accounted for 20.7% of consultations. In addition, most patients (97.9%) consulted with gastroenterologists; 8.6% also consulted with other departments including general physicians (9.5%), traditional Chinese medicine practitioners (6.8%), and gynecologists (4.6%).

In the past year, 49.1% of patients had more than three consultations. Patients with anxiety and depression underwent more consultations than patients without [anxiety, 3.0 (3.5) *vs* 2.0 (2.8), $P = 0.005$; depression, 3.0 (4.0) *vs* 2.0 (2.9), $P = 0.001$]. The number of consultations for patients with IBS in the past year was positively correlated with symptom score ($r = 0.271$, $P < 0.001$) but negatively correlated with IBS-QOL score ($r = -0.228$, $P < 0.001$).

Colonoscopies: During the whole disease course, 41.9% of patients underwent colonoscopies (average 1.7 ± 1.3); 76 patients (14.7%) underwent at least two colonoscopies, with the maximum being 10 (over 6 years). In the past year, 64.9% of patients underwent colonoscopies (average 1.1 ± 0.3); 19 patients (3.7%) had colonoscopies at least twice (maximum of three).

Medications and efficacy: In total, 89% of patients with IBS had taken medications during the whole disease course, with 54.7% reporting intermittent use and 16.9% long-term use. Consulters were more likely to take medications than non-consulters (93.7% *vs* 83.1%, $P < 0.001$). In the past year, the rate of medication was 88.8% and 14.8% of patients took more than three kinds of medications. Common medications used by patients with IBS-D were probiotics, traditional Chinese medicines, pinaverium bromide, loperamide, and traditional antispasmodics. Probiotics were most commonly used (52.2%), followed by traditional Chinese medicine (41.3%) (Table 1). Patient-reported medication response rates in the past year were over 50%. Although the overall response rate for pinaverium bromide was 73.1% and probiotics was 61.2%, "somewhat response" for the two medications was reported by 52.4% and 55.9%, respectively (Figure 1). Common medications used by those with IBS-C included traditional Chinese medicine, enemas, and prokinetics.

Table 1 Irritable bowel syndrome with diarrhea patients reported medication use ($n = 487$) *n* (%)

Medications	The whole disease course	The past year
Probiotics	240 (49.3)	254 (52.2)
Traditional Chinese Medicine	195 (40.0)	201 (41.3)
Pinaverium bromide	49 (10.1)	82 (16.8)
Loperamide	19 (3.9)	21 (4.3)
Traditional antispasmodic	11 (2.3)	10 (2.1)

Data were presented as number and percentage of patients who used medications.

Medical costs and overall satisfaction with medical care

Total direct medical costs estimated per patient per year for the whole disease course and for the past year were USD691.8 ± 1067.2 and USD762.7 ± 1146.0, respectively, with a maximum amount of USD7788.8. Degree of satisfaction with medical care was reported as complete satisfaction for 11.4% of patients, satisfaction for 31.8%, and dissatisfaction for 56.8%. Non-consulters reported a higher overall satisfaction rate (including complete satisfaction and satisfaction) than consulters (58.9% *vs* 30.5%, $P < 0.001$).

Variables influencing healthcare-seeking behavior and satisfaction

Univariate analysis: We investigated predictors for consultation, frequent consultations (≥ 3 times/year), frequent colonoscopies (≥ 2 times/year), long-term medications, multiple medications (≥ 3 kinds), and dissatisfaction with medical care in the whole disease course and the past year. Consulters were more likely to present with ordinary abdominal pain/discomfort, persistent symptoms, anxiety, and depression in the whole disease course. In the past year, consulters were more likely to have loose stools (Bristol Stool Form Scale type 6) and weight loss (Table 2). In addition, among frequent consulters over the whole disease course, the percentages of females, severe symptoms, weight loss, and coexisting functional dyspepsia (FD) were higher than among patients with < 3 consultations/year. In the past year, variables influencing healthcare-seeking behavior included severe symptoms, ordinary abdominal pain/discomfort, persistent symptoms, weight loss, and FD (Table 3). During the whole disease course, more females than males reported frequent colonoscopies (52.6% *vs* 38.6%, $P = 0.047$), sensation of incomplete evacuation (84.2% *vs* 65%, $P = 0.003$), and coexisting pain in other parts of the body (50% *vs* 33.6%, $P = 0.018$).

Table 2 Factors with significant difference between consulted and non-consulted patients with irritable bowel syndrome *n* (%)

	Consulters	Non-consulters	OR (95%CI)
During the whole disease course	*n* = 285	*n* = 231	
Ordinary abdominal pain/discomfort	174 (61.1)	101 (43.7)	2.02 (1.43-2.92)
Persistent symptoms	104 (36.5)	60 (26.0)	1.64 (1.12-2.40)
Disease course ⩾ 7 yr	121 (42.5)	77 (33.3)	1.48 (1.03-2.12)
Co-existed with GERD	157 (55.1)	107 (46.3)	1.42 (1.00-2.01)
Sleeping disorder	179 (62.8)	121 (52.4)	1.54 (1.08-2.18)
Anxiety[1]	128 (67.4)	98 (57.0)	1.56 (1.02-2.38)
Depression[1]	64 (33.7)	41 (23.8)	1.62 (1.02-2.58)
In the past year	*n* = 409	*n* = 107	
Mental labor	199 (48.7)	33 (30.8)	2.13 (1.35-3.35)
Severe abdominal pain	68 (16.6)	27 (25.2)	0.59 (0.36-0.98)
Loose stool	312 (83.6)	70 (72.9)	1.70 (1.07-2.69)
Weight loss	119 (29.1)	17 (15.9)	2.17 (1.24-3.81)

[1]The number of consulters and non-consulters were 190 and 172. IBS: Irritable bowel syndrome; GERD: Gastroesophageal reflux disease; OR: Odds ratio.

Table 3 Factors with significant difference between frequent and infrequent consulters in patients with irritable bowel syndrome *n* (%)

	Frequent consulters	Infrequent consulters	OR (95%CI)
During the whole disease course	*n* = 136	*n* = 149	
Female	76 (55.9)	65 (43.6)	0.55 (0.35-0.86)
Severe symptoms	39 (28.7)	24 (16.1)	1.93 (1.08-3.45)
Weight loss	45 (33.1)	26 (17.4)	2.34 (1.35-4.07)
Co-existed with FD	94 (69.1)	79 (53)	1.98 (1.22-3.22)
In the past year	*n* = 201	*n* = 208	
Severe symptoms	62 (30.8)	24 (11.5)	3.42 (2.03-5.75)
Ordinary abdominal pain/discomfort	123 (61.2)	97 (46.6)	1.8 (1.22-2.67)
Persistent symptoms	80 (39.8)	48 (23.1)	2.20 (1.44-3.38)
Weight loss	71 (35.3)	48 (23.1)	1.82 (1.18-2.81)
Co-existed with FD	131 (65.2)	114 (54.8)	1.54 (1.04-2.30)

Data were presented as *n* (%). χ^2 test. FD: Functional dyspepsia; OR: Odds ratio; CI: Confidence interval.

Table 4 Factors with significant difference of medication behaviors in patients with irritable bowel syndrome in the past year *n* (%)

	Long-term medication (*n* = 88)	Intermittent medication (*n* = 370)	OR (95%CI)	Medications ⩾ 3 kinds (*n* = 68)	Medications < 3 kinds (*n* = 390)	OR (95%CI)
Mental labor	26 (29.5)	169 (45.7)	0.59 (0.30-0.82)			
Severe symptoms	33 (37.5)	73 (19.7)	2.44 (1.48-4.03)			
Persistent symptoms	45 (51.1)	107 (28.9)	2.57 (1.60-4.13)	33 (48.5)	119 (30.5)	2.15 (1.27-3.62)
Weight loss	40 (45.5)	86 (23.2)	2.75 (1.70-4.47)	28 (41.2)	98 (25.1)	2.09 (1.22-3.56)
Anxiety[1]	46 (79.3)	163 (61.3)	2.42 (1.23-4.79)	40 (78.4)	169 (61.9)	1.87 (1.11-3.15)
Depression[1]	24 (41.4)	75 (28.2)	1.80 (1.00-3.23)			
Co-exist with FD				49 (72.1)	232 (59.5)	1.76 (1.00-3.10)

[1]The number of long-term medication and intermittent medication were 58 and 266. χ^2 test. IBS: Irritable bowel syndrome; FD: Functional dyspepsia; OR: Odds ratio.

Table 4 lists differences between patients with long-term medications and intermittent medications, multiple medications (⩾ 3 kinds) and fewer than three kinds of medications. Patients with persistent symptoms, weight loss, and anxiety were more likely to take long-term and multiple medications.

Comparison of degree of satisfaction with medical care in the whole disease course and in the past year showed that IBS symptoms, weight loss, sleeping disorders, and psychological disorders influenced patient-reported satisfaction rates (Table 5).

Multivariate analysis: We entered the above influencing factors into a multivariate logistic regression model, and found ordinary (not pre-defecation) abdominal pain/discomfort was an independent predictor for consultation in the whole disease course. Severe symptoms and persistent symptoms were independent predictors for frequent consultations in the past year. In the whole disease course, frequent colonoscopies were associated

Table 5 Factors with significant difference between irritable bowel syndrome patients with satisfaction and dissatisfaction to medical care *n* (%)

	Satisfaction	Dissatisfaction	OR (95%CI)
During the whole disease course	*n* = 293	*n* = 223	
Severe symptoms	76 (25.9)	38 (17.0)	1.71 (1.10-2.64)
Ordinary abdominal pain/discomfort	179 (61.1)	96 (43.0)	2.08 (1.46-2.96)
Persistent symptoms	112 (38.2)	52 (23.3)	2.04 (1.38-3.01)
Mucous stool	196 (66.9)	129 (57.8)	1.47 (1.03-2.11)
Weight loss	90 (30.7)	46 (20.6)	1.71 (1.13-2.57)
Co-existed with GERD	162 (55.3)	102 (45.7)	1.47 (1.03-2.08)
Co-existed with sleeping disorder	189 (64.5)	111 (49.8)	1.83 (1.29-2.62)
Anxiety[1]	145 (72.5)	81 (50.0)	2.64 (1.70-4.08)
Depression[1]	73 (36.5)	32 (19.8)	2.34 (1.44-3.78)
In the past year	*n* = 255	*n* = 261	
Severe symptoms	69 (27.1)	45 (17.2)	1.69 (1.11-2.58)
Ordinary abdominal pain/discomfort	155 (60.8)	120 (46.0)	1.66 (1.17-2.34)
Persistent symptoms	98 (38.4)	66 (25.3)	1.73 (1.19-2.52)
Mucous stool	172 (67.5)	153 (58.6)	1.46 (1.02-2.10)
Weight loss	81 (31.8)	55 (21.1)	1.65 (1.11-2.45)
Anxiety[1]	125 (72.7)	101 (53.2)	2.34 (1.51-3.64)
Depression[1]	61 (35.5)	44 (23.2)	1.82 (1.15-2.89)

[1]The number of satisfaction and dissatisfaction were 172 and 190. χ^2 test. IBS: Irritable bowel syndrome; GERD: Gastroesophageal reflux disease; OR: Odds ratio.

Table 6 Multivariate analysis of factors associated with healthcare seeking behaviors and satisfaction to medical care in patients with irritable bowel syndrome

	Adjusted OR (95%CI)
Consultation in the whole disease course	
Ordinary abdominal pain/discomfort	2.07 (1.31-3.27)
Consultation in the past year	
Mental labor	2.19 (1.35-3.55)
Weight loss	2.17 (1.22-3.89)
Frequent consultations in the whole disease course	
Severe symptoms	1.88 (1.12-3.15)
Weight loss	1.94 (1.09-3.47)
Frequent consultations in the past year	
Severe symptoms	1.74 (1.06-2.82)
Persistent symptoms	1.66 (1.01-2.72)
Frequent colonoscopies in the whole disease course	
Sensation of incomplete evacuation	2.76 (1.35-5.67)
Co-existed pain in other parts of the body	1.92 (1.07-3.45)
Long-term medication in the past year	
Persistent symptoms	2.02 (1.07-3.81)
Weight loss	2.58 (1.38-4.82)
Dissatisfaction with medical care in the whole disease course	
Ordinary abdominal pain/discomfort	1.99 (1.24-3.18)
Weight loss	1.73 (1.01-2.95)
Anxiety	2.08 (1.20-3.59)

Logistic regression analysis. IBS: Irritable bowel syndrome; OR: Odds ratio.

with sensation of incomplete evacuation. In the past year, long-term medications were associated with persistent symptoms and weight loss. In the whole disease course, coexisting anxiety was the strongest independent predictor for dissatisfaction with medical care (Table 6).

DISCUSSION

In the present study, we analyzed clinical medical care data for patients with IBS from a tertiary hospital, and found that IBS-D was most common in China. Most patients consulted with gastroenterologists in tertiary hospitals, and there was a high rate of colonoscopies. In patients with IBS-D, the most commonly used medications were probiotics. Conventional treatments were reported as partially effective, and patient-reported satisfaction rates were low. Ordinary abdominal pain/discomfort, severe and persistent symptoms, weight loss, and anxiety were independent predictors for healthcare-seeking behavior and satisfaction with medical care.

In our study, patients with IBS showed a long disease course, with 30% of patients having IBS for more than 10 years, which highlighted the importance of accurate diagnosis and effective management[16]. Most of our participants had IBS-D, with 5.5% having IBS-C/IBS-M; these rates are much lower than domestic epidemiological data[10]. This might be attributed to the fact that we enrolled patients with typical IBS symptoms, and suggests patients with IBS-D might be more likely to seek healthcare. In the whole disease course, the consultation rate for IBS symptoms (55.2%) was similar to that in Taiwan (47%)[17] and the United States (46%)[18], but was lower than in Australia (73%)[18]. Chinese patients with IBS mostly consulted with tertiary hospitals (78.9%) and gastroenterologists (97.9%), which differs from Western countries[9,19] and may be related to a lack of well-established referral systems. A small number of patients consulted with other departments because of coexisting headache and urogenital symptoms[20].

The Rome Ⅲ IBS diagnostic criteria emphasize improvement of abdominal pain/discomfort after defecation. However, our data showed more than half of participating patients presented with ordinary abdominal pain/discomfort (non-defecation). In addition, ordinary abdominal pain/discomfort was an independent predictor for healthcare seeking among patients with IBS. Previous published papers indicated the severity[7,16,21], frequency[21], and duration[7] of abdominal pain were predictors for seeking healthcare among patients with IBS. We demonstrated that the number of visits was positively correlated with intestinal symptom scores, and patients with severe symptoms and weight loss were more likely to frequently seek healthcare. In the past year, predictors for frequent consultations included persistent symptoms. Weight loss was one of the alarm features for patients with IBS[22] with a reported prevalence of 21%, which might be associated with

FD (especially postprandial distress syndrome[23] and psychological disorders[24]). The reported prevalence of gastrointestinal malignancies in the population with unintentional weight loss was 6%-38%[25]. Patients with IBS were more worried about having serious diseases than healthy controls[26], and 21% of healthcare seekers reported "fear that abdominal symptoms relate to cancer or other illness" as the most important reason for seeking healthcare[27]. Usually, patients attributed their symptoms to organic etiologies such as intestinal infection or ulcers[28]. Fear of organic diseases prompted frequent consultations[29].

A previous study in Hong Kong[30] showed a higher degree of anxiety was an independent factor associated with healthcare-seeking behavior in IBS, but that study did not show the exact degree of anxiety and odds ratios. Despite intestinal symptoms, we found patients with anxiety and depression had more visits. During the whole disease course, anxiety and depression were more common among consulters than non-consulters. However, multifactor analysis indicated that anxiety and depression were not independent predictors for healthcare-seeking behavior.

Before study enrollment, 64.9% of patients underwent colonoscopies and 14.7% of patients had colonoscopies at least twice. In an American cohort study, the detection rate of structural lesions of the colon in non-IBS-C patients fulfilling Rome Ⅱ criteria without alarm features was similar to healthy controls[31]. Akhtar et al. reviewed medical records of patients with IBS who underwent colonoscopies because of new gastrointestinal symptoms 15 years after diagnosis, and found that there was no difference in the prevalence of organic colonic lesions with non-IBS controls[32]. The newly established Rome IV criteria recommend appropriate diagnostic testing only if alarm symptoms are present[13]. The American College of Gastroenterology recommends colonoscopy should be performed in patients with IBS who have alarm features and in those aged over 50 years[22]. In China, the high colonoscopy rate may be associated with the increasing incidence of colorectal cancer[33] and the relatively low cost of examination. We demonstrated that the sensation of incomplete evacuation and pain in other parts of the body were independent predictors for frequent colonoscopies.

In total, 88% of patients had taken medications in the past year, and 14.8% had taken more than three kinds of medications. Probiotics were the most commonly used drugs. Despite multiple studies confirming the efficacy of probiotics in treating IBS[34,35], our results displayed a markedly low response rate and they are not the most commonly used drugs in Western countries. Most other investigated drugs were partially effective, which was similar to a study in the United States that showed only 19%, 18%, 15%, and 10% of patients with IBS reported medical therapy was completely effective in relieving constipation, diarrhea, abdominal pain, and bloating, respectively[11]. Psychological evaluations at enrollment showed a high prevalence of anxiety and depression, although few patients reported use of antidepressants or psychotherapy. Interestingly, 83.1% of non-consulters had taken medications, which might partially account for the low response rate. In the past year, patients with persistent symptoms and weight loss were more likely to take long-term medications.

IBS severely influenced patients' quality of life and caused considerable financial burden. In Germany[36], total costs for IBS were €994.97 per patient per year, 37% of which was for medications; in the past year, one in 15 patients was hospitalized for IBS. In the present study, average direct costs were estimated at USD762.7 per patient in the past year. Even so, the patient-reported rate of complete satisfaction was 11.4%, which was close to United States data (14%)[11] and indicates dissatisfaction with current treatment is a global issue. In addition, 41.1% of non-consulters reported dissatisfaction with medical care, which suggests they were unsatisfied with over-the-counter drugs. Coexisting anxiety was the strongest predictor for poor satisfaction with medical care, followed by ordinary abdominal pain/discomfort. The latter suggests that the pathogenesis of ordinary abdominal pain/discomfort differs from pre-defecation abdominal pain/discomfort, and may need higher level treatment (*e.g.*, centrally acting drugs).

There were some limitations in this study. First, we set strict inclusion criteria for patients with IBS, which excluded patients with light, atypical symptoms, and fewer examinations. In addition, some patients did not complete HAMA and HAMD evaluations. Patient-reported healthcare-seeking behavior was retrospective and we did not know whether their medications were prescription or over-the-counter medicines. Finally, our study was a single-center study and might not be representative of the overall situation in China.

In conclusion, Chinese patients with IBS were dominated by those with IBS-D. Patients most commonly consulted with tertiary hospitals and gastroenterologists, and there was a high rate of colonoscopies. Most conventional treatments were only partially effective and patients reported low satisfaction rates. Intestinal symptoms influenced healthcare-seeking behavior among patients with IBS from different levels, and coexisting anxiety was the strongest predictor for dissatisfaction with medical care.

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is a chronic recurrent functional bowel disorder which impairs patients' quality of life. Patients with IBS report poor treatment response and satisfaction rates for traditional treatments and undergo frequent consultations and referrals. In China, data for predictors of healthcare-seeking behavior and satisfaction with medical care are lacking. Studies regarding predictors for healthcare-seeking behavior among patients with IBS may provide evidence for IBS management strategies in this region.

Research motivation

The present study comprehensively summarized the characteristics of

healthcare-seeking behavior, medical costs, and satisfaction with care among Chinese patients with IBS. The authors also investigated predictors for frequent consultations, frequent colonoscopies, dissatisfaction with medical care, and long-term and multiple medications among Chinese patients with IBS. The authors' study provides a basis for future studies on healthcare-seeking behavior among patients with IBS, and may provide management guidance for clinicians.

Research objectives

The main objectives of this study were to investigate the characteristics of healthcare-seeking behavior, medical costs, and satisfaction with care among Chinese patients with IBS, and determine predictors for frequent consultations, frequent colonoscopies, dissatisfaction with medical care, and long-term and multiple medications in this population.

Research methods

The authors enrolled patients with IBS who met Rome Ⅲ diagnostic criteria and excluded organic diseases in a tertiary gastroenterology clinic from 2009 to 2016. Patients were administered IBS questionnaires in face-to-face interviews, which included intestinal and extra-intestinal symptoms, medical consultations and management. Data were collected and analyzed with SPSS version 19.0 software. Patients were divided into frequent consulters and infrequent consulters; frequent colonoscopies and infrequent colonoscopies; long-term medications and intermittent medications; medications $\geqslant$ 3 kinds and medications < 3 kinds; satisfaction with medical care and dissatisfaction with medical care. Univariate analysis was conducted with χ^2 test to detect factors with significant differences between groups and the significant different factors above were entered into a multivariate logistic regression model to determine independent predictors for their healthcare-seeking behavior.

Research results

The authors found Chinese IBS patient present high rates of frequent healthcare- seeking behavior, colonoscopies, medications and low satisfaction with medical care. Abdominal pain/discomfort during non-defecation period (ordinary abdominal pain/discomfort) instead of pre-defecation abdominal pain/discomfort was the independent predictor for their healthcare-seeking behavior. Sensation of incomplete evacuation was the independent predictor for frequent colonoscopies. Patients with anxiety were more likely to report "dissatisfaction to medical care". In the past year, patients with severe and persistent IBS symptoms sought medical care frequently. How to educate patients and obtain reasonable utilization of medical resources need to be solved.

Research conclusions

The results demonstrated that most patients with IBS were partially responsive to traditional treatment. Intestinal symptoms were major predictors for healthcare-seeking behavior, and patients with anxiety were more likely to be dissatisfied with medical care. The authors' results provided guidance for Chinese IBS management. Doctors should pay attention to patients with specific symptoms such as ordinary abdominal pain/discomfort and anxiety.

Research perspectives

From the study, The authors learned that patients with IBS tended to undergo frequent consultations and investigations. Physicians should give patients sufficient explanations and pay attention to their psychological status. Future researches might emphasize the reasons of low effective rate of routine treatments and investigate the efficacy of psychological treatment through prospective studies.

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SYSTEMATIC REVIEWS

Vaccinations in immunosuppressive-dependent pediatric inflammatory bowel disease

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Data sharing statement: De-identified data are available from the corresponding author at Patricia.Fulkerson@cchmc.org. This project was IRB approved (Study #2015-9400). The research was determined to be no more than minimal risk to participants so consent was waived. The data are anonymized and risk of identification is low.

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Abstract

AIM

To determine the vaccination rates in pediatric immunosuppression-dependent inflammatory bowel disease (IBD) and review the safety and efficacy of vaccinations in this population.

METHODS

The electronic medical records from October 2009 to December 2015 of patients diagnosed with IBD at 10 years of age or younger and prescribed anti-tumor necrosis factor alpha (anti-TNF-α) therapy were reviewed for clinical history, medication history, vaccination history, and hepatitis B and varicella titers. Literature discussing vaccination response in IBD patients were identified through search of the MEDLINE database and reviewed using the key words "inflammatory bowel disease", "immunization", "vaccination", "pneumococcal", "varicella", and "hepatitis B". Non-human and non-English language studies were excluded. Search results were reviewed by authors to

select articles that addressed safety and efficacy of immunizations in inflammatory bowel disease.

RESULTS

A total of 51 patients diagnosed with IBD prior to the age of 10 and receiving anti-TNF-α therapy were identified. Thirty-three percent of patients (17/51) had incomplete or no documentation of vaccinations. Sixteen case reports, cohort studies, cross-sectional studies, and randomized trials were determined through review of the literature to describe the safety and efficacy of hepatitis B, pneumococcal, and varicella immunizations in adult and pediatric patients with IBD. These studies showed that patients safely tolerated the vaccines without significant adverse effects. Importantly, IBD patients receiving immunosuppressive medications, particularly anti-TNF-α treatment, have decreased vaccine response compared to controls. However, the majority of patients are still able to achieve protective levels of specific antibodies.

CONCLUSION

Immunizations have been shown to be well-tolerated and protective immunity can be achieved in patients with IBD requiring immunosuppressive therapy.

Key words: Vaccination; Immunosuppression; Early-onset inflammatory bowel disease; Very early-onset inflammatory bowel disease

Core tip: Chronic immunosuppression and immune defects can contribute to increased susceptibility to infections in pediatric inflammatory bowel disease (IBD). Immunization rates among IBD patients are low due to concerns about vaccine efficacy while on immunosuppression and disease exacerbation with administration. The aim of this review was to determine the vaccination rates in pediatric immunosuppression-dependent IBD and the safety and efficacy of immunizations in this population.

Nguyen HT, Minar P, Jackson K, Fulkerson PC. Vaccinations in immunosuppressive-dependent pediatric inflammatory bowel disease. *World J Gastroenterol* 2017; 23(42): 7644-7652 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7644.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7644

INTRODUCTION

The prevalence of inflammatory bowel disease (IBD) is on the rise, particularly in the elderly population and very young children. Approximately 25% of patients with IBD will be diagnosed during childhood, and very early-onset IBD (VEOIBD) further classifies those children diagnosed before 6 years of age. VEOIBD comprises 15% of pediatric IBD cases and has an incidence rate of 4.37 per 100000 children and a prevalence of 14 per 100000 children[1]. The increasing incidence in the very young is of interest to immunologists as these patients often are referred for evaluation for immunodeficiency. Conventional, polygenic IBD predominates in patients aged 7 years and older at time of diagnosis, but approximately 20% of VEOIBD is monogenic-single gene defects that affect the gastrointestinal immune regulation. Mutations that result in chronic granulomatous disease, IL-10 signaling alterations, and defects in X-linked inhibitor of apoptosis function have been associated with VEOIBD[1]. These abnormalities in innate immunity are further compounded by immunosuppressive medications prescribed for IBD treatment, resulting in increased risk of infection. A cross-sectional analysis showed that bacterial pneumonia was one of the most common causes of hospitalizations for IBD patients on immunomodulators or anti-tumor necrosis factor alpha (anti-TNF-alpha) therapy with a prevalence of *S. pneumoniae* pneumonia at 82.6 per 100000 compared to 69.2 per 100000 in controls[2]. Adult IBD patients have an increased risk of pneumonia (OR = 1.54, 95%CI: 1.49-1.60) compared to matched individuals without IBD with use of immunosuppressive therapies like biologics (OR = 1.32, 95%CI: 1.11-1.57) and corticosteroids (OR = 1.91, 95%CI: 1.72-2.12) as a risk factor[3]. VEOIBD children in particular are at increased risk for vaccine-preventable infections, as many may have not yet completed their primary vaccination series prior to starting immunosuppressive therapies such as immunomodulators or anti-TNF-α.

The Infectious Diseases Society of America recommends that patients with chronic inflammatory diseases treated with long-term immunosuppression receive inactive vaccinations, such as pneumococcal vaccines, per standard immunization schedules[4]. Despite these recommendations, vaccination rates among IBD patients are lower than expected. In a study of 169 adult IBD patients, only 10% of participants received recommended pneumococcal vaccines[5]. Common reasons among patients for decreased adherence with vaccination recommendations have included belief in poor efficacy of vaccines, lack of knowledge about vaccine guidelines, and fear of disease exacerbation with vaccine administration[5].

The primary aim of our study was to determine the vaccination rates among pediatric patients with immunosuppression-dependent IBD at our institution by retrospectively reviewing the electronic medical records from October 2009 through December 2015 at Cincinnati Children's Hospital Medical Center (CCHMC).

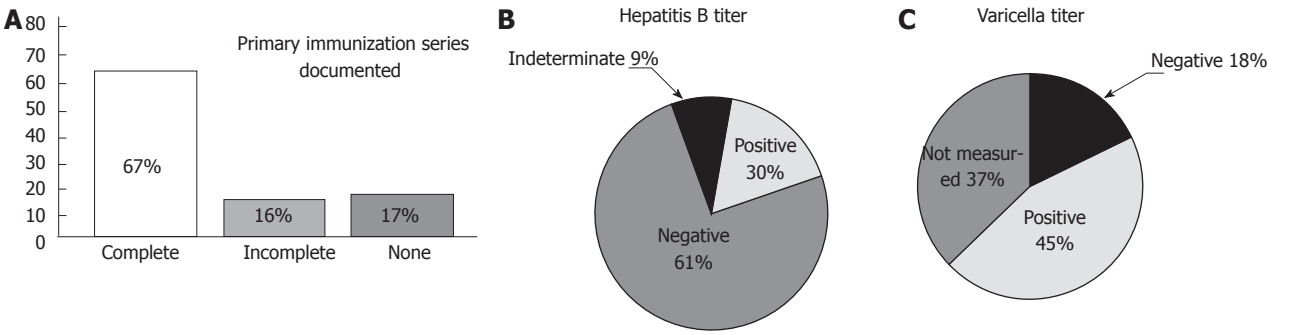

Figure 1 Completeness of primary immunization documentation (A) and immune response to hepatitis B (B) and varicella (C) vaccines. Primary immunizations evaluated include: diphtheria, tetanus, pertussis (DTaP), inactivated poliovirus (IPV), pneumococcal (PCV), *Haemophilus influenzae* type B (Hib), hepatitis B (HepB), measles, mumps, rubella (MMR), and varicella (VZV). These vaccines have been recommended for persons 18 years and younger in the United States by the Centers for Disease Controls' Advisory Committee on Immunization Practices[37]. Fifty-one patients were included in this study. Of the 17 patients who had incomplete or no immunization documentations, 3 had explanations for incomplete vaccinations-1 patient started on infliximab in infancy, 1 patient did not receive the 2nd MMR and VZV since infliximab started, and parents of 1 patient declined some vaccines.

Additionally, we systematically reviewed the literature.

MATERIALS AND METHODS

Determination of vaccination rates in pediatric IBD

Pediatric patients with a diagnosis of IBD made prior to the age of 10 years and receiving anti-TNF-alpha were identified at CCHMC. We included patients diagnosed prior to age 10 to ensure capture of patients with possible monogenic disease. The electronic medical records for the patients, dated from October 2009 to December 2015, were retrospectively reviewed. Information including clinical history, exam findings, patient's IBD status, results of esophagogastroduodenoscopy and/or colonoscopy with biopsies, vaccination records, medication records, vaccine titers, and infectious disease laboratory testing results were collected. This study was approved by the CCHMC Institutional Review Board.

Literature review

The MEDLINE database was searched through PubMed with search strategies as detailed in (Table 1). Search results were reviewed by the primary research participants to determine if articles addressed safety and efficacy of immunizations in inflammatory bowel disease and other immunomodulator-dependent diseases. Articles were limited to randomized trials, case-control studies, cohort studies, and reviews. Childhood and adult immunizations to pneumococcal, Hepatitis B, and varicella with any dose and any schedule were included. Non-human and non-English language studies were excluded.

RESULTS

Vaccination Rates in Immunosuppression-dependent pediatric IBD

A total of 51 pediatric patients with a diagnosis of IBD made prior to the age of 10 years and receiving anti-TNF-α were identified. The age at diagnosis for these 51 patients ranged from 15 mo to 9 years of age. Sixty-seven percent (34/51) had documentation of a completed primary vaccination series (Figure 1A). The remainder of the patients had no or incomplete documentation of immunizations.

Hepatitis B (HepB) serology has been recommended prior to initiation of immunosuppressive therapies due to the risk of reactivation of latent HepB infection with the start of treatment[6,7]. Additionally, it is recommended that non-immune HepB patients receive a vaccine booster[8]. In our retrospective study, serology specifically evaluating HepB surface antibody showed that 67% (27/44 patients who had documented serology) were non-responders to their initial HepB vaccine series (Figure 1B). Six of the non-responders (6/27) had documentation of HepB vaccine booster receipt and post-vaccination titers drawn. Four of the 6 achieved seroprotection following the booster vaccine. Eighteen of the non-responders (18/27) either did not receive HepB vaccine booster or did not have repeat HepB titers measured.

With increasing use of immunomodulatory therapy in the management of IBD, evaluation of varicella immunity is also recommended as primary infection can be severe and life-threatening in immunocompromised hosts. Twenty-eight percent (9/32) of patients who had varicella antibodies measured had negative titers (Figure 1C). We found that 4 of the 9 patients had only one varicella vaccine administration documented.

Safety and efficacy of inactive vaccines in pediatric IBD

The efficacy of immunizations in patients with chronic inflammatory diseases requiring immunosuppressive therapies has been an area of concern. Studies involving rheumatologic disorders, such as rheumatoid arthritis and systemic lupus erythematous, have shown that immunizations are well tolerated and do not exacerbate disease activity[9,10]. Further studies demonstrate that patients with rheumatologic

Table 1 Search strategies

Search terms	Search limitation	Number of search results
"inflammatory bowel disease" + "immunization"	Limited to human species and English language	436
"inflammatory bowel disease" + "vaccination"	Limited to human species and English language	284
"inflammatory bowel disease" + "pneumococcal"	Limited to human species and English language	44
"inflammatory bowel disease" + "Hepatitis B"	Limited to human species and English language	181
"inflammatory bowel disease" + "varicella"	Limited to human species and English language	68
"immunosuppression" + "pneumococcal"	Limited to human species and English language	191
"immunosuppression" + "Hepatitis B" + "vaccination"	Limited to human species and English language	141
"immunosuppression" + "varicella" + "vaccination"	Limited to human species and English language	71

diseases receiving immunosuppressive therapies may have a decreased response to immunizations but are still able to mount a specific-antibody response to vaccinations[11-15]. Interestingly, Kapetanovic et al. proposed the possibility of anti-TNF-α therapy enhancing the immune response as rheumatoid arthritis patients receiving anti-TNF monotherapy in this cohort had a serum response to pneumococcal polysaccharide vaccine (PPSV23) that was similar to that of healthy controls[16]. In addition, anti-pneumococcal protective titers were sustained as long as 10 years following administration of the PPSV23 in patients with autoimmune inflammatory disease[17]. In a randomized controlled study with 103 adult rheumatoid arthritis patients, the effects of systemic immunosuppression on vaccine responses were evaluated. Patients treated with both methotrexate and rituximab had decreased response to PPSV23, a T-independent antigen, compared to patients treated with methotrexate alone. However, over half of the patients receiving adjunctive therapy with rituximab responded to at least one of the pneumococcal serotypes. Additionally, there was no difference in response to the T-dependent antigens, such as the tetanus toxoid, between the two treatment groups[18]. These results support the increased antigenicity of and improved response to vaccines containing T-dependent antigens in patients with chronic inflammatory diseases receiving immunosuppressive therapy.

The vaccine response in adult IBD patients undergoing immunosuppressive therapies is similar to those of rheumatologic patients (Table 2). In general, adult IBD patients have decreased magnitude of response to PPSV23/pneumococcal conjugate (PCV13) and HepB vaccines, but most patients retain their specific antibody response and can attain protective titer levels. The main difference between the findings of vaccine efficacy studies in rheumatologic patients and IBD patients was that anti-TNF therapy was associated with a higher risk of reduced response. However, IBD patients receiving anti-TNF treatment were still able to achieve protective levels of specific antibodies. An accelerated, double-dose HepB immunization series has been shown to be efficacious, wherein patients receive double doses of the vaccine 3 times at one-month intervals[19]. When combined with booster immunization in non-responders, the majority of IBD patients can attain seroprotection[19]. Additionally, PCV13, a T-dependent vaccine, was associated with increased titers compared to PPSV23[20]. These findings also apply to the Hepatitis A vaccine[21]. Vaccinations were overall well-tolerated and were not associated with adverse reactions such as exacerbation of the underlying inflammatory disease[20,22-24].

The efficacy and safety of primary vaccinations in pediatric IBD patients has been investigated in a limited number of studies (Table 3). The Hepatitis A vaccine series is highly immunogenic in pediatric IBD patients with seroconversion rates over 90% and has no significant differences in response between case patients compared to healthy controls; the vaccine was also well tolerated[25-27]. The HepB series does not have the same immunogenicity as Hepatitis A (Table 3). Pediatric IBD patients were shown to have decreased seroconversion rate following the completion of the 3-dose series compared to controls, but the majority, over 70%, still seroconverted[27,28]. PCV13 is commonly encountered in pediatric clinics compared to PPSV23 since the conjugate vaccine is fundamental to the primary vaccine series. Banaszkiewics et al. demonstrated that pediatric IBD patients have a good response to PCV13, further supporting that T-cell immunity seems to be conserved in IBD patients receiving immunosuppressive therapy and that T-dependent vaccines may be preferential to T-independent vaccines in these patients[29].

Safety and efficacy of live vaccines in pediatric IBD

Live vaccines have long been contraindicated in immunocompromised hosts. However, with the immunocompromised state comes increased risk of contracting infections prevented by these vaccines. Herpes zoster can occur in 20%-50% of patients following bone marrow transplant[30]. Crohn's disease (varicella OR = = 12.75; 95%CI: 8.30-19.59; herpes zoster OR = = 7.91; 95%CI: 5.60-11.18) and ulcerative colitis (varicella OR = = 4.25; 95%CI: 1.98-9.12; herpes zoster OR = = 3.90; 95%CI: 1.98-7.67) in pediatric patients have an increased association with hospitalizations for varicella or herpes zoster[30]; thus, there is great

Table 2 Studies of efficacy and safety of pneumococcal and hepatitis B vaccines in adult inflammatory bowel disease patients receiving immunosuppressive therapy

Ref.	Study design	Subjects (*n*.)	Comparison groups	Outcome measured	Adverse events	Effects
Andrade *et al*[38], 2015	Retrospective cohort	217	IBD patients treated with infliximab and/or azathioprine	Hepatitis B antibodies 1-3 mo after HepB series completion	No comment on adverse effects	Receipt of vaccination while under infliximab or azathioprine treatment resulted in decreased seroconversion (OR = 17.6, 95%CI: 8.5-33.9 and OR = 3.3, 95%CI: 1.6-9.1)
Cosio-Gil *et al*[39], 2015	Retrospective cohort	172	IBD patients	Hepatitis B antibodies 1-3 mo after HepB series completion	No comment on adverse effects	50.6% patients responded to 1st series (95%CI: 42.9-58.3) 66.8% patients responded to 1st or 2nd series (95%CI: 59.3-73.8) Older age associated with decreased response (for patients > 55 yr, OR = 3.6, 95%CI: 1.3-10.2)
Cekic *et al*[40], 2015	Retrospective cohort	125	IBD patients	Hepatitis B antibodies 1 mo after HepB series completion	No comment on adverse effects	Age over 45 years, active disease, CD subtype, and immune suppression negatively impacted vaccine response
Ben Musa *et al*[41], 2014	Retrospective, cross-sectional	500	IBD patients	Hepatitis B antibodies	No comment on adverse effects	Younger age associated with increased HepB vaccine response
Sempere *et al*[24] 2013	Retrospective cohort	105	IBD patients	Hepatitis B antibodies 1-3 mo after HepB series completion	No significant adverse events associated with vaccination	Ileal CD (P = 0.01), long-standing IBD (P = 0.03), low albumin (P = 0.02), and systemic steroid use with more than one dose (P = 0.02) associated with decreased response
Altunoz *et al*[42], 2012	Retrospective cohort	211-159 patients with IBD, 52 healthy controls	IBD patients and healthy controls	Hepatitis B antibodies at least 1 month after HepB series completion	No comment on adverse events	Diagnosis of IBD overall (P < 0.001), male sex among IBD patients (P = 0.01), immunosuppressive therapy (P < 0.001), and active disease (P < 0.001) associated with decreased response
Gisbert *et al*[19], 2012	Prospective cohort	241	IBD patients	Hepatitis B antibodies 1-3 mo after HepB series (accelerated schedule or double dose) completion	No direct comment on adverse events	Older age (OR = 0.96, 95%CI: 0.94-0.98, P < 0.001) and anti-TNF therapy (OR = 0.39, 95%CI: 0.20-0.76, P < 0.01) associated with decreased rate of seroconversion 65% of participants responded after the 1st or 2nd series
Kantsø *et al*[20], 2015	Randomized trial	157	CD patients receiving PCV13 *vs* PPV23	Specific antibody response to 12 pneumococcal serotypes 1 mo after vaccination	No significant adverse events related to vaccination	PCV13 induced higher post-immunization titers for 5 serotypes (P < 0.05), regardless of treatment Immunosuppressive treatment with or without anti-TNF-α impaired immune response to both vaccines
Lee *et al*[23], 2014	Prospective cohort	197	CD patients	Antibody response 1 mo after PPSV23	No serious adverse effects in study	Female gender and anti-TNF therapy (monotherapy or combination with immunomodulator) associated with decreased response
Fiorino *et al*[22], 2012	Prospective cohort	96	IBD patients	Antibody response 3 wk after PPSV23	No serious adverse effects in the study	Infliximab only and combination therapy associated with decreased response (P = 0.009 and P = 0.038, respectively)
Melmed *et al*[43], 2010	Prospective cohort	64-45 patients with IBD, 19 healthy controls	A) IBD patients not receiving immunosuppressive therapy B) IBD patients receiving immunosuppression C) Healthy controls	Specific antibody response to 5 pneumococcal serotypes 4 wk after PPSV23	No comments on adverse effects	Combination immunosuppression associated with decreased response rate ($P \leqslant 0.01$)

CD: Crohn's disease; HepB: Hepatitis B; IBD: Inflammatory bowel disease; PCV13: Pneumococcal conjugate vaccine; PPSV23: Pneumococcal polysaccharide vaccine; TNF: Tumor necrosis factor.

interest in determining whether live vaccines are safe for pediatric patients with IBD on immunosuppressive therapy. Lu *et al*[32] illustrated the safety and efficacy of the varicella vaccine in IBD patients on immunosuppressive therapies in a case series report. Three of the patients were on 6-mercaptopurine and tolerated the varicella vaccine without issue, developing equivocal or greater immunity. Two patients received the varicella vaccine while on infliximab, albeit inadvertently, without issue and developed positive titers to the virus.

Table 3 Studies of efficacy and safety of pneumococcal and hepatitis B vaccines in pediatric inflammatory bowel disease patients receiving immunosuppressive therapy

Ref.	Study design	Subjects (*n*)	Comparison groups	Outcome measured	Adverse effects	Effects
Urganci *et al*[27], 2013	Prospective cohort	97-47 with IBD, 50 healthy controls	IBD patients and healthy controls	Hepatitis A and hepatitis B antibodies 1 month following hepatitis A vaccine and hepB series	No severe adverse reactions associated with vaccination	All participants seroconverted to hepatitis A IBD patients had decreased seroconversion to Hepatitis B (70.2% *vs* 90% in healthy controls, $P = 0.02$) No statistically significant association between treatment and vaccination response
Moses *et al*[27], 2012	Prospective, cross-sectional	100 IBD patients	IBD patients receiving infliximab	Hepatitis B immunity (anti-HBs $\geqslant$ 10 IU/mL)	No comments on adverse effects	Older age at diagnosis and study visit, pancolitis, and lower albumin levels associated with non-immunity ($P < 0.05$) Infliximab dose, duration, frequency did not affect baseline immunity; associated with decreased immunity to booster
Fallahi *et al*[44], 2014	Prospective cohort	38-18 with IBD; 20 healthy controls	A: IBD patients not receiving immunosuppressive therapy B: IBD patients receiving immunosuppression C: Healthy controls	Increase in total IgG 28 d after PPSV23 vaccination and percentage of switched memory B cells	No comments on adverse effects	IBD associated with decreased percentage of switched memory B cells and lower increase in total IgG level ($P = 0.007$ and $P = 0.001$, respectively)
Banaszkiewicz *et al*[29], 2015	Prospective cohort	178-122 with IBD; 56 healthy controls	A: IBD patients not receiving immunosuppressive therapy B: IBD patients receiving immunosuppression C: Healthy controls	Specific antibody response 6-8 wk following 1 dose of PCV13	No serious adverse effects related to PCV13	Adequate vaccine response achieved in all participants (90.4% in IBD patients *vs* 96.5% in controls) with no significant difference between IBD patients and controls ($P = 0.53$) Immunosuppressive therapy associated with decreased rise in geometric mean titers ($P = 0.04$)

anti-HBs: Anti-hepatitis B surface antibody; CD: Crohn's Disease; HepB: Hepatitis B; IBD: Inflammatory bowel disease; PCV13: Pneumococcal conjugate vaccine; PPSV23: Pneumococcal polysaccharide vaccine; TNF: Tumor necrosis factor.

The safety of live vaccines in other pediatric populations receiving immunosuppression has been investigated. Sauerbrei *et al*[30] studied the efficacy and safety of the varicella vaccine in children after bone marrow transplant. Fifteen patients received the varicella vaccine 12-23 mo (median 18 mo) after transplant. Notably, the study participants were within 1-2 years of transplantation during which time some degree of immune dysfunction is expected, but they were not receiving immunosuppression and their lymphocyte counts had to be greater than 1000/μL with T cell counts greater than 700/μL. Importantly, no study participant experienced adverse events related to the varicella vaccine. Nine of the participants were seronegative prior to the vaccine, and 8 of the 9 seroconverted within 6 mo of vaccine administration. The remaining patient required a second dose of the vaccine, after which seroconversion was achieved within 6 months. Only 3 study participants had unchanged titers. Machado *et al*[34] also demonstrated that the measles, mumps, rubella vaccine was overall well tolerated in bone marrow transplant patients[33]. In addition, the varicella vaccine was found to be safe in juvenile rheumatic patients receiving methotrexate or corticosteroids.

These results suggest that live vaccines may be tolerated in patients receiving long-term immunosuppressive therapies, particularly in those without severe immune defects. However, this topic remains controversial, and the support for administering live vaccines in patients receiving immunosuppressive therapies is very limited and consists of small cohort or case series studies. Further studies are needed to confirm the safety of live vaccinations in an immunosuppressed population, which would greatly benefit from them.

DISCUSSION

Children with a diagnosis of IBD early in life are at significant risk of infection due to their immunosuppression from both their underlying disease and treatment.

These patients will require years, if not a lifetime, of immunosuppressive therapy, and such regimens may be started prior to completion of their primary vaccination series due to their young age at diagnosis, augmenting their risk of infection. IBD patients in general have decreased vaccinations rates[35]. Working with allergists and immunologists, a thorough auditing of immunizations and measurement of antibodies to vaccine-preventable microbes at time of diagnosis can be achieved. Further, immunologists can update immunizations and ensure appropriate antibody response to provide protection in this growing, vulnerable population. To assess seroconversion and seroprotection to an immunization, specific serum antibody levels measured prior to and approximately four to eight weeks following vaccine administration are recommended[36]. Although booster vaccinations or completion of immunizations may not be possible prior to starting immunosuppressive treatment, studies have shown that these patients can still mount an immune response to vaccines, particularly to T-dependent antigens, until seroprotective status is achieved. Optimal vaccination schedules and long-term immunogenicity of these vaccines remain to be studied in pediatric IBD patients. In addition, considering their unique immune dysregulation, further studies in the efficacy of immunizations in pediatric IBD patients, especially in the very young, are needed.

COMMENTS

Background

The population of young children affected by inflammatory bowel disease (IBD) is growing. This disease is oftentimes complicated by immune defects and long-term immunosuppressive therapy, resulting in an increased susceptibility to infections. Updating vaccines at diagnosis or before initiating chronic immunosuppressive therapy would be ideal; however, this is often not achievable due to young age and the necessity to initiate treatment imminently. Immunization rates among IBD patients are lower with the efficacy of immunizations while receiving immunosuppression and the fear of disease exacerbation after vaccine administration negatively impacting rates. The aim of this review is to determine the vaccination rates in pediatric immunosuppression-dependent IBD and the safety and efficacy of immunizations in this population.

Research frontiers

The prevalence of IBD is on the rise, particularly in very young children. Approximately 25% of patients with IBD will be diagnosed during childhood, and very early-onset IBD (VEOIBD) further classifies those children diagnosed before 6 years of age and comprises 15% of pediatric IBD cases. As elucidated by Uhlig *et al* in 2014, VEOIBD has been associated with single gene defects affecting the gastrointestinal immune regulation in 20% of cases. This emerging population has posed diagnostic and management challenges for both gastroenterologists and immunologists. In addition to innate immunity abnormalities, these patients require long-term immunosuppression, including anti-tumor necrosis factor alpha (anti-TNF-α) therapies, for treatment. These factors contribute to an increased risk of infection.

Innovations and breakthroughs

Due to the young age at the time of diagnosis, patients with VEOIBD may not be able to complete their primary vaccination series prior to initiation of immunosuppressive therapies, which further exacerbates the increased risk of infection. The rate of vaccinations in addition to the safety and efficacy of immunizations has been studied in adult and, to a lesser extent, pediatric IBD patients. Literature discussing vaccination response in IBD patients were identified through search of the MEDLINE database and reviewed by the authors.

Applications

This review shows that vaccinations are well-tolerated in IBD patients, and protective immunity can be achieved in those receiving immunosuppression. Immunologists can help provide an auditing of immunizations and can ensure appropriate antibody response to provide protection in this vulnerable population.

Terminology

VEOIBD classifies children diagnosed with IBD at age 6 years or younger and is associated with single gene defects affecting gastrointestinal immune regulation in 20% of cases. Anti-TNF-α therapies, which include infliximab and adalimumab, are monoclonal antibodies that inhibit the inflammatory cytokine tumor necrosis factor-alpha. Pneumococcal conjugate vaccine (PCV13) contains thirteen serotypes of pneumococcus and elicits an immune response dependent on T-cells. Pneumococcal polysaccharide vaccine (PPSV23) contains 23 pneumococcal serotypes and incites production of specific antibodies independent of T-cells.

Peer-review

In this systematic review, the authors detailed the safety and efficacy of vaccinations in pediatric IBD.

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