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World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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Melatonin attenuates high fat diet-induced fatty liver disease in rats

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Abstract

AIM: To investigate melatonin's preventive action in oxidative stress in a rat model with high fat diet-induced non-alcoholic fatty liver disease (NAFLD).

METHODS: NAFLD was induced by high fat diet (HFD) in adult, male, Wistar rats, weighing 180-230 g. After acclimatization for one week, they were randomly assigned to 6 experimental groups that comprised animals on regular diet plus 5 or 10 mg/kg melatonin, for 4 or 8 wk; animals on HFD, with or without 5 or 10 mg/kg melatonin, for 4 or 8 wk; and animals on HFD for 8 or 12 wk, with melatonin 10 mg/kg for the last 4 wk. Liver damage was assessed biochemically by the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and histologically. Lipid peroxidation and oxidative stress were assessed by malondialdehyde and glutathione levels in liver tissue. Lipidemic indices and portal vein pressure were also measured.

RESULTS: Compared to rats not receiving melatonin, rats on 5 or 10 mg/kg of melatonin had lower mean liver weight (-5.0 g and -4.9 g) ($P < 0.001$) and lower liver weight to body weight ratio (-1.0%) ($P < 0.001$), for the two doses, respectively. All rats fed HFD without melatonin developed severe, grade Ⅲ, steatosis. Rats on HFD with concurrent use of melatonin showed significantly less steatosis, with grade Ⅲ steatosis observed in 1 of 29 (3.4%) rats on 10 mg/kg melatonin

and in 3 of 27 (11.1%) rats on 5 mg/kg melatonin. Melatonin was ineffective in reversing established steatosis. Melatonin also had no effect on any of the common lipidemic serum markers, the levels of which did not differ significantly among the rats on HFD, irrespective of the use or not of melatonin. Liver cell necrosis was significantly less in rats on HFD receiving melatonin than in those not on melatonin, with the AST levels declining by a mean of 170 U/L (P = 0.01) and 224 U/L (P = 0.001), and the ALT levels declining by a mean of 62.9 U/L (P = 0.01) and 93.4 U/L (P < 0.001), for the 5 and 10 mg/kg melatonin dose, respectively. Melatonin mitigated liver damage due to peroxidation and oxidative stress in liver tissue as indicated by a significant decline in MDA production by 12.7 (P < 0.001) and 12.2 (P < 0.001) μmol/L /mg protein /mg tissue, and a significant increase in glutathione by 20.1 (P = 0.004) and 29.2 (P < 0.001) μmol/L /mg protein /mg tissue, for the 5 and 10 mg/kg melatonin dose, respectively.

CONCLUSION: Melatonin can attenuate oxidative stress, lessen liver damage, and improve liver histology in rats with high fat diet-induced NAFLD, when given concurrently with the diet.

Key words: Melatonin; Non-alcoholic fatty liver disease; Oxidative stress; Metabolic syndrome; Portal vein pressure; Lipid peroxidation; Antioxidants

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INTRODUCTION

Non-alcoholic fatty liver (NAFL) and its progressive form nonalcoholic steatohepatitis (NASH) is a common non-alcoholic fatty liver disease (NAFLD). The NAFL-NASH complex can be viewed as a pathological spectrum, with steatosis without inflammation or necrosis at one end (NAFL), and its progression with inflammation, necrosis and fibrosis or cirrhosis at the other end (NASH)[1].

Recent data show that NAFLD is a hepatic manifestation of the metabolic syndrome that is associated with insulin resistance and encompasses a cluster of disorders, such as obesity, dyslipidemia, type 2 diabetes mellitus, and hypertension[2]. Many of the risk factors for the NAFL-NASH complex are well defined, but the underlying pathogenesis is not well understood. At present, therapy is aimed at modifying the risk factors, but there are no proven therapies for the prevention or even treatment of NAFLD. Work on the pathogenesis of NAFLD suggests that the amelioration or prevention of oxidative stress may be an effective treatment of NAFLD in humans eschewing a healthy lifestyle[2].

Of the many biological targets of oxidative stress, lipids figure most prominently. Lipid peroxidation generates a number of byproducts, with malondialdehyde (MDA) being the principal product of fatty acid peroxidation, a highly toxic molecule that is used as a biomarker of lipid peroxidation and oxidative stress[3]. The biological effects of oxidative stress are neutralized *in vivo* by antioxidative defense mechanisms that include vitamins C and E, carotenoids, antioxidant enzymes, and glutathione (GSH). The latter in its reduced form is the single most important protective and regulatory antioxidant. Since lipid peroxidation markedly lowers its level, glutathione together with MDA are used as indicators of oxidative stress in the liver[4].

Melatonin is a serotonin-derived neurohormone formed primarily in the brain by the pineal gland of all mammals, including man[5]. In humans, melatonin is secreted mostly nocturnally. Melatonin exerts its many physiological actions *via* specific cell membrane and nuclear receptors, although many of its actions are receptor-independent, including the scavenging of free radicals and the interaction with cytosol proteins, like calmodulin and tubulin-associated proteins[6,7]. More importantly, melatonin as an active substance in the neuro-immune-endocrine system expresses numerous biological functions concerning the circadian rhythm, sleep, the stress response, the process of aging, and immunity[8]. Furthermore, melatonin has natural direct or indirect antioxidant effects[6,9].

We hypothesized that the use of melatonin could complement and amplify the antioxidant-defense system and thereby reduce or prevent the severity of liver damage associated with NAFLD. We tested our hypothesis, in a rat model of NAFLD induced by a high fat diet, by studying the effects of pharmacological doses of melatonin on liver function, liver histopathology, portal vein pressure, lipid metabolism, and oxidative stress.

MATERIALS AND METHODS

Animals and their treatment

Adult, male, Wistar rats weighing 180-230 g were supplied by the Hellenic Pasteur Research Institute. They were housed in a climate-controlled room at 24 ± 1.5 ℃, with a 12-h light (8 am-8 pm)/dark (8 pm-8 am) cycle, and free access to food and tap water. The animals were treated according to the "Principles of Laboratory Animal Care" of the National Society for Medical Research, and the Guidelines for the Care and Use of Laboratory Animals, prepared by the Academy of Sciences and published by the National Institutes of Health (Institute of Laboratory Animal Resources Commission on Life Sciences, 1996). At the end of the study, the rats were anaesthetized with ether to measure the portal vein pressure, collect blood *via* cardiac puncture, and remove the liver upon sacrifice.

Melatonin and special diet

Melatonin was purchased from Sigma Aldrich Chemical Co. (St Louis, MO, United States), dissolved in a minimum volume of absolute ethanol, and diluted to 5 mg/mL with 0.9% NaCl. The ethanol concentration in the final solution was 0.8%.

Melatonin was injected intraperitoneally, consistently between 7 pm and 8 pm, at a dose of 5 or 10 mg/kg body weight. Rats that did not receive melatonin were injected with an equivalent amount of saline/alcohol fluid (0.9% NaCl containing 0.8% ethanol).

High fat diet (HFD) in pellet form was obtained from Mucedola s.rl (Milano, Italy). It contains 19% protein, 17.5% fat, 3.5% fibre, 3.5% ashes, vitamins and minerals. Regular diet (RD) was obtained from Kounker Keramaris Bros and Co, (Athens, Greece). It contains 16.5% protein, 8% fibre, 8% ashes, 2% fat, 1% NaCl, vitamins and minerals.

Experimental protocol

Eighty-two rats were used in the study. After acclimatization for one week, they were randomly assigned to 6 experimental groups. Group 1 rats (n = 12) were fed HFD for 4 wk (subgroup 1A, n = 6) or 8 wk (subgroup 1B, n = 6). Group 2 rats (n = 10) were fed RD plus 5 mg/kg melatonin for 4 wk (subgroup 2A, n = 5) or 8 wk (subgroup 2B, n = 5). Group 3 rats (n = 10) were fed RD plus 10 mg/kg melatonin for 4 wk (subgroup 3A, n = 5) or 8 wk (subgroup 3B, n = 5). Group 4 rats (n = 20) were fed HFD plus 5 mg/kg melatonin for 4 wk (subgroup 4A, n = 10) or 8 wk (subgroup 4B, n = 10). Group 5 rats (n = 20) were fed HFD plus 10 mg/kg melatonin for 4 wk (subgroup 5A, n = 10) or 8 wk (subgroup 5B, n = 10). Group 6 rats (n = 10) were fed HFD for 8 wk (subgroup 6A, n = 5) or 12 wk (subgroup 6B, n = 5) plus 10 mg/kg melatonin for the last 4 wk.

Measurement of portal vein pressure

The abdomen was entered *via* a midline incision and the portal vein was cannulated through the mesenteric vein with a PE-50 catheter. The portal pressure was recorded on a multichannel recorder through a highly sensitive transducer (SpaceLabs Medical Inc. Model 11-14-15)[10]. The zero reference point was determined to be 1 cm above the operating table.

Measurement of plasma and serum biochemical markers

Blood was collected *via* cardiac puncture in vacutainer tubes (Becton Dickinson Hellas, Athens, Greece), centrifuged at room temperature for 15 min at 3000 *g*, and serum stored in cryotubes at -70 ℃. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a multianalyzer (Cobas Miras, Roche, Basel, Switzerland). Serum total cholesterol (TC), triglycerides (TG), low-density (LDL), and high-density (HDL) lipoproteins were measured using an online dual-enzymatic method for the simultaneous quantification of TC and TG, employing high-performance liquid chromatography.

Tissue preparation

Upon sacrifice, livers were quickly removed, washed with ice-cold 0.9% NaCl, dried on a filter paper, weighed, and samples were taken from the left anterior lobe. These were divided in two parts, one part was fixed immediately in 10% neutral-buffered solution with 4% formaldehyde for 24 h before being embedded in paraffin, the other part was immediately stored at -70 ℃ for measurement of tissue MDA and GSH levels.

Liver histopathological evaluations

Serial liver sections, 4 μm thick, were processed routinely for hematoxylin-eosin staining. The histopathological features were scored by one pathologist who was unaware of the specific dietary regimen of the animals. Each section was evaluated for grading of steatosis according to the criteria reported by Brunt *et al*[11].

Measurement of liver MDA

Before the assay, tissue samples were washed in ice-cold NaCl 0.9%, blotted on absorbent paper, and weighed. Each sample was then minced in a small volume of ice-cold 20 mm Tris-HCl buffer, pH 7.4, in a 1:10 w/v ratio, and homogenized. After centrifugation at 3000 *g* for 10 min at 4 ℃, the clear homogenate supernatant was used for biochemical assay. For the determination of MDA, 0.65 mL of 10.3 mmol/L N-methyl-2-phenyl-indole in acetonitrile was added to 0.2 mL of tissue sample. After vortexing for 3-4 s, 0.15 mL of 15.4 mol/L methanesulfonic acid was added and samples were mixed well, closed with a tight stopper, and incubated at 45 ℃ for 40 min. The samples were then cooled on ice, centrifuged, and the absorbance was measured spectrophotometrically at 586 nm. A calibration curve, comprising accurately prepared standard MDA solutions (from 2 to 20 nmol/mL), was also run for quantitation[12]. Measurements were performed in triplicate. MDA levels were expressed as μmol/L/mg protein/mg tissue.

Measurement of liver GSH levels

GSH levels were measured in homogenized liver tissue using the colorimetric assay for GSH, according to the manufacturer's instructions (Bioxytec GSH-400, Oxis Research, Portland United States)[13]. GSH concentrations were determined using a standard curve of absorbance units versus GSH concentrations. GSH levels were expressed as μmol/L /mg protein /mg tissue.

Statistical analysis

In the analysis of data, continuous variables were reported as mean ± SD and compared using the Student t test. Categorical variables were reported as relative frequencies (%) and compared using the χ^2 test. Treatment effects on histology grading were estimated using logistic regression models.

The odds ratios (ORs) were estimated and presented

Table 1 Univariate analysis of diet and melatonin use and its duration

	Liver weight (g)	*P* value	Liver weight/ body weight (%)	*P* value	Portal vein pressure (mmHg)	*P* value
Diet						
HFD	13.3 ± 2.8	< 0.001	3.5 ± 0.5	< 0.001	11.9 ± 2.7	< 0.001
RD	9.1 ± 1.3	BV	2.4 ± 0.3	BV	8.0 ± 1.7	BV
Melatonin						
10 mg/kg	11.2 ± 2.5	< 0.001	3.0 ± 0.5	< 0.001	10.5 ± 2.6	0.01
5 mg/kg	11.3 ± 2.3	< 0.001	3.0 ± 0.6	< 0.001	10.3 ± 2.8	0.03
0 mg/kg	16.2 ± 2.3	BV	4.0 ± 0.5	BV	13.0 ± 3.5	BV
Duration						
8 wk	12.0 ± 3.6	0.86	3.2 ± 0.7	0.71	10.1 ± 2.5	0.02
4 wk	12.2 ± 2.6	BV	3.2 ± 0.7	BV	12.0 ± 3.3	BV

Variables are expressed as mean ± SD. HFD: High fat diet; RD: Regular diet; BV: Baseline value.

Table 2 Effect of melatonin after adjusting for high fat diet and duration of its use

Melatonin	5 mg/kg	*P* value	10 mg/kg	*P* value
Liver weight (g)	-3.9	< 0.001	-3.9	< 0.001
Liver weight/body weight ratio (%)	-0.7	< 0.001	-0.7	< 0.001
Portal vein pressure (mmHg)	-1.6	0.09	-1.4	0.14

Results are expressed as the mean difference between rats on melatonin by comparison to those not on melatonin.

with their 95%CI. A two-sided $P < 0.05$ was considered statistically significant. Stata v8 package was used for data analysis.

RESULTS

The results pertain to seventy-eight rats, since four of the eighty-two used rats (two, one, and one from subgroups 4A, 4B and 5A, respectively) died upon completion of the study during anaesthesia.

Liver weight, liver weight over body weight ratios, portal vein pressure

Rats on HFD (groups 1, 4 and 5) had a significantly higher mean liver weight (+4.2 g) ($P < 0.001$), higher liver weight over body weight ratio (+1.1%) ($P < 0.001$), and higher mean portal vein pressure (+3.9 mmHg) ($P < 0.001$) compared to rats on RD (groups 2 and 3). Rats receiving melatonin (groups 2, 3, 4 and 5) had lower mean liver weight [-5.0 g at 10 mg/kg ($P < 0.001$) and -4.9 g at 5 mg/kg melatonin ($P < 0.001$)], lower liver weight over body weight ratio (-1.0%) ($P < 0.001$) for both melatonin doses, and lower mean portal vein pressure [-2.5 mmHg ($P = 0.03$) and -2.7 mmHg for 5 and 10 mg/kg melatonin, respectively], compared to rats not receiving melatonin (group 1). All comparisons between subgroups with 5 mg/kg *vs* 10 mg/kg melatonin were not statistically significant. The duration of melatonin use (4 wk *vs* 8 wk) had an insignificant effect on liver weight and liver weight over body weight ratio; however it was associated with a statistically significant decline in mean portal vein pressure (-1.9 mmHg) ($P = 0.02$) in groups treated for 8 wk compared to 4 wk (Table 1). After adjusting for HFD and duration of treatment, melatonin was associated with a significant decline in liver weight and liver weight over body weight ratio, but had no significant effect on portal vein pressure (Table 2). Comparisons between groups on high (10 mg/kg) *vs* low (5 mg/kg) melatonin yielded statistically insignificant results for both liver weight and liver weight over body weight ratio.

Effects of melatonin use on liver steatosis

Of the 48 rats on HFD (groups 1, 4 and 5), all but one (in subgroup 5B) (47/48, 98%) developed steatosis grade I to grade Ⅲ, against none among the 20 rats on RD (groups 2 and 3) ($P < 0.001$). All 12 rats (100%) on HFD without melatonin (group 1) developed grade Ⅲ steatosis. By contrast, rats on HFD for 4 or 8 wk with concurrent use of 5 or 10 mg/kg melatonin (groups 4 and 5) showed a significant mitigation of liver steatosis, with only 4 of 36 rats developing grade Ⅲ steatosis ($P < 0.001$). When all rats receiving melatonin were grouped together, grade Ⅲ steatosis was evident in 1 of 29 (3.4%) rats receiving 10 mg/kg melatonin (groups 3 and 5) ($P < 0.001$) and in 3 of 27 (11.1%) rats (groups 2 and 4) ($P < 0.001$) receiving 5 mg/kg melatonin (Figure 1).

Overall, 1 mg/kg increase in melatonin dose had a protective effect on steatosis in rats on HFD, reducing its risk (grade I or worse steatosis) by OR 0.85 (95%CI: 0.72-0.99). The duration of melatonin use (4 wk *vs* 8 wk) had no significant effect on the severity of steatosis (Table 3). After adjusting for duration of melatonin use, melatonin showed again a protective effect toward grade I or worse steatosis in rats on HFD, reducing the risk by OR 0.69 per 1 mg/kg increase in melatonin dose (95%CI: 0.59-0.83) (Table 4).

All rats (10/10, 100%) on HFD for 8 or 12 wk receiving 10 mg/kg melatonin in the last 4 wk (subgroups 6A and 6B) showed grade Ⅲ steatosis, as against 4 of 36 rats (11.1%) on HFD that concurrently received 5 mg/kg or 10 mg/kg melatonin (groups 4 and 5) ($P < 0.001$).

Serum biochemical parameters and liver tissue markers of oxidative stress

The levels of TC, LDL, HDL and TG did not differ sig-

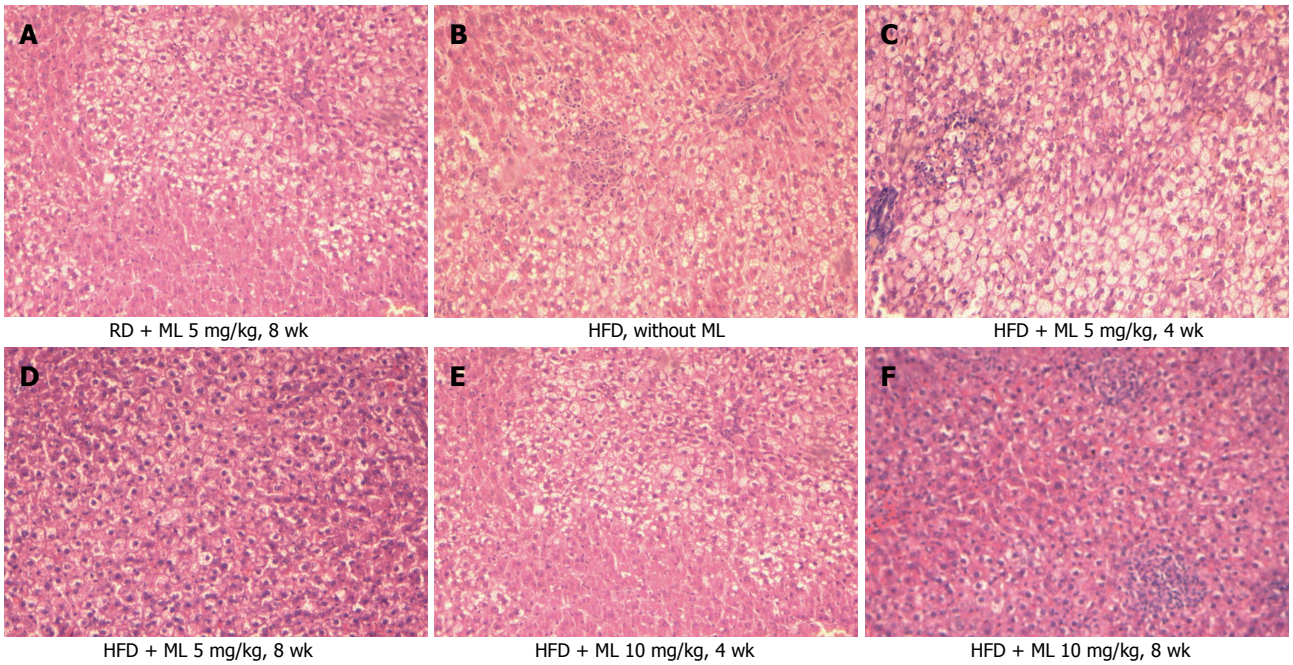

Figure 1 Histopathological features of liver samples. A: Normal liver architecture in rats on regular diet plus melatonin; B: Severe fatty liver disease in rats on high fat diet without melatonin; C-F: Attenuated steatosis in rats on high fat diet and melatonin. (Hematoxylin-eosin staining was used, ×100). RD: Regular diet; HFD: High fat diet; ML: Melatonin.

Table 3 Univariate analysis of diet, melatonin use and its duration *n* (%)

n = 68	Grade 0	Grade 1	Grade 2	Grade 3	OR (95%CI)	*P* value
Diet						
HFD (*n* = 48)	1 (2.10)	19 (39.60)	12 (25)	16 (33.30)	NE	< 0.001
RD (*n* = 20)	20 (100)	0 (0)	0 (0)	0 (0)	BV	
Melatonin						
10 mg/kg (*n* = 29)	11 (37.90)	9 (31)	8 (27.60)	1 (3.40)	0.85[1] (0.72-0.99)	0.04
5 mg/kg (*n* = 27)	10 (37)	10 (37)	4 (14.90)	3 (11.10)		
0 mg/kg (*n* = 12)	0 (0)	0 (0)	0 (0)	12 (100)		
Duration						
8 wk (*n* = 35)	11 (31.40)	10 (28.60)	7 (20)	7 (20)	0.95 (0.34-2.66)	0.92
4 wk (*n* = 33)	10 (30.30)	9 (27.30)	5 (15.10)	9 (27.30)	BV	

[1]Per 1 mg/kg increase in melatonin. HFD: High fat diet; NE: Not estimated; RD: Regular diet; BV: Baseline value.

Table 4 Ordinal logistic regression analysis of melatonin effect in rats on high fat diet

n = 48	OR (95%CI)	*P* value
Melatonin (per 1 mg/kg increase)	0.69 (0.59-0.83)	< 0.001
Duration (8 wk *vs* 4 wk)	0.65 (0.20-2.10)	0.47

nificantly among the rats on HFD, irrespective of the use or not of melatonin (Figure 2). After adjusting for HFD and duration of melatonin use, melatonin had no effect on any of the common lipidemic serum markers (Table 5).

There was a significant aminotransferase decrease in rats on HFD receiving melatonin as compared with those not on melatonin (Figure 3A and B). Specifically, AST and ALT levels declined by a mean of 170 and 62.9 U/L, respectively, in the 5 mg/kg melatonin group, and by 224 and 93.4 U/L, respectively, in the 10 mg/kg melatonin group (Table 5). However, differences between the 5 mg/kg *vs* 10 mg/kg melatonin dose were not significant for either the AST ($P = 0.30$) or the ALT ($P = 0.12$) (Table 5).

Compared to animals not on melatonin, melatonin use led to a significant decline in MDA production in liver tissue by 12.7 and 12.2 for the 5 and 10 mg/kg melatonin dose, respectively, and a significant increase in GSH in liver tissue by 20.1 and 29.2 for the 5 and 10 mg/kg melatonin dose, respectively, in multivariable analysis adjusting for diet and duration of melatonin use (Figure 3C and D) (Table 5). The MDA and GSH differences in liver tissue between rats receiving 5 mg/kg *vs* 10 mg/kg melatonin were not significant.

DISCUSSION

The validity of our experimental model of NAFLD in

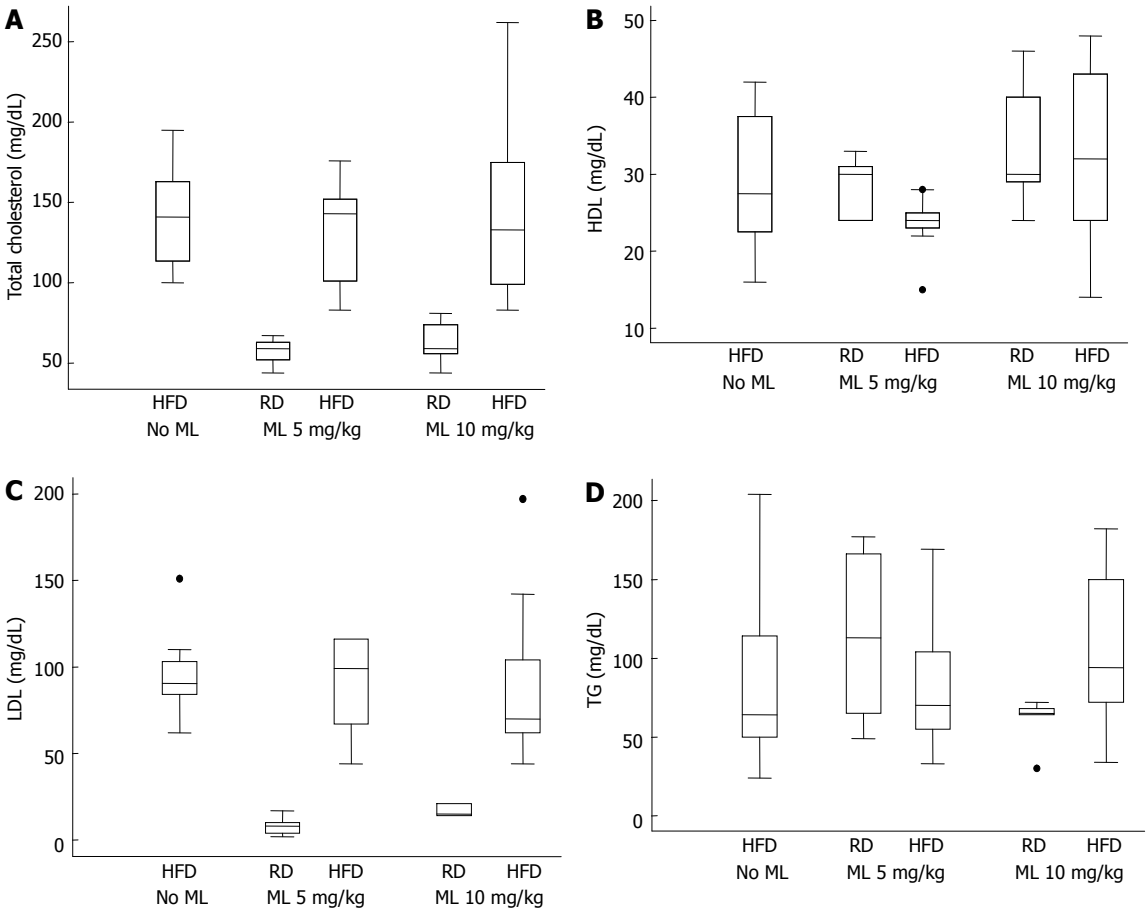

Figure 2 Lipidemic profiles of rats on regular diet or high fat diet, with or without melatonin. A: Serum total cholesterol; B: High-density lipoproteins; C: Low-density lipoproteins; D: Triglycerides. HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TG: Triglycerides; HFD: High fat diet; RD: Regular diet; ML: Melatonin; ●: Indicates outliers.

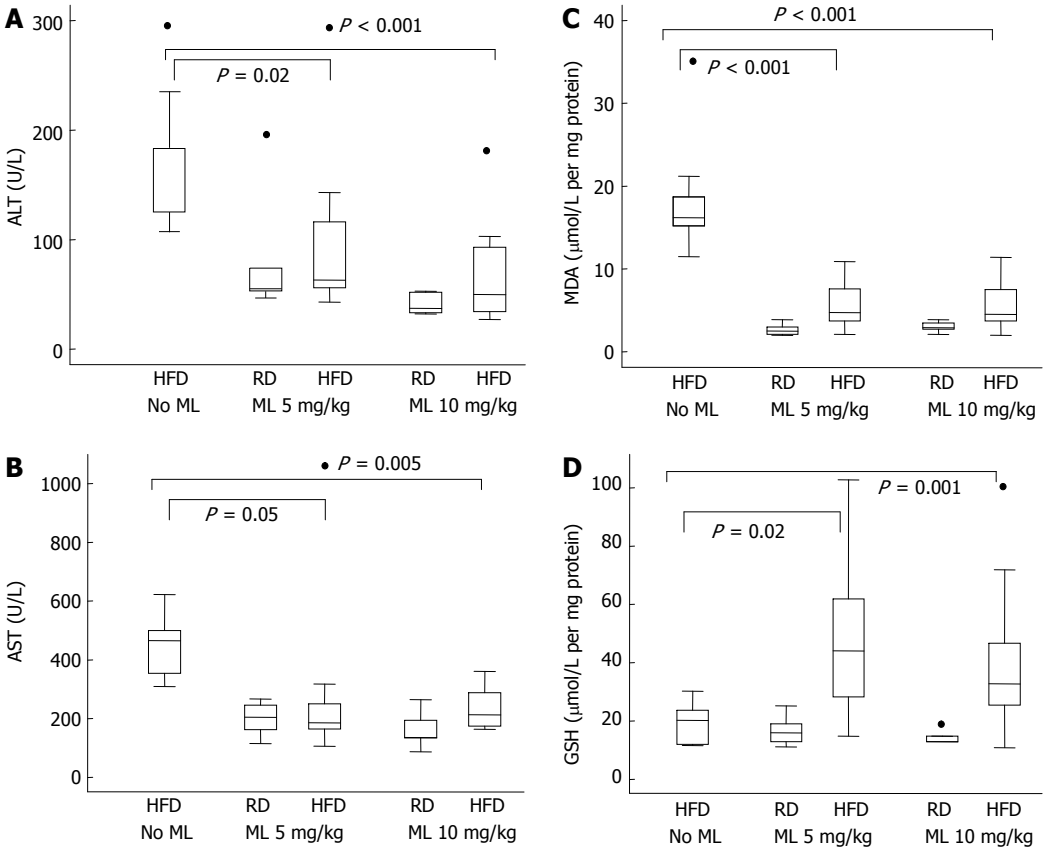

Figure 3 Hepatic inflammation and oxidative stress assessment. A: Hepatic inflammation represented by serum alanine aminotransferase; B: Aspartate aminotransferase; C: Oxidative stress represented by tissue levels of malondialdehyde; D: Glutathione, in rats on regular or high fat diet, with or without melatonin. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; MDA: Malondialdehyde; GSH: Glutathione; HFD: High fat diet; RD: Regular diet; ML: Melatonin; ●: Indicates outliers.

Table 5 Effect of melatonin on laboratory indices

	Melatonin (5 mg/kg)	*P* value	Melatonin (10 mg/kg)	*P* value
Lipidemic profile				
TC (mg/dL)	-10.7	0.45	-2.2	0.87
LDL (mg/dL)	-6.3	0.60	-3.9	0.74
HDL (mg/dL)	-5.3	0.10	1.9	0.52
TG (mg/dL)	6.7	0.71	-5.2	0.76
Hepatic function				
AST (U/L)	-170	0.01	-224	0.001
ALT (U/L)	-62.9	0.01	-93.4	< 0.001
Oxidative profile				
MDA	-12.7	< 0.001	-12.2	< 0.001
GSH	20.1	0.004	29.2	< 0.001

Multivariate linear regression analysis of melatonin effect after adjusting for duration of its use and high fat diet (HFD). Results are expressed as the mean difference between rats on melatonin by comparison to those not on melatonin. TC: Total cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglycerides; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MDA: Malondialdehyde; GSH: Glutathione.

rats on HFD was confirmed by the histopathological findings of liver steatosis. Liver steatosis was absent in rats on regular diet.

In this rat NAFLD model, administration of 5 or 10 mg/kg melatonin, for 4 or 8 wk, was effective in mitigating the course of NAFLD. In particular, melatonin showed a strong tendency to attenuate liver steatosis and to curtail the rise of liver weight, portal vein pressure, and serum aminotransferases. By contrast, rats on HFD without melatonin showed significantly higher liver weight, liver weight over body weight ratio, portal vein pressure, serum aminotransferases, and higher TC, TG, LDL and HDL.

These results are in keeping with previous studies indicating a hepatoprotective effect of melatonin in rat models of diet-induced NAFLD[14,15].

The putative mechanism of the beneficial effect of melatonin on NAFLD in rats on HFD is the decrease of lipid peroxidation and the limitation or prevention of oxidative stress. Melatonin's antioxidant repertoire includes: its stimulation of antioxidant enzymes[16], the regulation of gene transcription for antioxidant enzymes[17], its direct free radical scavenger action[18], the stimulation of glutathione synthesis[19], its ability to augment the activities of other antioxidants[20], the protection of antioxidative enzymes from oxidative damage[21], its action on the mitochondrial respiratory chain activity whereby melatonin lowers the electron leakage and reduces the generation of free radicals[22], and finally its significant attenuation of LPS-induced sterol regulatory element-binding protein (SREBP)-1c activation and expression of SREBP-1c target genes that prevents LPS-induced hepatic lipid accumulation[23].

Lipid peroxidation, and peroxidation of membrane lipids, links steatosis to steatohepatitis, with its attendant necroinflammation, liver cell necrosis, increased ALT and AST levels, and fibrosis[24]. The aldehyde product of lipid peroxidation, MDA, induces hepatic stellate cell activation[25], these being the main collagen-producing cells within the liver, leading to enhanced extracellular matrix protein deposition. MDA may also contribute to inflammation by activating nuclear factor-kappaB (NF-κB), a transcription factor regulating the expression of several proinflammatory cytokines and adhesion molecules, including tumour necrosis factor-alpha, intercellular adhesion molecule 1, and E-selectin[26,27].

However, steatosis by itself (first hit) is not sufficient for the development of steatohepatitis and fibrosis, as the latter would require the action of additional factors (second hit)[28]. Such factors would include a source of free radicals capable of inducing oxidative stress[29], the inhibition of electron transfer along the respiratory chain that would lead to the generation of superoxide anions capable of initiating lipid peroxidation[30], and enhanced formation of reactive oxygen species (ROS) leading to increased oxidative stress. In this respect, melatonin has additive or multiplier effects on the reduction of lipid peroxidation[16,21], being twice as effective as vitamin E at protecting cell membranes from lipid peroxidation[31]. The observation in our study of significantly reduced MDA levels in livers of rats on HFD and melatonin, but not in rats on HFD alone might suggest that melatonin may have efficiently reduced lipid peroxidation and its end products, such as MDA.

In addition, melatonin, as a free radical scavenger, is effective against oxidative stress by reducing the production of free radicals and ROS and by improving the function of the mitochondrial respiratory chain[18,22,32]. Furthermore, melatonin increases the levels of several antioxidative enzymes, including superoxide dismutase, glutathione peroxidase, and glutathione reductase.

Significantly, our study provided support for the protective effect of melatonin against oxidative stress by showing increased glutathione levels in the liver of rats on HFD and melatonin when compared to rats on HFD without melatonin. It would appear that melatonin does stimulate the production of glutathione, with the ensuing protective effect against oxidative stress.

Moreover, besides its free radical scavenging and antioxidative functions, melatonin's receptor-mediated local functions may contribute to its ability to preserve cell function and limit cell death from apoptosis or necrosis due to oxidative damage[33-35]. Melatonin's protective effect against liver cell necrosis was supported in our study by the significantly lower level of serum aminotransferases in rats on HFD receiving melatonin.

In contrast to the protective action of melatonin when given synchronously with HFD, this effect was lost in rats receiving melatonin at a late phase of the experiment, with melatonin failing to reverse or reduce the severity of steatosis. Our study is the first to indicate melatonin's possible ineffectiveness at reversing established steatosis. Clearly, further study is needed to test if delayed melatonin administration for more than 4 wk might be effective in reversing steatosis.

In our study, melatonin had no significant effect on the lipidemic markers. After adjusting for HFD and duration of melatonin use, the lipidemic serum markers (TC, TG, LDL and HDL) were higher in rats on HFD without melatonin than in rats on HFD receiving melatonin, but this difference did not reach statistical significance. This is in contrast to the findings of Pan *et al*[14] and Chen *et al*[23] who found significantly reduced TC and TG levels in rats given a moderate or high dose of melatonin (5 or 10 mg/kg), and also to the findings of Hoyos *et al*[36] who found significantly reduced levels of TC and LDL in the melatonin groups. However, our findings match those of the latter study as it concerns the TG levels.

We don't have a clear explanation for this discrepancy. Suffice to say that melatonin does not protect against the consequences of a high fat diet, including increased intestinal lipid absorption leading to an increased supply of free fatty acids to the liver, the increased de novo synthesis of fatty acids in the liver, or the increased VLDL-TG discharge from the liver. On the other hand, melatonin may have a hypocholesterolemic[36] and hypolipidemic effect[23,37]. This may partly explain our findings of no statistical difference in the lipidemic profile between the groups. Or, possibly, the conflicting results may correlate with the duration of melatonin administration or the dose used.

The extrapolation of our data to humans requires further investigation. Recently, Gonciarz *et al*[38] conducted a pilot study of a 3-mo course of melatonin treatment of patients with non-alcoholic steatohepatitis, with encouraging results. Based on our experimental evidence, the case might be made for the use of melatonin in conditions with characteristics of the metabolic syndrome, where excessive free radical generation and oxidative stress may occur. This view is supported by the findings of a recent study where melatonin improved the metabolic syndrome induced by high doses of fructose in rats, as showed by a decrease in the insulin resistance, together with a decrease in the concentration of serum tumour necrosis factor-α, hepatic lipid peroxide, and hepatic reduced glutathione[39].

Oral administration of melatonin is safe, and without serious acute or chronic toxicity[38,40]. However, its daytime use may cause feelings of sleepiness and fatigue, which can adversely affect performance[41].

In summary, melatonin was shown to counter lipid peroxidation and oxidative stress, thus providing a degree of protection against the development or the severity of NAFLD in rats concurrently on HFD and melatonin. However, in our experimental model, melatonin was not effective in reversing a state of established steatosis. Interestingly, melatonin administration was not associated with a significant change in the lipid metabolic profile.

COMMENTS

Background

The non-alcoholic fatty liver and its progressive form, nonalcoholic steatohepatitis, is a common non-alcoholic fatty liver disease (NAFLD). Recent data show that non-alcoholic fatty liver disease is a hepatic manifestation of the metabolic syndrome that is associated with insulin resistance and encompasses a cluster of disorders, such as obesity, dyslipidemia, type 2 diabetes mellitus, and hypertension. NAFLD is widely prevalent and is clinically being seen with greater regularity because of the high fat and calorie diets consumed in many countries, the disease being present in approximately 30% of the United States population, with the risk that as many as 15% to 20% of subjects with non-alcoholic steatohepatitis will develop cirrhosis. While much new information on the pathogenesis and natural history of non-alcoholic fatty liver disease is available, proven therapies for the prevention or even treatment of this common disease remain to be established.

Research frontiers

Work on the pathogenesis of NAFLD suggests that lipid peroxidation, peroxidation of membrane lipids and oxidative stress, are its main risk factors. In the area of prevention of NAFLD, the research hotspot is the use of melatonin, with its direct or indirect antioxidant effects, to complement and amplify the natural antioxidant-defenses-system of humans and thereby reduce or prevent the severity of liver damage associated with non-alcoholic liver disease.

Innovations and breakthroughs

Recent data show that lipid peroxidation, fatty acid peroxidation, and oxidative stress generate a number of byproducts that are highly toxic to liver cells leading to NAFLD. At present, therapy is aimed at modifying the risk factors, but there are no proven therapies for the prevention or even treatment of NAFLD. The biological effects of oxidative stress are neutralized *in vivo* by antioxidative defense mechanisms that include vitamins C and E, carotenoids, antioxidant enzymes, and glutathione. The use of melatonin could complement and amplify the natural antioxidant-defenses-system and thereby reduce or prevent the severity of liver damage associated with oxidative stress, in NAFLD. In the present experimental study, the authors provide ample evidence that melatonin counters against lipid peroxidation and oxidative stress, thus providing a degree of protection against the development or the severity of NAFLD. However, in this experimental model it was shown for the first time that melatonin was not effective in reversing a state of already established NAFLD.

Applications

Melatonin counters against oxidative stress and its use can ameliorate or prevent oxidative stress and may prevent or retard the development of NAFLD in humans not adopting a healthy life-style, by decreasing lipid peroxidation and by limiting or preventing oxidative stress. Based on the experimental evidence of this study, the case might be made for the use of melatonin in conditions with characteristics of the metabolic syndrome, where excessive free radical generation and oxidative stress may occur.

Terminology

Melatonin is a neurohormone formed primarily in the brain of all mammals, being secreted mostly nocturnally in humans. Melatonin expresses numerous actions such as natural direct or indirect antioxidant effects and scavenging of free radicals. Humans can benefit from melatonin's actions, especially if they have a tendency to develop the metabolic syndrome.

Peer review

This is a well designed and conducted study. Results are clearly presented, and represent a possible contribution for the prevention and management of NAFLD. This report documents that melatonin prevents or retards the development of fatty liver in rats fed a high fat diet. Moreover, the authors report that once fatty liver develops, melatonin does not reverse this condition. These are important findings with clear clinical implications.

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ORIGINAL ARTICLE

Hypothermia predicts hepatic failure after extensive hepatectomy in mice

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Abstract

AIM: To investigate the effect of hypothermia on the function of the liver remnant (LR) after extended hepatectomy.

METHODS: We performed a 75% partial hepatectomy (PH) in male C57BL/6J mice. Body temperature was measured with a rectal probe. The study mice were prospectively grouped as hypothermic (HT) or normothermic (NT) if their body temperature was < 34 ℃ *vs* ⩾ 34 ℃, respectively. Blood and liver samples were obtained at 24 and 48 h after 75% PH. Various factors during and after 75% PH were compared at each time point and the most important factor for a good outcome after 75% PH was determined.

RESULTS: At 24 and 48 h after 75% PH, LR weight was decreased in HT mice compared with that in NT mice and the assay results in the HT mice were consistent with liver failure. NT mice had normal liver regeneration. Each intra- and post-operative factor which showed statistical significance in univariate analysis was evaluated by multivariate analysis. The most important factor for a good outcome after 75% PH was body temperature at both 24 and 48 h after surgery.

CONCLUSION: Hypothermia after an extensive hepatectomy predicts impending liver failure and may be a useful clinical marker for early detection of liver failure after extended hepatectomy.

Key words: Hypothermia; Predictor; Hepatectomy; Liver failure; Mortality

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INTRODUCTION

Liver failure (LF) occurs in various conditions, including acute LF, LF of an insufficient liver remnant (LR) after extended hepatectomy (EH) and LF of transplanted whole or split liver grafts[1-6]. Without a suitable liver replacement, death imminently ensues. When LF occurs

in an insufficient LR, it becomes clinically apparent by postoperative days 3-7 or later[2,7,8]. Biochemically, transaminases and bilirubin levels are markedly elevated, with prolonged coagulopathy. Liver histology shows destruction of sinusoids and hepatocellular parenchyma. Clinically, ascites and possibly encephalopathy can develop in patients[6,8]. Hepatic failure can have lethal consequences but the mechanisms responsible for LF in an insufficient LR are unknown.

In early studies, terminal LF was associated with hypothermia[9]. Hypothermia is also common in advanced stages of acute LF[10,11]. Similarly, earlier studies that conducted EH in rodent models demonstrated that the animals that failed to survive after EH had significant hypothermia, whereas those that survived maintained normothermia[12,13]. In a recent outcome analysis of living-donor liver transplantation, hypothermia < 34 ℃ was one of the significant predictors of hospital death in adult recipients[14]. In our laboratory, preliminary results showed that mice which failed to survive after 75% partial hepatectomy (PH) had significant hypothermia and the surviving animals remained normothermic. Therefore, we hypothesized that hypothermia predicts imminent LF after EH. In this study, we investigated body temperature (BT) after 75% PH and prospectively evaluated the correlation of hypothermia to biochemical, histological and molecular factors of LF in mice after 75% PH. Our results suggest that hypothermia predicts LF after EH.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (10-14 wk old), purchased from Jackson Laboratory (Bar Harbor, ME), were housed in a conventional mouse room with a 12 h light/dark cycle and were given food and water. The study was institutionally approved in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

75% PH

Hepatectomy models in mice have been well documented[15-17] and our surgical procedures have been described in detail elsewhere[17]. Left posterior, left and right anterior and right posterior lobes were resected[15-17]. After the mice underwent isoflurane general anesthesia, the liver lobes were mobilized. A hemostatic clip (Hemoclip, Edward Weck and Co., Research Triangle Park, NC) was applied across the pedicle at the base of the liver lobes, which were cut distally to the applied clip. For the sham operation performed on control subjects, laparotomy without liver resection was performed. Before closing the abdominal incision, 2 mL of warm saline was administered intraperitoneally. Cefalexin (30 mg/kg) and buprenorphine (0.1 mg/kg) were given subcutaneously. Postoperatively, the mice were housed under controlled temperature, humidity and light, with free access to food and water. No dextrose was provided. At the end of the procedure, all LRs were pink. Control mice had a normal BT and no postoperative mortality.

Measurement of BT after 75% PH

No heating pad or lamp was used after surgery. BT was measured with a rectal probe (RET-3, Physitemp Instrument Inc, Clifton, NJ) at 24 h after 75% PH.

In a preliminary study, the mice that failed to survive had hypothermia of < 34 ℃ after 75% PH (0 of 20 mice after 75% PH). None of the mice that had a BT ⩽ 30 ℃ survived (0 of 6 mice after 75% PH). In contrast, all but one mouse that had a BT over 34 ℃ survived beyond 5 d, consistent with previous reports[18,19].

In a separate preliminary study, after 75% PH in hypothermic mice, we achieved normothermia with a heating pad, as previously described[20]. However, a heating pad did not offer any benefits for survival ($P > 0.05$), consistent with a previous report[12]. Therefore, we did not employ a heating pad after surgery in this study.

Survival curves after 75% PH

To simulate clinical conditions[3,4], 75% PH was reproduced according to a previous description[15] in 27 mice ($n = 27$) and sham operations were performed in 13 control mice ($n = 13$).

Group classification

Based on the preliminary studies and other reports[8], we conducted the following experiments prospectively to test the hypothesis that hypothermia precedes LF after 75% PH in mice. Since LF occurs between 3 and 7 d postoperatively, we sacrificed the study mice at 24 and 48 h after 75% PH. Rectal temperature was measured at 24 h after 75% PH and we divided the mice into the following groups based on their BT: normothermic (NT) with a BT ⩾ 34 ℃ or hypothermic (HT) with a BT < 34 ℃.

Blood and liver sampling after 75% PH

When mice were sacrificed at 24 and 48 h after 75% PH, liver and blood samples were collected ($n = 10$ in each group). Excised LR was weighed and snap-frozen at -80 ℃. The sera were used for biochemical analysis.

Biochemical analysis and coagulation profiles

Serum levels of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) were determined by a colorimetric kit (BioTron Diagnostics Co., Hemet, CA). Total bilirubin (T-Bil) levels were determined by the QuantiChrom™ Bilirubin Assay Kit (BioAssay Systems, Heyward, CA). The prothrombin time-international normalized ratio (PT-INR) was measured by i-STAT analysis (Abbott Laboratories, North Chicago, IL).

Western blot

LR samples were homogenized in a buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L ethylene diamine tetra-acetic acid, 1 mmol/L ethylene glycol tetra-acetic acid, 1 mmol/L phenyl-methyl-sulfonyl

fluoride and protease and phosphatase inhibitors. Homogenates were centrifuged at 105000 *g* for 1 h at 4 ℃. Supernatants were collected. Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Samples were stored at -80 ℃ until use. Forty micrograms of protein was separated *via* SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBS-T) [20 mmol/L Tris (pH 7.4), 500 mmol/L NaCl, and 0.05% Tween-20] and probed using the following antibodies: phospho-STAT3, STAT3, phospho-Akt, Akt, phospho-p44/42MAPK, p44 42 MAPK, phospho-SAPK/JNK, SAPK/JNK, phospho-p38 MAPK, p38 MAPK, cyclin D1, Met, caspase 3 (Cell Signaling, Danvers, MA) and vascular endothelial growth factor (VEGF) (Abcam, Cambridge, MA). Immunoblots were incubated with peroxidase-conjugated secondary antibodies (Southern Biotech, Birmingham, AL) followed by enhanced chemi-luminescence or ECL-plus reagent (Amersham Biosciences, Piscataway, NJ). Blots were reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase primary antibody (IMGENEX, San Diego, CA) followed by mouse secondary-HRP antibody for confirmation of equal loading. Signals were quantified using an ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

Enzyme-linked immunosorbent assay

Serum samples were analyzed using an anti-rat hepatic growth factor (HGF) enzyme-linked immunosorbent assay kit (B-Bridge International, Inc. Mountain View, CA) according to the manufacturer's instructions.

Adenosine triphosphate assay

LR tissues were homogenized in 0.6 mol/L trichloroacetic acid and centrifuged at 9000 *g* for 5 min at 4 ℃. The supernatant was neutralized with 5 mol/L KOH mixed with 0.4 mol/L imidazole using color pHast (EMD, Gibbstown, NJ). Adenosine triphosphate (ATP) was measured using an ATP Determination Kit (Invitrogen, Carlsbad, CA).

Proliferation determination with Ki-67 and proliferating cell nuclear antigen immunohistochemical staining

Formalin-fixed liver specimens were embedded in the paraffin and cut into 5-μm sections and stained with hematoxylin and eosin. Immunohistochemical proliferation analyses for Ki-67 and proliferating cell nuclear antigen (PCNA) were performed after antigen retrieval with citric acid (pH 6.0) and 3% H_2O_2. Rabbit anti-Ki-67 and PCNA antibodies (Abcam, Cambridge, MA) were incubated at 4 ℃ overnight. An Elite ABC kit (Vector Laboratories Inc, Burlingame, CA) was used for immunostaining detection according to the manufacturer's instructions. The number of Ki-67-positive and PCNA-positive cells was counted in 10 random fields at ×200. Immunohistochemical analysis for c-Met was performed using mouse anti-c-Met antibody (Cell Signaling). Blocking and detection were performed with the Vectastain MOM Kit (Vector Laboratories, Burlingame, CA).

Apoptosis assay

Activation of caspase-3 was assayed by western blot, as described above. TdT-mediated DUTP-biotin nick end labeling (TUNEL) staining was performed using the ApopTag Apoptosis Detection kit (Chemicon, Billerica, MA) on formalin-fixed liver sections. DNA laddering detection was performed with extracted DNA samples using the TACS DNA Laddering Kit (R and D, Minneapolis, MN).

Univariate analyses for factors during and after 75% PH

Surgery time (minutes), anesthesia time (minutes), estimated % hepatectomy (%), LR weight/body weight (%), BT (°C), AST (U/L), ALT (U/L), T-Bil (mg/dL), Hb (g/dL), hematocrit (%), glucose (mg/dL) and PT-INR were assessed at 24 and 48 h after 75% PH. A total of 152 mice at 24 h and 108 mice at 48 h after 75% PH were evaluated.

Important factors for a good outcome after 75% PH

Some differences in the behavior between survivors and mice that eventually died were observed from the early postoperative period after 75% PH. We divided postoperative mice into two groups, based on the postoperative behavior (*i.e.*, asymptomatic and sick mice at 24 and 48 h after 75% PH). Postoperative behavior was consistent with outcomes. A total of 152 mice at 24 h and 108 mice at 48 h after 75% PH were evaluated. Each factor, which showed statistical significance in univariate analysis, was evaluated by multivariate analysis and the most important factor for a good outcome after 75% PH was determined.

Statistical analysis

Data are presented as the mean ± SD. Statistical comparisons were performed using analysis of variance followed by *t* tests with a Bonferroni adjustment for continuous unpaired variables, the Kaplan-Meier method (the log-rank) for survival rates and logistic regression analysis for important factors for survival. Statistical calculations were performed using SPSS Software Version 16.0 (SPSS Inc., Chicago, IL). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Hypothermia is associated with mortality in mice after 75% PH

No deaths were observed at 24 h among the mice that had 75% PH. However, after 48 h, the survival rate was decreased (Figure 1A). The overall 5 d mortality rate was 60%, consistent with previous reports[18,19]. The survival rate of HT mice (BT < 34 ℃) was significantly lower than that of NT mice after 75% PH (Fisher's exact test, $P = 0.003$) (Figure 1B). This finding suggested that BT

Table 1 Univariate analysis of the factors before and after 75% partial hepatectomy

	24 h after PH			48 h after PH		
	HT	NT	*P* value	HT	NT	*P* value
Surgery time (min)	12.5 ± 0.6	11.9 ± 0.4	NS	12.0 ± 0.4	12.0 ± 0.4	NS
Anesthesia time (min)	16.0 ± 0.6	15.2 ± 0.6	NS	15.3 ± 0.5	15.4 ± 0.3	NS
Estimated % of hepatectomy (%)	79.5 ± 1.8	77.1 ± 2.4	NS	76.1 ± 1.2	78.0 ± 0.1	NS
Remnant liver weight/body weight (%)	1.50 ± 0.03	1.73 ± 0.05	0.0002	1.74 ± 0.05	2.02 ± 0.05	< 0.0001
Last measured body temperature (℃)	24.4 ± 0.6	36.3 ± 0.2	< 0.0001	25.7 ± 0.7	36.1 ± 0.6	< 0.0001
AST (U/L)	1627 ± 722	342 ± 241	0.0124	1354 ± 514	51 ± 34	< 0.0001
ALT (U/L)	1389 ± 681	182 ± 34	0.0567	1465 ± 497	22 ± 7	0.0252
T-Bil (mg/dL)	12.9 ± 3.0	3.0 ± 0.5	0.0041	23.9 ± 3.1	3.1 ± 0.2	< 0.0001
Hb (g/dL)	9.5 ± 0.7	12.4 ± 0.5	0.0031	13.5 ± 0.8	12.5 ± 0.4	NS
Hematocrit (%)	28.0 ± 2.1	36.5 ± 1.4	0.0028	39.7 ± 2.3	36.7 ± 1.4	NS
Glucose (mg/dL)	133.9 ± 18.9	136.4 ± 5.1	NS	92.9 ± 11.7	128.9 ± 5.6	0.0117
PT- INR	0.9 ± 0.0	0.9 ± 0.0	NS	1.37 ± 0.24	1.84 ± 0.17	NS

The study mice were divided prospectively into two study groups according to their body temperature, *i.e.*, hypothermic (HT) or normothermic (NT), at 24 and 48 h after surgery. NS: Not significant; AST: Aspartate aminotransferase; ALT: Alanine aminotransaminase; T-Bil: Total bilirubin; Hb: Hemoglobin; PT-INR: Prothrombin time-international normalized ratio; PH: Partial hepatectomy.

Table 2 Multivariate analysis of the factors before and after 75% partial hepatectomy

24 h after 75% PH (n = 152)	*P* value
Remnant liver weight/body weight	0.0829
Body temperature (℃)	0.0372
AST (U/L)	0.1263
ALT (U/L)	0.3826
T-Bil (mg/dL)	0.8753
Hb (g/dL)	0.6974
Hematocrit (%)	0.7365
48 h after 75% PH (n = 108)	*P* value
Remnant liver weight/body weight	0.0012
Body temperature (℃)	0.0161
AST (U/L)	0.9182
ALT (U/L)	0.8827
T-Bil (mg/dL)	0.7322
Glucose (mg/dL)	0.9913

PH: Partial hepatectomy; AST: Aspartate aminotransferase; ALT: Alanine aminotransaminase; T-Bil: Total bilirubin; Hb: Hemoglobin.

might be a useful marker for impending hepatic failure after EH.

HT mice have a decreased liver mass at 24 and 48 h after 75% PH

After 75% PH, the animals were monitored prospectively. HT and NT mice were sacrificed at 24 and 48 h. Although HT mice had decreased physical activity, they were still active and not moribund. There were no differences in surgery time, anesthesia time or the ratio of resected liver weight to body weight between HT and NT mice (Table 1).

At the time of surgery there was no difference in BT between the two groups. However, at 24 and 48 h, we found that some mice were HT compared with others that were clearly NT. The mice that were HT at 24 h were sacrificed, as well as some of the NT mice. Similarly, at 48 h, the mice were divided into groups according to BT. The mean BT of HT mice was significantly lower than that of NT mice (24.7 ± 0.8 ℃ *vs* 36.4 ± 0.2 ℃, $P < 0.0001$) at 24 h and at 48 h (24.9 ± 1.1 ℃ *vs* 36.2 ± 0.4 ℃, $P < 0.0001$).

Perioperatively, there was no difference in the amount of liver resected (Table 1), but at the time of sacrifice, the LR weight was significantly less in HT mice compared with that in NT mice (24 h, 0.36 ± 0.00 mg *vs* 0.44 ± 0.07 mg, $P = 0.0100$; and 48 h, 0.45 ± 0.04 mg *vs* 0.51 ± 0.09 mg, $P = 0.0270$). Similarly, the liver remnant weight/body weight ratio was significantly lower in HT mice than that in NT mice (24 h, 1.50 ± 0.03 *vs* 1.73 ± 0.05, $P = 0.0002$; and 48 h, 1.74 ± 0.05 *vs* 2.02 ± 0.05, $P < 0.0001$). These results indicate that liver growth was decreased in HT mice.

Paradoxically, after 75% PH, some differences in behavior between survivors and mice that finally died were observed from the early postoperative period. To determine the most important factor, multivariate analyses at each time point were performed for the significant factors in univariate analysis (Table 1). BT had a significant effect at each time point on murine symptoms after 75% PH and the LR weight/body weight also had a significant effect at 48 h after 75% PH (Table 2). In this preliminary study, we speculated that BT is a critical factor for the postoperative course and/or survival and may be a reliable predictor from the early postoperative period.

Hypothermic mice have altered liver function and hepatic histology

On gross inspection at 24 h, the LR of HT mice appeared relatively normal (Figure 2A) but it was pale and severely abnormal at 48 h (Figure 2E). There was no intraperitoneal bleeding. Histologically, there was a significant number of hemorrhagic nodules throughout the liver parenchyma at 24 h. Significant microvesicular steatosis was observed at 24 h (Figure 2B) and it was worse at 48 h (Figure 2F). These findings are consistent with previous reports of LF in insufficient LRs[12,19,21]. In

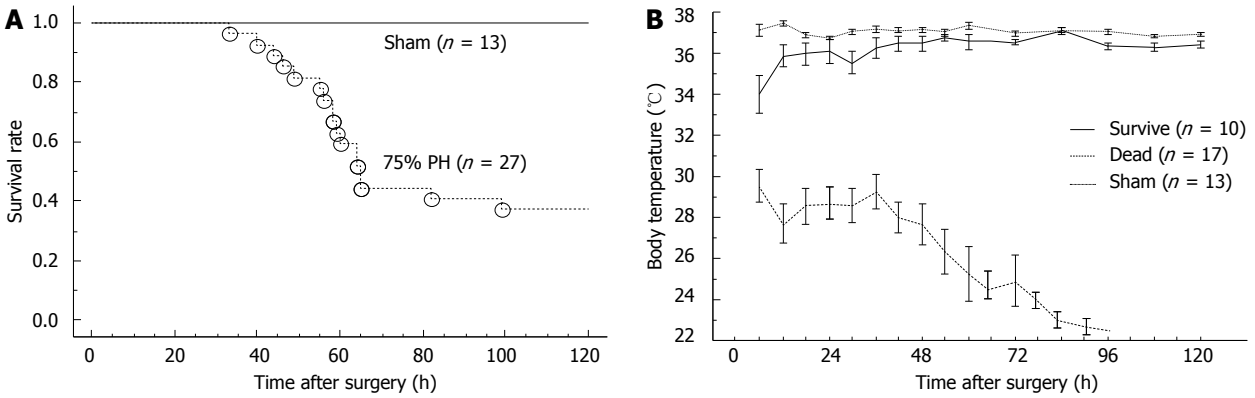

Figure 1 Hypothermia is associated with mortality in mice after 75% partial hepatectomy. A: Survival outcome of mice that underwent either sham laparotomy (top solid line) or 75% partial hepatectomy (PH) (line with circles); B: Change in body temperature (BT) after 75% PH separated by survival outcome retrospectively. At 24 and 48 h after surgery, the BT of mice that finally died was significantly lower than that of mice that survived (P = 0.0001).

contrast, in NT mice, the LR appeared normal at both 24 and 48 h (Figure 2C and G). Although there was significant microvesicular steatosis in livers of NT mice at 24 h (Figure 2D), it had largely resolved by 48 h (Figure 2H). The parenchymal architecture was essentially preserved in NT mice, as reported elsewhere[12,19,21].

HT mice had markedly elevated T-Bil levels at 24 h (12.9 ± 3.0 mg/dL *vs* 3.0 ± 0.5 mg/dL, P = 0.0008) and at 48 h (23.9 ± 3.1 mg/dL *vs* 3.1 ± 0.2 mg/dL, P < 0.0001) compared with those in NT mice. Serum AST levels were significantly higher in HT mice at 24 h (1627 ± 722 U/L *vs* 342 ± 241 U/L, P = 0.0124) and 48 h (1354 ± 514 U/L *vs* 51 ± 34 U/L, P < 0.0001) compared with those in NT mice. ALT levels were similar in both groups at 24 h but became significantly elevated at 48 h in HT mice compared with those in NT mice (1465 ± 497 U/L *vs* 22 ± 7 U/L, P = 0.0016). Hemoglobin (Hb) and hematocrit were significantly lower at 24 h in HT mice compared with those in NT mice (P < 0.0001). The anemia in HT mice was probably related to intrahepatic hemorrhage, consistent with a previous report[22]. At 48 h, Hb was the same in both groups (Table 1).

Serum glucose levels were similar in HT and NT mice at 24 h. However, serum glucose levels in HT mice were significantly lower than those in NT mice at 48 h (92.9 ± 11.7 mg/dL *vs* 128.9 ± 5.6 mg/dL, P = 0.0117), which is consistent with early hepatic insufficiency[18]. The PT-INR was measured in a separate study. There was no significant difference in the PT-INR between HT and NT mice at either 24 or 48 h. However, HT mice had a trend for an increased PT-INR at 48 h (1.84 ± 0.17 *vs* 1.37 ± 0.24, P = 0.06) (Table 1).

These results collectively showed that at 24 and 48 h, HT mice had gross, histological and biochemical evidence consistent with early stages of LF after 75% PH. In contrast, NT mice showed preserved liver function.

Hypothermic mice have a diminished proliferative index after 75% PH

In mice, DNA synthesis starts at approximately 24 h and peaks at approximately 36 to 48 h after standard 2/3 PH[23]. The proliferative capability was assessed using Ki-67 and PCNA[18,24,25] (Figure 3A). Ki-67 staining showed minimal Ki-67 activity in HT and NT mice at 24 h. HT mice continued to have a minimal Ki-67 index at 48 h. In contrast, NT mice showed significantly more Ki-67 immunostaining at 48 h compared with HT mice (P < 0.0001) (Figure 3B). Using PCNA, HT mice showed no proliferative activity at 24 h but proliferative activity was observed at 48 h. There were significant differences between HT and NT mice at 24 h (P < 0.0001) (Figure 3C). In contrast, NT mice had elevated proliferative activity at 24 and 48 h compared with HT mice, indicating normal progression and proliferation of hepatocytes. The initial lack of proliferative activity in HT mice is consistent with LF[18,19,26].

Hypothermic mice lack cyclin D1 expression

Hepatocytes progress through the cell cycle to proliferate. Cyclin D1 is a marker for when hepatocytes enter the G1 phase and continue to proliferate[27,28]. In NT mice, we observed a significant increase in cyclin D1 expression at 24 h (P < 0.0001) and an even more pronounced increase in cyclin D1 at 48 h (P = 0.0002) compared with HT mice (Figure 3E). These results suggested that proliferation was normal in NT mice. In contrast, HT mice had no detectable cyclin D1 at 24 h and minimal activity at 48 h (Figure 3D). These results are consistent with LF in HT mice[26,29].

Necrosis occurs in the LR of HT mice after 75% PH

Apoptosis is prevalent in LF after extensive PH[30,31]. However, necrosis is a major mechanism of liver failure in certain models of PH[12,19,21,32]. To determine the contribution of apoptosis, we performed TUNEL, DNA laddering and caspase-3 assays. The TUNEL assay showed no differences between HT and NT mice at 24 and 48 h (Figure 4A). No DNA laddering patterns were detected at each time point (Figure 4B). No cleaved caspase-3 expression was found (Figure 4C).

There was a large amount of hemorrhagic/necrotic nodules and their total area was large throughout the liver

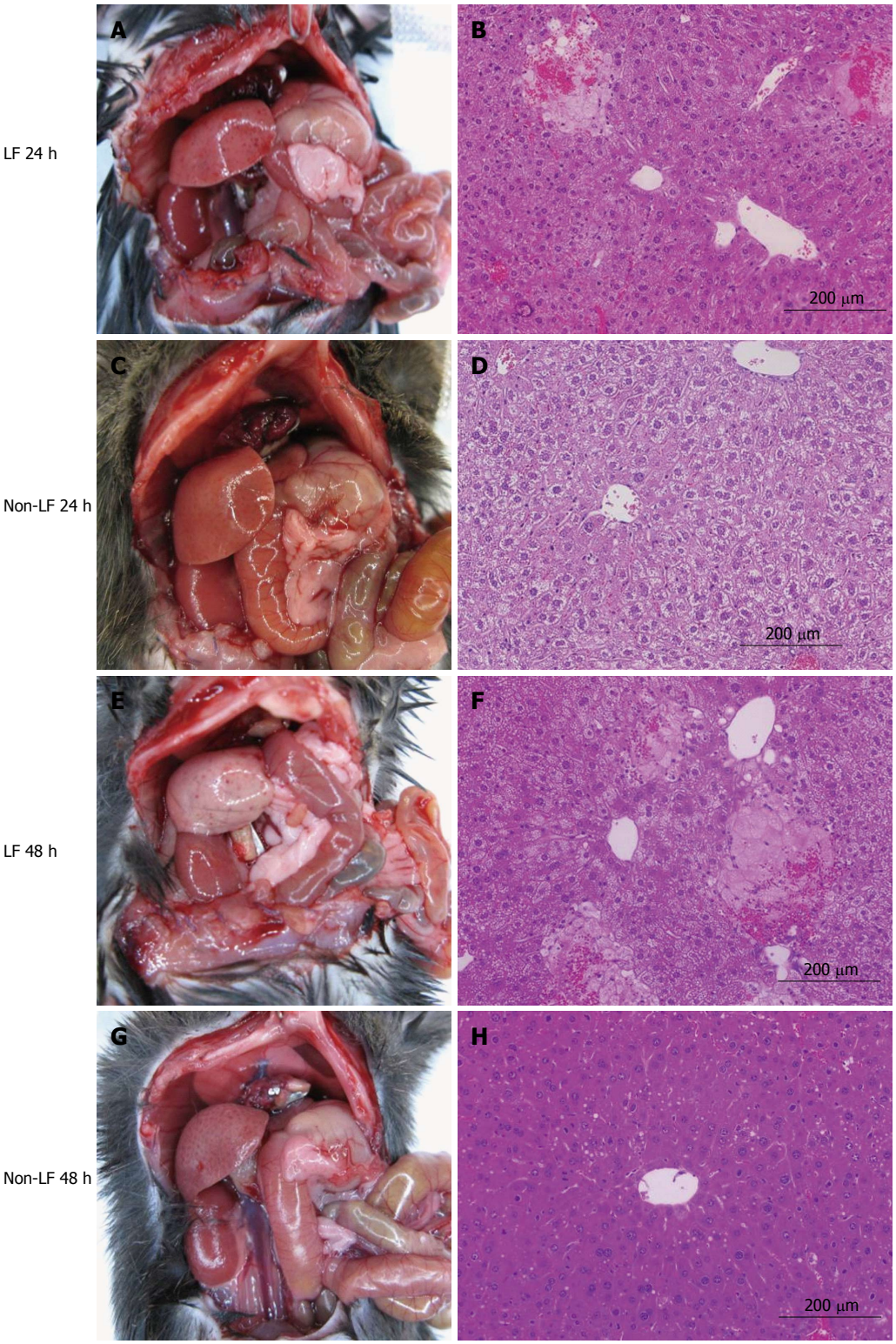

Figure 2 The gross appearance and hematoxylin-eosin-stained pathohistology of hypothermic and normothermic mice at 24 h (A-D) and 48 h (E-H) after 75% partial hepatectomy. LF: Liver failure.

in HT mice at 24 and 48 h. In contrast, minimal or no hemorrhage and necrosis were found in NT mice (Figure 5). These results suggested that necrosis but not apoptosis was the main pathway for cell death in LR, consistent with previous reports[12,19,21,32].

Signal transduction and energy production in the LR after 75% PH

By Western blot, c-Met expression was the same in both study groups at 24 h but was significantly lower in HT mice compared with that in NT mice at 48 h ($P = 0.0008$) (Figure 6). By immunohistochemistry, there was no significant difference in c-Met expression per cell between the groups, suggesting that the decrease in c-Met expression in HT mice was due to a loss of hepatocytes in the hemorrhagic and necrotic nodules.

We found no significant differences in phosphorylated levels of STAT3, Akt and MAP kinases, including

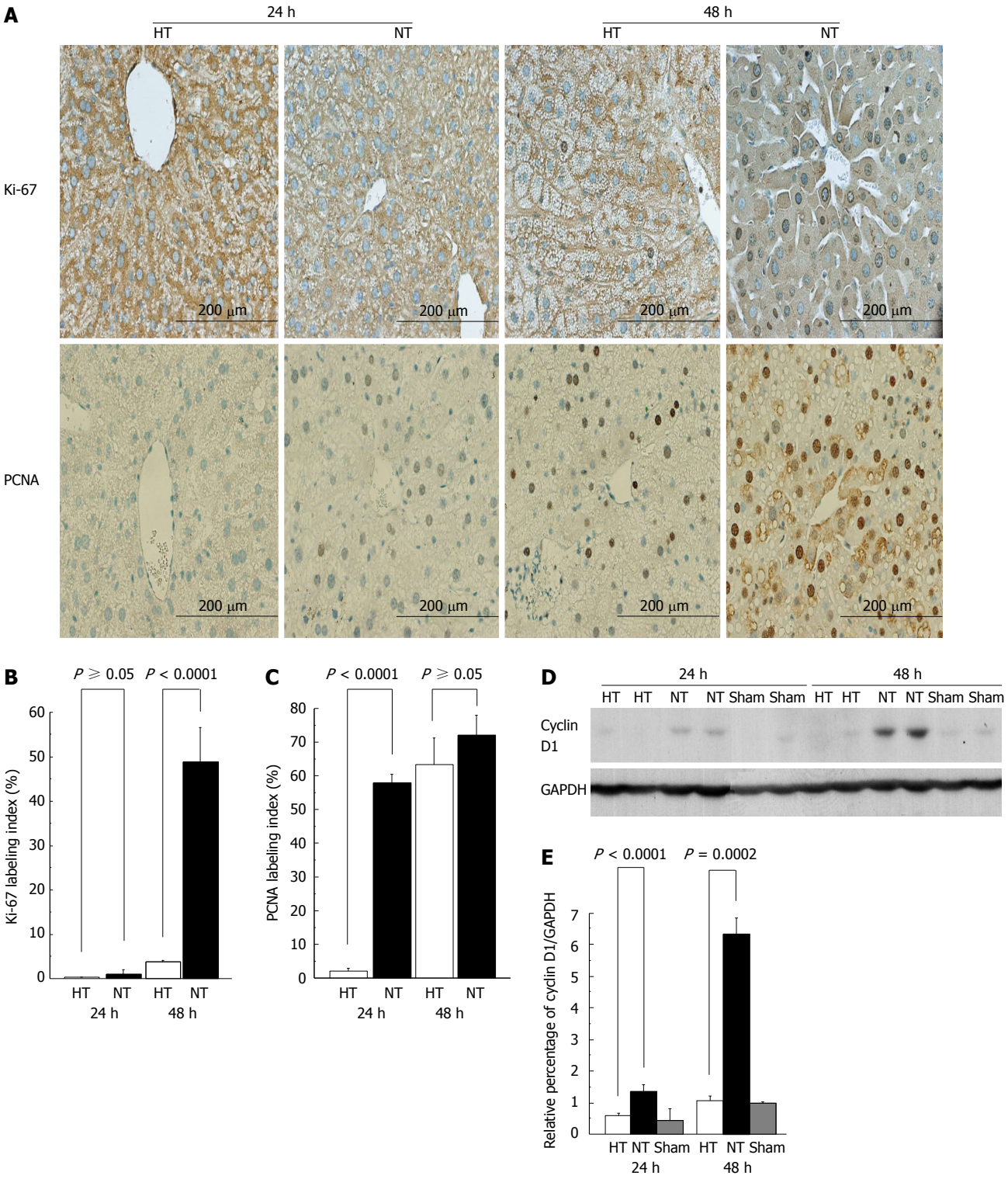

Figure 3 Hypothermic mice have a diminished proliferative index after 75% partial hepatectomy. A: Ki-67 and proliferating cell nuclear antigen (PCNA) labeling at 24 and 48 h; B: Ki-67 labeling index; C: PCNA labeling index; D: Expression of cyclin D1; E: The ratio of cyclin D1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH). HT: Hypothermic; NT: Normothermic.

ERK1/2, SAPK/JNK and p38 MAPK, between HT and NT mice at 24 and 48 h, although the levels were higher than baseline levels found in the sham animals (Figure 7). These results are consistent with a previous report demonstrating that Akt and STAT3 are elevated in LR[19]. However, an increase in signal transduction in an insufficient LR might not be adequate for liver regeneration, as previously reported[33].

We also found that ATP concentrations were significantly lower at 48 h in HT mice compared with those in NT mice ($P = 0.0100$)[19,29] but we did not observe any difference in ATP levels at 24 h ($P = 0.0758$) (Figure 8).

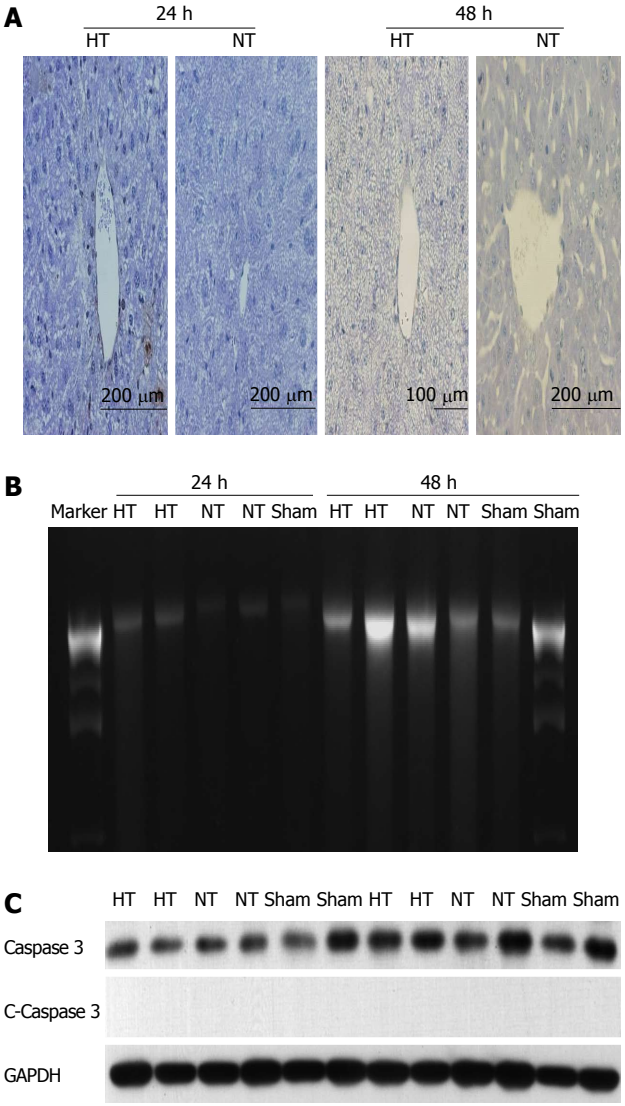

Figure 4 TdT-mediated DUTP-biotin nick end labeling, DNA laddering and caspase-3 assays to determine the contribution of apoptosis. A: The TdT-mediated DUTP-biotin nick end labeling (TUNEL) immunostaining. TUNEL assay shows no differences between hypothermic (HT) and normothermic (NT) mice at 24 and 48 h; B: The DNA laddering. No DNA laddering patterns were detected at each time point; C: Caspase-3 expression. Cleaved (C) caspase-3 is not expressed. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

We did not find a difference in cytochrome c among the groups. We also did not observe a difference in VEGF levels between the groups at 24 h but HT mice had significantly higher VEGF levels than those in NT mice at 48 h ($P < 0.0001$) (Figure 9). We also found no differences in HGF in the sera of the study mice.

DISCUSSION

The clinical indicators for early detection of LF in an insufficient LR after EH are unknown. Most current studies on the mechanisms of LF have focused only on surviving animals because there have been no reliable indicators of the onset of LF in an insufficient LR[19].

In this study, we prospectively demonstrated that hypothermia is directly correlated with biochemical, histological

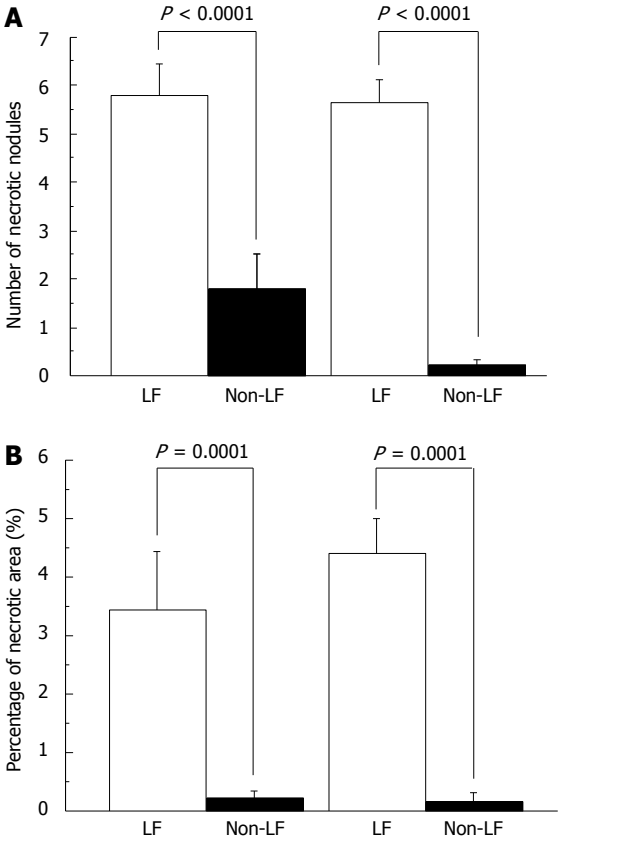

Figure 5 Necrosis occurs in the liver remnant of hypothermic mice after 75% partial hepatectomy. A: Number of necrotic nodules in a ×100 microscope field in the liver remnant (LR); B: Percentage area of necrotic nodules in a LR section. LF: Liver failure.

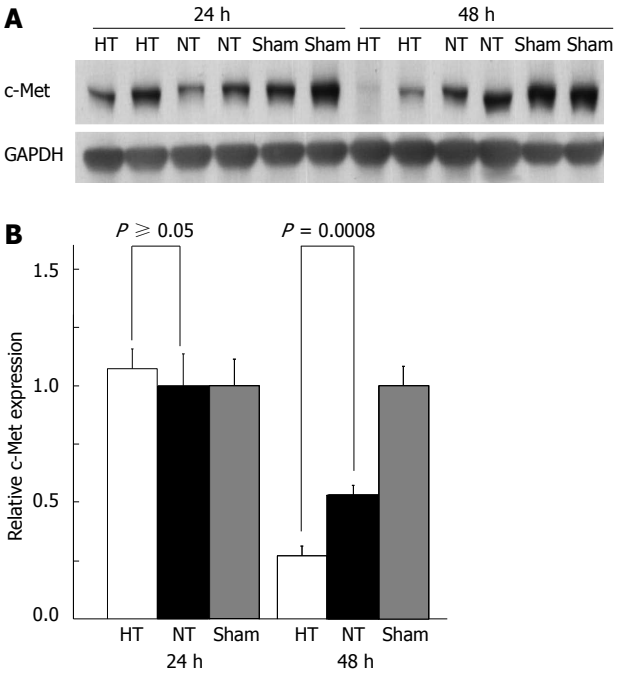

Figure 6 Signal transduction and energy production in the liver remnant after 75% partial hepatectomy. A: Expression of c-Met; B: The expression of c-Met was the same in both study groups at 24 h but it was significantly lower in hypothermic mice compared with that in normothermic mice at 48 h ($P = 0.0008$). HT: Hypothermic; NT: Normothermic; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

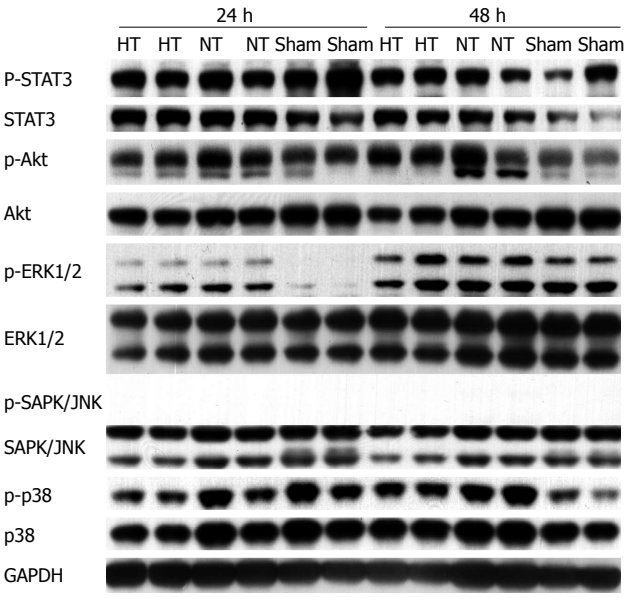

Figure 7 Actual findings of Western blot for p-STAT3, STAT3, p-AKT, AKT, p-ERK1/2,ERK1/2, p-SAPK/JNK, SAPK/JNK, p-p38, p38 and anti-glyceraldehyde-3-phosphate dehydrogenase. Using Western blot, signal transduction in the liver remnant at 24 and 48 h in hypothermic (HT) and normothermic (NT) mice was examined, with sham animals serving as controls. Although p-STAT3, p-AKT, p-ERK1/2 and p-p38 were up-regulated in the liver remnant after 75% partial hepatectomy in both HT and NT mice compared with the sham controls, there was no significant difference between the HT and NT groups. There was no change in p-SAPK/JNK. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control.

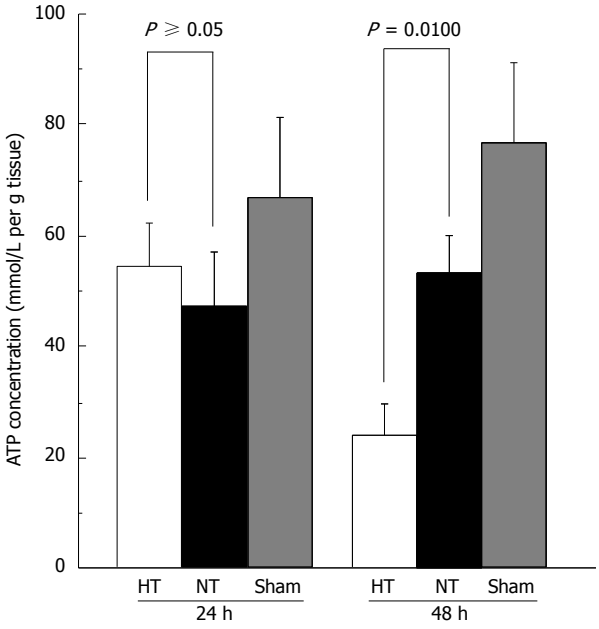

Figure 8 Adenosine triphosphate concentrations. Adenosine triphosphate concentrations were determined in the liver remnant at 24 and 48 h in hypothermic and normothermic mice. Sham animals served as controls. HT: Hypothermic; NT: Normothermic.

and molecular factors of LR after 75% PH in mice. Consistent with previous studies[12-14], HT mice in this study had ongoing LF. Their LRs appeared abnormal, showed hemorrhage and necrosis, had a severely disrupted liver

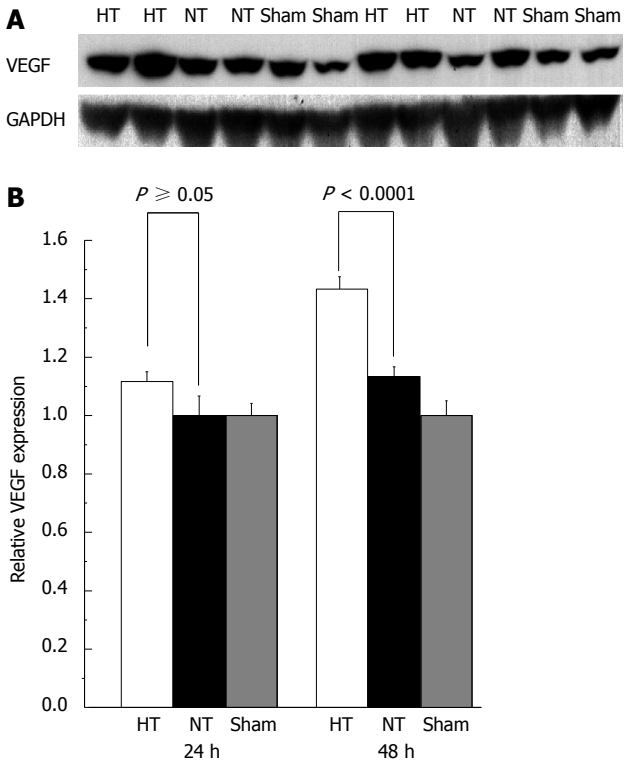

Figure 9 Vascular endothelial growth factor levels. A: Expression of vascular endothelial growth factor (VEGF); B: Hypothermic mice had significantly higher VEGF levels than those in normothermic mice at 48 h but not at 24 h. HT: Hypothermic; NT: Normothermic; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

function profile, and demonstrated markedly suppressed proliferative indices and cyclin D1. The lack of cyclin D1 in HT mice is consistent with the absence of liver regeneration and inadequate liver regeneration results in high mortality rates[29]. In addition, in our model, LF was due to hepatocellular necrosis rather than apoptosis[19,21].

Two-thirds PH (2/3 PH) in rodents is the standard model for investigating LF and hepatic regeneration[34,35]. With 2/3 PH, all animals survive. A > 70% PH is routinely performed as a model of LF. Longo *et al*[18] showed an increased mortality after 24 h with ⩾ 75% PH in mice. Emond *et al*[12] used ⩾ 90% PH in rats, resulting in 90% mortality. The rats that died after 90% PH failed to achieve a BT above 34 ℃. Moreover, maintaining normothermia by external warming does not improve survival[12]. Similarly, Eguchi *et al*[13] found suppressed LR growth and an altered biochemical liver profile in 92% PH rats and those rats had hypothermia of 29 ℃ at 30 h. Moreover, in those animals, maintaining normothermia by external warming led to increased mortality[13]. These data are consistent with our results, which collectively support the hypothesis that hypothermia is predictive of LF after EH.

In a retrospective analysis, a prothrombin time < 50% and T-Bil level > 50 μmol/L at day 5 after hepatectomy predicted a mortality rate of over 50%[36]. Similarly, a low platelet count (< 100000/μL), PT-INR >2.0 and T-Bil level > 6.6 mg/dL at 48 h were correlated with LF and

death unless a liver transplant was performed[37]. Therefore, LF is lethal and usually has reached an advanced stage when it becomes clinically and biochemically evident. Moreover, these retrospectively derived prognostic factors may not be applicable universally and remain to be validated[7].

Our findings of hemorrhagic and necrotic nodules in the liver of HT mice suggest mechanisms of injury and potential therapy similar to those previously reported[12,18,19,21,32]. For example, specific blockade of tumor necrosis factor prevents LF after EH[32]. Interleukin-6 (IL-6) might protect against oxidative injury and necrosis by promoting STAT3 and Akt pathways[19]. Mitochondrial injury and dysfunction with increased free radical production could be a focus of therapy[21,29]. In addition, portal hyperperfusion is implicated in an insufficient LR and small-for-size graft (SFSG)[38,39]. Attenuating portal hyperperfusion has been shown to ameliorate liver dysfunction in residual extreme small LRs[40], as well as in liver transplantation with SFSGs[41-44]. However, the exact mechanisms for LR in an insufficient LR remain incompletely understood. The role of free radical scavengers, tumor necrosis factor blockage, IL-6 and portal diversion in clinical practice require further evaluation.

After 75% PH, MAPK cascades were activated above baseline levels in the sham-operated mice in this study but we did not observe any difference in the MAPK cascades, including MAPK p38, ERK1/2, JNK, Akt and STAT3, in HT and NT mice. The lack of liver regeneration in HT mice in the presence of activated MAPK cascades indicates severe hepatic dysfunction, as previously suggested[33]. These results suggest that understanding the mechanisms responsible for hemorrhage and necrosis, which might be independent of MAPKs, is essential for preventing and treating LF.

Our study is limited by a potentially confounding factor, namely, whether hypothermia was the cause rather than the result of the observed LF. It has been suggested that hypothermia impedes liver regeneration[45,46]. However, hypothermia is a well-known sequel of acute LF[10]. Mild hypothermia can ameliorate brain edema as well as liver injury in acute LF[46-48]. In our study, we monitored BT prospectively and separated the study animals by BT before the animals became moribund[19]. Although we could not completely rule out the potential contribution of hypothermia as a cause of LF after EH, the prospective characterizations of the mice in this study indicate that hypothermia has an important role as an early indicator of LF.

In conclusion, hypothermia after EH portends impending LF. Hypothermia might be a clinically useful marker for investigators to focus on the molecular pathways that are important during the early development of LF in an insufficient LR. Further study to validate the role of hypothermia as a predictor of LF in an insufficient LR is warranted.

ACKNOWLEDGMENTS

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COMMENTS

Background

Liver failure occurs in various conditions, including acute liver failure and an insufficient liver remnant after extended hepatectomy. Without a suitable liver replacement, death imminently ensues. Hepatic failure can have lethal consequences but the mechanisms responsible for liver failure in an insufficient liver remnant are unknown.

Research frontiers

In early studies, terminal liver failure was associated with hypothermia. Hypothermia is also common in advanced stages of acute liver failure. Similarly, earlier studies that conducted extended hepatectomy in rodent models demonstrated that the animals that failed to survive after extended hepatectomy had significant hypothermia, whereas those that survived maintained normothermia.

Innovations and breakthroughs

In the laboratory, preliminary results showed that mice which failed to survive after extended hepatectomy had significant hypothermia and the surviving animals remained normothermic. Therefore, the authors hypothesized that hypothermia predicts imminent liver failure after extended hepatectomy. In this study, the authors investigated body temperature after extended hepatectomy and evaluated the hypothermia as a predictor of liver failure after extended hepatectomy.

Applications

Hypothermia might be a clinically useful predictor for the early development of liver failure in an insufficient liver remnant after extended hepatectomy.

Terminology

Here, the authors focused only on the liver surgery field. In the near future, the authors will investigate the impact of hypothermia on the liver failure in the split liver transplantation model with portal hypertension and cold ischemia/warm reperfusion injury.

Peer review

There is merit in describing the differences between hypothermic and normothermic mice in terms of the pathological, biochemical and immunological findings, which are novel. The manuscript is well-written.

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ORIGINAL ARTICLE

Platelet count and sustained virological response in hepatitis C treatment

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Abstract

AIM: To examine the epidemiological data, hematological safety and treatment responses of peginterferon-alpha 2a plus ribavirin therapy for hepatitis C.

METHODS: Between March 2008 and February 2011, 196 hepatitis C virus (HCV) genotype 1 infected Japanese (127 treatment-naive and 69 treatment-experienced patients) patients treated with peginterferon-alpha 2a plus ribavirin were enrolled. We examined the epidemiological data and treatment responses were retrospectively analyzed in terms of hematological safety. HCV RNA was measured by the COBAS TaqMan HCV test.

RESULTS: Overall sustained virological response (SVR) rates of treatment-naive and treatment-experienced patients were 56% and 39%, respectively. Multivariate logistic regression analysis showed that SVR was attained independently of early virological response in both treatment-naive and treatment-experienced patients. SVR rates did not differ between the pretreatment hemoglobin < 13 g/dL and ⩾ 13 g/dL groups. However, in treatment-naive patients, the SVR rate of the pretreatment platelet count < 130000/μL group was significantly lower than that of the pretreatment platelet count ⩾ 130000/μL group.

CONCLUSION: Attention should be paid to potential thrombocytopenia in the treatment of chronic hepatitis C patients.

Key words: Anemia; Antiviral treatment; Chronic hepatitis C; Platelet count; Sustained virological response

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection leads to cirrhosis and hepatocellular carcinoma[1]. The combination of pegylated interferon alpha-2a or alpha-2b plus ribavirin is the standard of care (SOC) for HCV-infected patients[2]. This therapy leads to sustained virological response (SVR) in approximately 50% of patients[2]. In 2011, two HCV NS3/4A protease inhibitors, boceprevir and telaprevir, became available for HCV genotype 1 patients in United States and some other countries[3-6]. The addition of boceprevir or telaprevir to standard therapy with pegylated interferon plus ribavirin, compared with standard therapy alone, significantly increased the SVR rates in patients infected with HCV genotype 1[3-6].

Thrombocytopenia occasionally accompanies advanced chronic liver diseases[7] and is associated with the natural history of HCV infection and anti-viral therapy[8]. Thrombocytopenia is also one of the major obstacles when treating patients infected with HCV by pegylated interferon plus ribavirin with or without direct-acting antivirals, including boceprevir and telaprevir[9,10]. Diagnosis of thrombocytopenia in chronic hepatitis C patients is associated with increased incidences of certain comorbidities, complications and medical interventions, significantly increasing medical resource utilization[11].

Genome-wide association studies have recently revealed that interleukin 28B (IL28B) single nucleotide polymorphisms are significantly associated with the response to pegylated interferon-alpha plus ribavirin therapy for chronic hepatitis C[12-15] and that inosine triphosphatase (*ITPA*) gene variant protects against anemia during pegylated interferon-alpha plus ribavirin therapy for chronic hepatitis C[16]. However, severe hemoglobin decline, which is mainly found in ITPA-CC patients, was inversely correlated with thrombocytopenia, contributing to the association between severe anemia and a relative reactive increase in platelet count[17,18].

It is well known that improved adherence to medication will favorably affect SVR rates in pegylated interferon-alpha 2a plus ribavirin therapy for chronic hepatitis C[19,20]. Because the use of erythropoietin or hematopoietic growth factors was not allowed in these treatments by Japanese health insurance plans, hematological adverse events are the most common laboratory abnormalities, leading to dose modification or discontinuation. In the present study, we retrospectively analyzed the epidemiological data and treatment responses were retrospectively analyzed in terms of hematological safety.

MATERIALS AND METHODS

Patients

From March 2008 through October 2011, patients were recruited from Chiba University and 30 hospitals in Chiba, Ibaraki and Saitama prefectures[21-23]. Patients were eligible if they met the following inclusion criteria: (1) infected with HCV genotype 1 alone; (2) age $\geqslant$ 20 years; (3) diagnosis of chronic hepatitis C based on positive HCV RNA; (4) negative for HBs antigen; (5) negative for human immunodeficiency viral antibody; (6) no high autoantibody titers; (7) no severe renal disease; (8) no severe heart disease; (9) no mental disorders; (10) no current intravenous drug abuse; and (11) no pregnancy[21].

Study design

The design of this study has been partly described[21-23]. 196 patients, who could be judged with SVR or non-SVR, were enrolled. In this study, 180 μg of pegylated interferon-alpha 2a per week plus 400-1200 mg of ribavirin daily comprised the usual treatment protocol for as long as 48 or 72 wk. Clinical and laboratory assessments were performed at least every 4 wk during treatment and a 24 wk follow-up period[22]. Adverse reactions were documented by oral inquiry, physical examinations and laboratory tests.

Measurement of HCV RNA in serum

The COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan), with a range from 1.2 to 7.8 log IU/mL, was used for the measurement of HCV RNA levels every 4 wk before, during and for 24 wk after the end of treatment.

Measurement of serum alanine aminotransferase levels, other liver function and hematological tests

Serum alanine aminotransferase, other liver function and hematological tests were carried out by standard methods every 4 wk before, during and for 24 wk after the end of treatment.

Definition of treatment response

SVR was defined as undetectable serum HCV RNA at 24 wk after the end of treatment. Patients with undetectable HCV RNA within the initial 4 wk of treatment were considered to have demonstrated a rapid virological response (RVR). Patients who had undetectable HCV RNA within the initial 12 wk of treatment were considered to have had a complete early virological response (cEVR) (described as EVR here).

Ethics

This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The Ethics Committee of Chiba University School of Medicine approved the study protocol. Informed consent was obtained from all patients prior to enrollment.

Statistical analysis

Data were expressed as mean ± SD. Differences were evaluated by Student's *t* test, χ^2 test or Fisher's exact test. $P < 0.05$ was considered statistically significant. Multivariate logistic regression analysis was used to determine

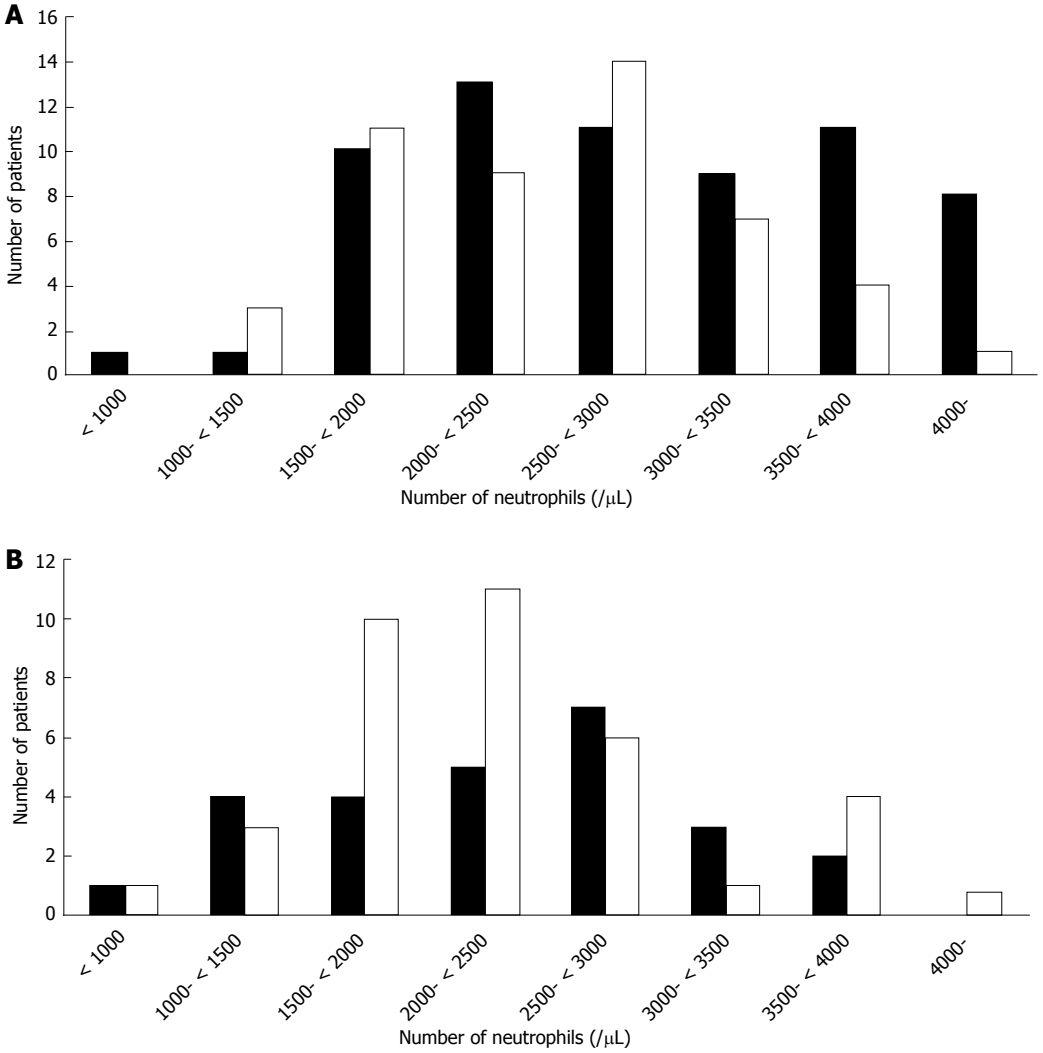

Figure 1 Distribution of pre-treatment neutrophil counts in treatment-naive and treatment-experienced patients. A: Results from 64 sustained virological response (SVR, black column) and 49 non-SVR (white column) of 113 treatment-naive patients are shown; B: Results from 26 SVR (black column) and 41 non-SVR (white column) of 67 treatment-experienced patients are shown.

Table 1 Clinical characteristics of chronic hepatitis C patients treated with pegylated interferon alpha-2a plus ribavirin in the present study

	Previous treatment		*P* value[1]
	(-)	(+)	
Number of patients	127	69	
Age (yr)	56.1± 10.7	59.0 ± 10.1	0.064
Gender (male/female)	62/65	34/35	NS
Body mass index (kg/m^2)	23.4 ± 3.2	23.3 ± 3.9	NS
LDL cholesterol (mg/dL)	107 ± 52.3	102 ± 31.5	NS
ALT (IU/L)	72.0 ± 53.7	66.4 ± 48.5	NS
Gamma-glutamyl transferase (IU/L)	55.6 ± 74.8	67.0 ± 77.2	NS
Alpha-fetoprotein (ng/mL)	12.7 ± 30.0	25.9 ± 79.5	NS
HCV RNA (log IU/mL)	6.5 ± 0.7	6.4 ± 0.7	NS
White blood cells (/mL)	5213 ± 1519	4619 ± 1273	0.006
Neutrophils (/mL)	2752 ± 924	2474 ± 981	0.058
Hemoglobin (g/dL)	14.0 ± 1.4	13.7 ± 1.6	NS
Platelets (× 10^4/mL)	16.5 ± 5.0	15.6 ± 5.2	NS
RVR (+/-)	18/109	7/62	NS
EVR (+/-)	66/61	24/45	0.021

Values are expressed as mean ± SD. [1]*P* value indicates those between two groups with and without pretreatment by Student's *t* test or χ^2 test. NS: Not significant; LDL: Low-density lipoprotein; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; RVR: Rapid virological response; EVR: Early virological response.

factors that significantly contributed to SVR. Statistical analysis was performed using the Excel statistics program for Windows ver.7 (SSRI, Tokyo, Japan) and DA Stats software (O. Nagata, Nifty Serve: PAF01644).

RESULTS

Patients' baseline background factors

Baseline characteristics of the patients are shown in Figure 1 and Table 1. Of 196 patients, 127 were treatment-naive and 69 had a history of interferon therapy with or without ribavirin. Higher HCV viral load (HCV RNA ⩾ 5.0 log IU/mL) was seen in 95.2% (121/127) treatment-naive and 98.5% (68/69) treatment-experienced patients. In the 69 patients previously treated, 4 had received pegylated interferon-alpha 2a monotherapy, 14 standard interferon monotherapy, 6 standard interferon plus ribavirin, 38 pegylated interferon-alpha 2b plus ribavirin, 2 pegylated interferon-alpha 2a plus ribavirin, and 5 with unknown details. Concerning the virological response of the 69 patients to their previous treatment, 25 were relapsers, 26 were null-responders, and 18 were unknown. In the present study, of the 127 treatment-naive patients, 89 and 38 patients were treated for as long as 48 and 72

Table 2 Comparison of factors between chronic hepatitis C patients with and without sustained virological response in the present study

	Previous treatment					
	(-)			(+)		
	SVR	Non-SVR	*P* value[1]	SVR	Non-SVR	*P* value[1]
Number of patients	72	55		27	42	
Age (yr)	54.6 ± 10.9	58.0 ± 10.3	NS	58.5 ± 9.9	59.3 ± 10.3	NS
Gender (male/female)	41/31	21/34	0.036	11/16	23/19	NS
Body mass index (kg/m^2)	23.4 ± 3.2	23.3 ± 3.9	NS	23.6 ± 3.6	22.8 ± 2.6	NS
LDL cholesterol (mg/dL)	107 ± 52.3	102 ± 31.5	NS	100 ± 31.3	92.8 ± 26.7	NS
ALT (IU/L)	67.6 ± 42.0	77.8 ± 65.9	NS	52.9 ± 35.4	75.1 ± 54.0	NS
Gamma-glutamyl transferase (IU/L)	45.1 ± 43.1	69.8 ± 102	NS	50.2 ± 51.1	76.9 ± 88.0	NS
Alpha-fetoprotein (ng/mL)	8.8 ± 9.7	17.4 ± 43.1	NS	13.3 ± 26.3	32.6 ± 96.5	NS
HCV RNA (log IU/mL)	6.4 ± 0.7	6.6 ± 0.6	NS	6.3 ± 0.9	6.4 ± 0.6	NS
White blood cells (/mL)	5363 ± 1582	5015 ± 1423	NS	4561 ± 1175	4657 ± 1345	NS
Neutrophils (/mL)	2910 ± 1006	2546 ± 767	NS	2337 ± 813	2562 ± 1074	NS
Hemoglobin (g/dL)	14.2 ± 1.5	13.8 ± 1.4	NS	13.8 ± 1.1	13.7 ± 1.9	NS
Platelets (× 10^4/mL)	17.5 ± 4.9	15.3 ± 4.8	0.013	16.5 ± 4.9	15.0 ± 5.3	NS
RVR (+/-)	18/54	0/55	< 0.001	7/20	0/42	< 0.001
EVR (+/-)	56/16	10/45	< 0.001	8/19	5/37	< 0.001

Values are expressed as mean ± SD. [1]*P* value indicates those between two groups with and without Sustained virological response (SVR) by Student's *t* test or χ^2 test. NS: Not significant; LDL: Low-density lipoprotein; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; RVR: Rapid virological response; EVR: Early virological response.

wk, respectively, and of the 69 treatment-experienced patients, 36 and 33 patients were treated for as long as 48 and 72 wk, respectively.

Characteristics of SVR and non-SVR patients

In the 127 treatment-naive patients, SVR was achieved in 56.6% (72/127) and non-SVR was seen in 43.3% (55/127) (27 relapsers, 11 null-responders and 17 stopped treatment due to adverse events) (Table 2). In these treatment-naive patients, there were significantly more male patients and higher neutrophil and platelet counts in the SVR group than in the non-SVR group at baseline (Table 2). Lower HCV viral load (HCV RNA < 5.0 log IU/mL) was seen in 8.3% (6/72) treatment-naive and 0% (0/55) treatment-experienced patients. RVR and EVR were significantly higher in the SVR group [25.0% (18/72) and 77.7% (56/72), respectively] than in the non-SVR group [0% (0/55) and 18.1% (10/45), respectively].

In the 69 patients previously treated, SVR was achieved in 39.1% (27/69) and non-SVR was seen in 60.8% (42/69) (23 relapsers, 14 null-responders and 5 stopped treatment due to adverse events) (Table 2). In these previously treated patients, the baseline backgrounds between the SVR and non-SVR groups did not differ (Table 2). RVR and EVR were significantly higher in the SVR group [25.9% (7/27) and 70.3% (19/27), respectively] than in the non-SVR group [0% (0/42) and 11.9% (5/42), respectively].

Multivariate analysis showed that EVR was significantly associated with SVR in treatment-naive patients and in treatment-experienced patients. For the EVR in the treatment-naive patients, odds ratio (OR) is 15.01 (95%CI: 5.72-44.56, $P < 0.001$); for the EVR in the treatment-experienced patients, OR is 21.7 (95%CI: 6.12-96.35, $P < 0.001$). Category is the same in the two groups. In treatment-naive patients, platelet count tended to be an independent factor in multivariate analysis. For the platelet counts, category > 16.1 × 10^4/μL, OR is 2.79 (95%CI: 0.98-8.57, $P = 0.061$).

Effects of anemia on SVR

Hematological adverse events are the most common laboratory abnormalities leading to dose modification or discontinuation[24]. First, we examined the effects of hemoglobin at baseline on the SVR rates.

In the 127 treatment-naive patients, there were no differences in SVR rates between the hemoglobin < 13 g/dL and hemoglobin ≥ 13 g/dL groups [55.2% (16/29) and 57.1% (56/98), respectively]. We also did not observe any difference in SVR rates between the hemoglobin < 13 g/dL and hemoglobin ≥ 13 g/dL groups in 48 wk treatment [55.6% (10/18) and 57.7% (41/71), respectively] or 72 wk treatment [54.5% (6/11) and 55.6% (15/27), respectively] in these patients.

In the 69 previously treated patients, there were no differences in SVR rates between the hemoglobin < 13 g/dL and hemoglobin ≥ 13 g/dL groups [30.0% (6/20) and 42.9% (21/49), respectively]. We also did not observe any difference in SVR rates between the hemoglobin < 13 g/dL and hemoglobin ≥ 13 g/dL groups in 48 wk treatment [38.5% (5/13) and 39.1% (9/23), respectively] or 72 wk treatment [14.3% (1/7) and 46.2% (12/26), respectively] ($P = 0.126$) in these patients.

Effects of thrombocytopenia on SVR

Next, we examined the effects of platelet counts at baseline on the SVR rates. In the 127 treatment-naive patients, the SVR rate of the platelet count < 130000/μL group

Table 3 Treatment outcomes in treatment-naive and treatment-experienced patients according to platelet counts at baseline

	< 130000/μL	⩾ 130000/μL	*P* value
Proportion of SVR-archived in treatment-naive patients			
Total patients	10/27 (37.0%)	62/100 (62.0%)	0.020
48 wk treatment	7/20 (35.0%)	44/69 (63.8%)	0.022
72 wk treatment	3/7 (42.9%)	18/31 (58.1%)	NS
Treatment-experienced patients			
Total patients	7/24 (29.2%)	20/45 (44.4%)	NS
48 wk treatment	7/20 (33.3%)	9/21 (42.9%)	NS
72 wk treatment	2/9 (22.2%)	11/24 (45.8%)	NS

NS: Not significant; SVR: sustained virological response.

[37.0% (10/27)] was significantly lower than that of the platelet count ⩾ 130000/μL group [62.0% (62/100)] (Table 3). We also observed a significantly lower SVR rate in the platelet count < 130000/μL group [35.0% (7/20)] than in the platelet count ⩾ 130000/μL group [63.8% (44/69)] with 48 wk treatment. The RVR rate of the platelet count < 130000/μL group [15.0% (3/20)] was similar to that of the platelet count ⩾ 130000/μL group [18.8% (13/69)] with 48 wk treatment, but the EVR rate of the platelet count < 130000/μL group [30.0% (6/20)] was significantly lower than that of the platelet count ⩾ 130000/μL group [69.5% (48/69)] with 48 wk treatment ($P = 0.0033$). In contrast, there were no differences in SVR rates between the platelet count < 130000/μL [42.9% (3/7)] and ⩾ 130000/μL groups with 72 wk treatment [58.1% (18/31)] (Table 3). The RVR and EVR rates of the platelet count < 130000/μL group [14.2% (1/7) and 57.1% (4/7), respectively] were similar to those of the platelet count ⩾ 130000/μL group [3.2% (1/31) and 25.8% (8/31), respectively] with 72 wk treatment.

In the 69 previously treated patients, there were no differences in SVR rates between the platelet count < 130000/μL and ⩾ 130000/μL groups [29.2% (7/24) and 44.4% (20/45), respectively] (Table 3). We also did not observe any differences in SVR rates between the platelet count < 130000/μL and ⩾ 130000/μL groups with 48 wk [33.3% (5/15) and 42.9% (9/21), respectively] or 72 wk treatment [22.2% (2/9) and 45.8% (11/24), respectively] in these patients (Table 3).

DISCUSSION

After HCV NS3/4A protease inhibitors began to be used in clinical practice, resulting side effects of their application for chronic hepatitis C were also expected to appear[6,24]. In fact, hematological adverse events became the most common laboratory abnormalities, leading to dose modifications or even discontinuation during SOC for chronic hepatitis C[25-30]. In the present study, we observed that EVR was significantly associated with SVR in both treatment-naive and treatment-experienced patients. According to multivariate analysis, RVR was not associated with SVR in either of the patient types, although the reason for this might be that RVR was obtained in only 25 patients. We also examined the epidemiological data and treatment responses were retrospectively analyzed in terms of hematological safety. We observed that the SVR and EVR rates of the platelet count ⩾ 130000/μL group were better than those of the platelet count < 130000/μL group in treatment naive-patients (Table 3).

Unexpectedly, we did not observe any difference in SVR rates between the hemoglobin < 13 and ⩾ 13 g/dL groups (see Results section) or according to neutrophil counts (Figure 1), although the hemoglobin level or WBC level was supposedly an important factor affecting adherence to treatment. We did not observe any association between baseline platelet count below 130000/μL and SVR in the treatment-experienced patients in the present study (Table 3). It may be possible that thrombocytopenia reflects the fact that patients with low platelet counts are more prone to being cirrhotic and therefore should have a lower response rate to therapy.

The significant lower SVR in patients with baseline platelet counts below 130000/μL in treatment-naive patients treated for 48 wk, but not for 72 wk, likely reflects the major role of liver stage in patients with presumably favorable viral kinetics (considering that patients treated for 48 wk will have had an EVR), whereas in patients with slower decay of viral load and presumably treated for 72 wk, liver stage could have had a lower impact on SVR. In the present study, liver biopsy was performed in 67 patients and fibrosis stages 1, 2, 3 and 4 were seen in 30, 11, 5 and 3 of the platelet count ⩾ 130000/μL group and 3, 7, 6, 2 of the < 130000/μL group, respectively. We also observed that 3 of 5 cirrhotic patients obtained SVR. Perhaps IL28B polymorphism played a major role in these patients, possibly explaining why some cirrhotics achieved SVR. Further studies will be needed to clarify this point.

So far, anemia and neutropenia are well-recognized effects of higher-dose peginterferon alpha plus ribavirin regimens, but it was also reported that no patient had to discontinue therapy owing to thrombocytopenia[27]. We observed a greater number of lower SVR rates in the low platelet group [35.2% (12/34); $P = 0.037$] of the higher hemoglobin group than in the high platelet group [57.5% (65/113)] of the higher hemoglobin group (hemoglobin ⩾ 13 g/dL). We also observed that lower SVR rates tended to occur more in the low platelet group [29.4% (5/17)] than in the high platelet group [53.1% (17/32)] of the lower hemoglobin group (hemoglobin < 13 g/dL). Thus, the present study suggested that thrombocytopenia is an important factor for SVR.

In the present study, we also experienced only two treatment-naive patients discontinuing treatment due to neutropenia. One was a 70-year old male who discontinued treatment at 1 wk after its commencement, and the other was a 51-year old female who discontinued treatment at 9 wk (Figure 1). Further study will be needed as the numbers of samples in the current study were limited.

In conclusion, SVR was attained independently of EVR in both treatment-naive and treatment-experienced patients. The SVR rates between the pretreatment hemoglobin < 13 and ⩾ 13 g/dL groups did not differ. However, in treatment-naive patients, the SVR rate of the pretreatment platelet count < 130000/μL group was significantly lower than that of the pretreatment platelet count ⩾ 130000/μL group. Patients with low platelet counts were subject to dose and/or treatment duration reductions. In fact, if these subjects required such treatment adjustments, this may provide a partial explanation for the difference in SVR rates between naive and experienced patients. Attention should be paid to thrombocytopenia in the treatment of chronic hepatitis C patients.

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COMMENTS

Background

It is well known that improved adherence to medication will favorably affect sustained virological response (SVR) rates in peginterferon-alpha 2a plus ribavirin therapy for chronic hepatitis C. Because the use of erythropoietin or hematopoietic growth factors was prohibited in these treatments by Japanese health insurance plans, hematological adverse events are the most common laboratory abnormalities, leading to dose modification or discontinuation.

Research frontiers

Peginterferon-alpha 2a plus ribavirin therapy for chronic hepatitis C leads to hematological adverse events, some of which are unknown. The authors examined the epidemiological data and treatment responses were retrospectively analyzed in terms of hematological safety. In this study, the authors demonstrate that attention should be paid to potential thrombocytopenia in the treatment of chronic hepatitis C patients.

Innovations and breakthroughs

Recent reports have highlighted the importance of inosine triphosphatase gene variants that protect against anemia in patients treated for chronic hepatitis C. In treatment-naive patients, the SVR rate of the pretreatment platelet count < 130000/μL group was significantly lower than that of the pretreatment platelet count ⩾ 130000/μL group. The authors also observed that 3 of 5 biopsy-proven cirrhotic patients obtained SVR.

Applications

With the use of standard of care, attention should be paid to thrombocytopenia in the treatment of chronic hepatitis C patients.

Peer review

It is well known that improved adherence to medication will favorably affect SVR rates in pegylated interferon-alpha 2a plus ribavirin therapy for chronic hepatitis C. In the present study, the authors retrospectively analyzed the epidemiological data and treatment responses were retrospectively analyzed in terms of hematological safety. In treatment-naive patients, the SVR rate of the pretreatment platelet count < 130000/μL group was significantly lower than that of the pretreatment platelet count ⩾ 130000/μL group, despite the existence of cirrhosis. Of particular interest was the fact that the results suggested that attention should be paid to thrombocytopenia in the treatment of chronic hepatitis C patients.

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ORIGINAL ARTICLE

CD14 upregulation as a distinct feature of non-alcoholic fatty liver disease after pancreatoduodenectomy

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Abstract

AIM: To investigate the pathogenesis of non-alcoholic fatty liver disease (NAFLD) after pancreatoduodenectomy (PD).

METHODS: A cohort of 82 patients who underwent PD at Okayama University Hospital between 2003 and 2009 was enrolled and the clinicopathological features were compared between patients with and without NAFLD after PD. Computed tomography (CT) images were evaluated every 6 mo after PD for follow-up. Hepatic steatosis was diagnosed on CT when hepatic attenuation values were 40 Hounsfield units. Liver biopsy was performed for 4 of 30 patients with NAFLD after PD who consented to undergo biopsies. To compare NAFLD after PD with NAFLD associated with metabolic syndrome, liver samples were obtained from 10 patients with NAFLD associated with metabolic syndrome [fatty liver, n = 5; non-alcoholic steatohepatitis (NASH), n = 5] by percutaneous ultrasonography-guided liver biopsy. Double-fluorescence immunohistochemistry was applied to examine CD14 expression as a marker of lipopolysaccharide (LPS)-sensitized macrophage cells (Kupffer cells) in liver biopsy specimens.

RESULTS: The incidence of postoperative NAFLD was 36.6% (30/82). Univariate analysis identified cancer of the pancreatic head, sex, diameter of the main pancreatic duct, and dissection of the nerve plexus as factors associated with the development of NAFLD after PD. Those patients who developed NAFLD after PD demonstrated significantly decreased levels of serum albumin, total protein, cholesterol and triglycerides compared to patients without NAFLD after PD, but no glucose intolerance or insulin resistance. Liver biopsy was performed in four patients with NAFLD after PD. All four patients showed moderate-to-severe steatosis and NASH was diagnosed in two. Numbers of cells positive for CD68 (a marker of Kupffer cells) and CD14 (a marker of LPS-sensitized Kupffer cells) were counted in all biopsy specimens. The number of CD68+ cells in specimens of NAFLD after PD was significantly increased from that in specimens of NAFLD associated with metabolic syndrome specimens, which indicated the presence of significantly more Kupffer cells in NAFLD after PD than in NAFLD associated with metabolic syndrome. Similarly, more CD14+ cells, namely, LPS-sensitized Kupffer cells, were observed in NAFLD after PD than in NAFLD associated with metabolic syndrome. Regarding NASH, more CD68+ cells and CD14+ cells were observed in NASH after PD specimens than in NASH associated with metabolic syndrome. This showed that more Kupffer cells and more LPS-sensitized Kupffer cells were present in NASH after PD than in NASH associated with metabolic syndrome. These observations suggest that

after PD, Kupffer cells and LPS-sensitized Kupffer cells were significantly upregulated, not only in NASH, but also in simple fatty liver.

CONCLUSION: NAFLD after PD is characterized by both malnutrition and the up-regulation of CD14 on Kupffer cells. Gut-derived endotoxin appears central to the development of NAFLD after PD.

Key words: Non-alcoholic fatty liver disease; Pancreatoduodenectomy; CD14; Endotoxin; Kupffer cells

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INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) associated with metabolic syndrome is increasing worldwide[1,2]. NAFLD associated with metabolic syndrome is considered to be associated with chronic over-nutrition that results in the accumulation of visceral fat and obesity and is one of the most common forms of chronic liver disease[3]. NAFLD associated with metabolic syndrome is related to states such as severe obesity, impairment of glucose tolerance and hyperlipidemia[4]. Indeed, several studies have shown strong relationships among NAFLD associated with metabolic syndrome, hepatic insulin resistance and type 2 diabetes mellitus[5-7].

On the other hand, NAFLD sometimes develops after pancreatoduodenectomy (PD)[8]. Tanaka *et al*[9] recently reported that NAFLD after PD was characterized by non-obesity and a lack of both hyperlipidemia and insulin resistance. The pathogenesis of NAFLD after PD may thus differ from the pathogenesis of NAFLD associated with metabolic syndrome.

Recently, Tilg *et al*[10] proposed a new model, suggesting that many hits may act in parallel to finally result in liver inflammation, with gut and adipose tissue-derived factors in particular playing a central role. It was reported that the clinical features of the patients with NAFLD after PD are similar to those found in the murine non-alcoholic steatohepatitis (NASH) model induced by a methionine-choline-deficient (MCD) diet[9]. In mice fed the MCD diet, portal endotoxemia due to an impaired gut barrier caused by the MCD diet was observed[11]. We therefore hypothesized that gut-derived lipopolysaccharide (LPS) was associated with NAFLD after PD. LPS might trigger the release of inflammatory cytokines from Kupffer cells, in turn mediating severe hepatic steatosis and liver injury after PD. The process by which LPS activates Kupffer cells seems to be mediated by LPS-binding protein, CD14 and toll-like receptor 4[12]. We therefore focused on the CD14 expression on Kupffer cells from liver specimens in cases of NAFLD after PD.

We examined the prevalence, clinical and histological features, and expression of CD14 as a marker of LPS-sensitized Kupffer cells in liver tissues obtained from patients with either NAFLD associated with metabolic syndrome or NAFLD after PD.

MATERIALS AND METHODS

Between February 2003 and August 2009, a total of 100 patients underwent PD at Okayama University Hospital, Okayama, Japan. Of these, 18 patients were excluded from the study because they were unavailable for regular follow-up computed tomography (CT) of the abdomen. The remaining 82 patients (49 men and 33 women) were enrolled in this study. There were no patients with preoperative NAFLD, based on CT and laboratory findings. The mean age at the time of surgery was 63 years (range 31-85 years). Histological diagnosis was pancreatic carcinoma in 32 patients, intraductal papillary-mucinous neoplasm in 27, bile duct carcinoma in 3, and others in 20. In terms of surgical procedures, conventional PD was employed for 73 patients and pylorus-preserving PD for 9 patients. For patients with adenocarcinoma of the pancreatic head, we routinely performed dissection of the nerve plexus around the superior mesenteric artery (SMA), leaving the left side of the SMA near the origin intact. A modification of Child's method was employed for digestive reconstruction.

Body mass index (BMI) was determined for all patients and blood examinations were performed before and every 3 mo after PD. Follow-up continued for more than 12 mo in all cases. Routine blood examinations included fasting lipid, blood glucose and insulin levels, and levels of hemoglobin (HbA1c), asparate aminotransferase (AST), alanine aminotransferase, total protein, albumin and cholinesterase.

CT images were obtained using a 16-multidetector CT scanner (GE Yokogawa, Tokyo, Japan) without intravenous contrast medium. The raw data set was reconstructed at 5 mm thickness. CT images were evaluated every 6 mo after PD for follow-up. Hepatic steatosis was diagnosed on CT when hepatic attenuation values were 40 Hounsfield units. According to this criterion, 30 of 82 patients who underwent PD were found to have newly appearing hepatic steatosis.

Liver biopsy was performed for 4 of 30 patients with NAFLD after PD who consented to undergo biopsies (fatty liver, $n = 2$; NASH, $n = 2$); in these two men and two women, the mean age was 61 (52-73) years, histological diagnosis was pancreatic carcinoma in one patient, intraductal papillary-mucinous neoplasm in two, and serous cyst adenoma in one, and all of them underwent PD. To compare NAFLD after PD with NAFLD associated with metabolic syndrome, liver samples were obtained from

Table 1 Comparison between pre- and intra-operative data between patients with and without non-alcoholic fatty liver disease after pancreatoduodenectomy

	Non-NAFLD (n = 52)	NAFLD (n = 30)	P value[1]
BMI (kg/m^2)	21.7 ± 3.3	21.4 ± 2.6	0.622
Age (yr)	63.6 ± 10.9	63.4 ± 11.9	0.940
Pancreatic head cancer (yes)	31%	53%	< 0.001[2]
Sex (male)	69%	43%	0.035[2]
Diameter of MPD (mm)	3.9 ± 3.2	5.6 ± 3.2	0.025
Total cholesterol (mg/dL)[3]	188.2 ± 53.3	202.0 ± 39.9	0.238
Triglycerides (mg/dL)[3]	119.8 ± 69.2	139.9 ± 74.8	0.363
Total protein (g/dL)[3]	8.0 ± 0.9	8.3 ± 0.5	0.322
Albumin (g/dL)[3]	4.0 ± 0.4	4.0 ± 0.5	0.761
Cholinesterase (U/L)[3]	230.6 ± 88.2	244.5 ± 104.9	0.531
Insulin (μU/mL)[3]	5.5 ± 2.3	7.7 ± 3.5	0.500
HOMA-IR[3]	1.6 ± 1.0	3.2 ± 3.4	0.324
HbA1c (%)[3]	5.5 ± 0.8	6.2 ± 1.8	0.060
ALT[3]	65.6 ± 0.8	79.9 ± 107.3	0.506
Operation (PPPD)	15%	10%	0.738[2]
Operation time (min)	400.9 ± 49.0	394.6 ± 37.8	0.544
Intraoperative blood loss (mL)	425.2 ± 171.9	419.3 ± 155.6	0.878
Patients with nerve plexus dissection	15%	93%	< 0.001[2]
Pancreatic resection line (SMA)	19%	37%	0.081[2]

[1]P values calculated using the χ^2 test; [2]P values calculated using Fisher's exact test; [3]Values represent mean ± SD unless otherwise indicated. NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; MPD: Main pancreatic duct; HOMA-IR: Homeostasis model assessment for insulin resistance; HbA1c: Hemoglobin A1c; ALT: Alanine aminotransferase; PPPD: Pylorus preserving pancreaticoduodenectomy; SMA: Superior mesenteric artery.

10 patients with NAFLD associated with metabolic syndrome (fatty liver, n = 5; NASH, n = 5) by percutaneous ultrasonography-guided liver biopsy; these four men and six women had a mean age of 52 (40-64) years. In 10 patients, NAFLD associated with metabolic syndrome was diagnosed by the following criteria: (1) the absence of regular intake of alcohol and past history of abdominal surgery; (2) negative results for hepatitis B virus surface antigen and anti-hepatitis C virus antibodies; and (3) the absence of other types of chronic liver disease.

Specimens were fixed in 40 g/L neutral-buffered formaldehyde, cut at 4 μm thickness and stained using hematoxylin and eosin or the Azan-Mallory method. Histological findings were assessed in a blinded fashion by an independent pathologist. Histological diagnosis of NASH was made based on the presence of macrovesicular steatosis, hepatocyte ballooning and lobular inflammation.

For double immunofluorescence, tissue array slides were deparaffinized and soaked in 0.01 mol/L citrate buffer (pH 6.0) at 90 ℃ for 30 min for antigen retrieval. Samples were treated with 10 mg/mL bovine serum albumin (BSA) to inhibit non-specific antibody binding, then incubated with primary murine monoclonal antibody to CD68 (Kp-1, dilution 1:500; Dako, Glostrup, Denmark) for 1 h at 37 ℃. After washing three times with phosphate-buffered saline (PBS) (pH 7.2), samples were incubated with Cy5-labeled secondary rabbit polyclonal antibody to murine immunoglobulin G for 30 min at 37 ℃. For the second immunoreaction, a similar procedure was used:

Table 2 Comparison of postoperative data between patients with and without non-alcoholic fatty liver disease after pancreatoduodenectomy

	Non-NAFLD (n = 52)	NAFLD (n = 30)	P value[1]
BMI (kg/m^2)[2]	20.1 ± 3.5	17.4 ± 2.8	0.004
Total cholesterol (mg/dL)[2]	159.2 ± 40.4	133.3 ± 36.4	0.011
Triglycerides (mg/dL)[2]	102.2 ± 36.4	84.9 ± 49.0	0.169
Total protein (g/dL)[2]	7.0 ± 0.7	6.1 ± 0.7	< 0.001
Albumin (g/dL)[2]	4.0 ± 0.5	3.1 ± 0.7	< 0.001
Cholinesterase (U/L)[2]	215.5 ± 78.5	156.9 ± 88.1	0.006
Insulin (μU/mL)[2]	9.7 ± 9.8	4.1 ± 3.8	0.009
HOMA-IR[2]	3.1 ± 3.3	1.3 ± 1.2	0.117
HbA1c (%)[2]	5.5 ± 0.9	5.6 ± 0.9	0.589
ALT[2]	31.6 ± 18.5	52.0 ± 45.1	0.033
Insulin treatment	12%	27%	0.126

[1]P values calculated using the χ^2 test; [2]Measured 6 mo postoperatively. Values represent mean ± SD unless otherwise indicated. NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; HOMA-IR: Homeostasis model assessment for insulin resistance; HbA1c: Hemoglobin A1c; ALT: Alanine aminotransferase.

samples were treated with 10 mg/mL BSA, then incubated with another primary antibody to CD14 (dilution 1:100; Zymed Laboratories, San Francisco, CA), then incubated with fluoresceinisothiocyanate isomer (FITC)-labeled secondary antibody. After washing with PBS, Cy5-labeled and FITC-labeled samples were examined using a fluorescence microscope (SZX12; Olympus, Tokyo, Japan).

Frequencies of double-positive cells were determined by histology experts counting these cells in entire specimens. Double-positive cells in the hepatic lobule were counted in five high-power fields (original magnification ×60). Cell counts are expressed as the mean ± SD in each specimen.

Statistical analysis

Statistical analysis were performed using SPSS for Windows version 11.0 software (SPSS, Chicago, IL, United States). Continuous variables are expressed as mean ± SD and the statistical significance of differences was determined using Student's t test. Comparisons between groups were made using the χ^2 test for categorical variables. Values of $P < 0.05$ were considered statistically significant.

RESULTS

The median follow-up for the 82 patients who underwent PD was 840 d (range, 183-2553 d). The frequency of NAFLD after PD was 36.6% (30/80). Clinical pre- and intraoperative data for patients with NAFLD (n = 30, 36.6%) and patients without NAFLD (n = 52, 63.4%) are summarized in Table 1. Univariate analysis identified cancer of the pancreatic head, sex, diameter of the main pancreatic duct (MPD) and dissection of the nerve plexus as factors associated with the development of NAFLD after PD.

Clinical postoperative data for patients with and without NAFLD are summarized in Table 2. The BMI, serum

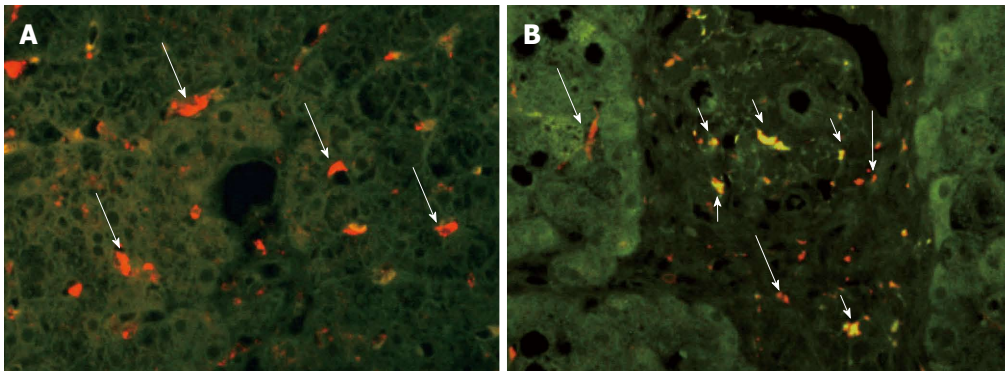

Figure 1 Immunohistochemical staining of liver biopsy specimens for CD68 and CD14 (×60). Kupffer cells (CD68+) and lipopolysaccharide-sensitized Kupffer cells (CD14+) were compared between samples from conventional non-alcoholic fatty liver disease (NAFLD) and NAFLD after pancreatoduodenectomy (PD). Long arrows represent CD68+ Kupffer cells; Short arrows represent CD68+ and CD14+ Kupffer cells. A: Conventional NAFLD; B: NAFLD after PD.

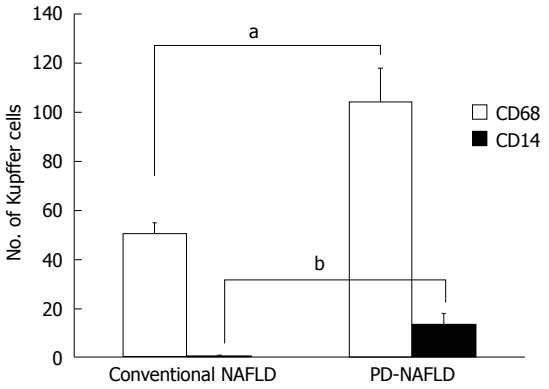

Figure 2 Number of cells positive for CD68 (a marker of Kupffer cells) and CD14 (a marker of lipopolysaccharide-sensitized Kupffer cells). Conventional non-alcoholic fatty liver disease (NAFLD) and NAFLD after pancreatoduodenectomy (PD). Conventional NAFLD and NAFLD after PD specimens showed mean cell counts of 50.6 ± 4.0 and 104.3 ± 13.3 CD68+ Kupffer cells per individual, respectively ($^aP < 0.05$). Cell counts for CD14+ Kupffer cells were 0.6 ± 0.3 and 13.5 ± 4.2 cells per individual, respectively ($^bP < 0.001$).

levels of total protein and albumin, and levels of cholinesterase, total cholesterol and insulin were significantly lower in patients with NAFLD after PD than in patients without NAFLD. Homeostasis model assessment for insulin resistance values, as an indicator of insulin resistance, and serum levels of triglycerides and HbA1c did not differ between these two groups.

At 6 mo postoperatively, 17 of the 30 patients (57%) with NAFLD after PD showed increased blood levels of AST. Of these 17 patients, we performed liver biopsies to pathologically examine specimens from four patients [mean serum levels of AST was 67 (54-80) U/L] who consented to undergo biopsies. Histopathological examination diagnosed two cases as stage 2 NASH, according to the Brunt criteria[13], and two cases as moderate-to-severe macrovesicular steatosis.

In the 10 patients with NAFLD associated with metabolic syndrome, the mean serum level of AST was 56 (35-112) U/L. Five of 10 patients showed moderate-to-severe macrovesicular steatosis and the remaining five patients showed stage 2 NASH in histological examination.

Numbers of cells positive for CD68 (a marker of Kupffer cells) and CD14 (a marker of LPS-sensitized Kupffer cells) were counted in all biopsy specimens (Figure 1). The mean number of CD68+ cells per individual was 50.6 ± 4.0 in specimens from NAFLD associated with metabolic syndrome and 104.3 ± 13.3 in specimens from NAFLD after PD, indicating the presence of significantly more Kupffer cells in NAFLD after PD than in NAFLD associated with metabolic syndrome ($P < 0.001$) (Figure 2). CD14+ cells were observed in specimens from NAFLD associated with metabolic syndrome and in specimens from NAFLD after PD, with mean positive cell counts of 0.6 ± 0.3 and 13.5 ± 4.2, respectively, showing more LPS-sensitized Kupffer cells in NAFLD after PD than in NAFLD associated with metabolic syndrome ($P < 0.001$) (Figure 2). We also attempted to classify NAFLD into simple fatty liver or NASH. As for simple fatty liver, mean numbers of CD68+ cells and CD14+ cells per individual were 51.0 ± 6.8 and 0.4 ± 0.2, respectively, in specimens from fatty liver associated with metabolic syndrome and 116.0 ± 10.7 and 20.5 ± 2.5, respectively, in specimens from fatty liver after PD. This indicated the presence of more Kupffer cells and more LPS-sensitized Kupffer cells in specimens from simple fatty liver after PD than in specimens from simple fatty liver associated with metabolic syndrome ($P < 0.001$, CD68+ cells; $P < 0.001$, CD14+ cells). Regarding NASH, the mean numbers of CD68+ cells and CD14+ cells per individual were 50.2 ± 7.4 and 0.8 ± 0.6, respectively, in specimens from NASH associated with metabolic syndrome and 92.5 ± 19.5 and 6.5 ± 0.5, respectively, in specimens from NASH after PD. This showed that more Kupffer cells and more LPS-sensitized Kupffer cells were present in specimens from NASH after PD than in specimens from NASH associated with metabolic syndrome (CD68+ cells, $P < 0.05$; CD14+ cells, $P < 0.05$) (Figure 3). These observations suggest that after PD, Kupffer cells and LPS-sensitized Kupffer cells were significantly upregulated, not only in NASH, but also in simple fatty liver.

DISCUSSION

Conventional NAFLD is thought to be caused by exces-

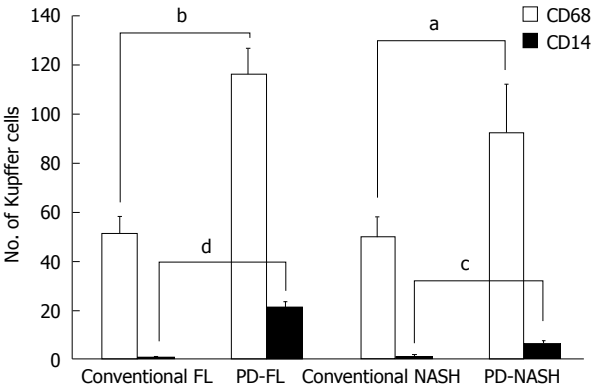

Figure 3 Number of cells positive for CD68 (a marker of Kupffer cells) and CD14 (a marker of lipopolysaccharide-sensitized Kupffer cells). Conventional fatty liver and fatty liver after pancreatoduodenectomy (PD), in conventional non-alcoholic steatohepatitis (NASH) and in NASH after PD. For simple steatosis, mean counts of CD68+ and CD14+ cells were 51.0 ± 6.8 and 0.4 ± 0.2 cells per individual in conventional fatty liver specimens, respectively, and 116.0 ± 10.7 and 20.5 ± 2.5 cells per individual, respectively, in fatty liver after PD specimens. This indicates more Kupffer cells and more lipopolysaccharide-sensitized Kupffer cells in simple fatty liver after PD specimens than in conventional simple fatty liver specimens ($^{b}P < 0.001$; $^{d}P < 0.001$). Regarding NASH, mean cell counts for CD68+ cells and CD14+ cells were 50.2 ± 7.4 and 0.8 ± 0.6 cells per individual, respectively, in conventional NASH specimens, and 92.5 ± 19.5 and 6.5 ± 0.5 cells per individual, respectively, in NASH after PD specimens. This showed that more Kupffer cells and more LPS-sensitized Kupffer cells were present in NASH after PD specimens than in conventional NASH specimens ($^{a}P < 0.05$; $^{c}P < 0.05$).

sive nutrition, lipid metabolic disorder and insulin resistance, as a part of metabolic syndrome[4]. However, the present study demonstrated that NAFLD after PD was related to non-obese status, malnutrition and lack of hyperlipidemia and insulin resistance. These findings suggest that the mechanisms underlying NAFLD after PD differ from those causing NAFLD associated with metabolic syndrome. Recent studies have also suggested that pancreatic exocrine insufficiency may cause NAFLD after PD[8,9]. Although only univariate analyses were examined in our study, pancreatic cancer was associated with the development of NAFLD, which is considered to lead to impaired pancreatic exocrine functions due to obstruction of the MPD, in turn resulting in obstructive distal pancreatic atrophy and fibrosis.

The clinical features of patients with NAFLD after PD resemble those in mice with MCD-induced NASH, in terms of the absence of obesity, insulin resistance and hypocholesterolemia[14]. Increased fatty acid uptake and decreased hepatic export of triglycerides in the form of very-low-density lipoprotein (VLDL) represent two important mechanisms contributing to MCD-induced NASH[14,15]. Malabsorption of essential amino acids such as choline due to pancreatic exocrine insufficiency may result in the development of NAFLD after PD.

NAFLD is often observed in patients showing hypoinsulinemia. Insulin inhibits lipolysis in adipose tissue and decreases the flux of free fatty acids (FFA) into plasma. In the absence of adequate insulin secretion, plasma FFA levels could conceivably be elevated due to increased adipose tissue lipolysis and the liver would then be unable to adequately couple triglycerides to apoprotein B or secrete VLDL-triglyceride, resulting in hepatic steatosis[16]. In this study, serum insulin concentrations were significantly lower in NAFLD after PD compared with non-NAFLD after PD. Insufficiency of insulin may be another factor contributing to the development of NAFLD after PD.

We performed liver biopsy in four patients with NAFLD after PD, diagnosing two cases of NASH. Kupffer cell counts have been shown to play important roles in the pathogenesis of NASH[17]. Within the liver, Kupffer cells are major sources of tumor necrosis factor-alpha (TNF-α) produced in response to LPS[12,18,19]. The process by which LPS activates Kupffer cells seems to be mediated by LPS-binding protein, CD14 and toll-like receptor 4[20]. We therefore focused on CD14 expression in liver specimens from NAFLD after PD because the promoter polymorphism of CD14 has been reported as a risk factor for both alcoholic and non-alcoholic steatohepatitis[21]. CD14 expression on Kupffer cells is low in the healthy human liver[22,23] but increases in the presence of inflammatory liver disease[24]. Expression of CD14 on Kupffer cells can be upregulated with LPS[25,26]. A previous study reported that TNF-α production was decreased in genetically engineered CD14-deficient mice by down-regulating sensitivity to LPS[27]. In contrast, the CD14 transgenic mice that overexpress CD14 on monocytes showed increased sensitivity to LPS[28]. These changes in CD14 expression could represent a mechanism regulating liver sensitivity to LPS toxicity.

The present study demonstrated that Kupffer cells were significantly more common and LPS-induced CD14+ Kupffer cells were upregulated in NAFLD after PD specimens compared to NAFLD associated with metabolic syndrome specimens. Furthermore, even in simple fatty liver after PD specimens, more Kupffer cells and more LPS-sensitized Kupffer cells were present than in specimens from simple fatty liver associated with metabolic syndrome. These findings indicate that LPS plays a significant role in the occurrence of NAFLD after PD, even from an early stage.

Previous reports have hypothesized that the overgrowth of small intestinal bacteria might play a contributory role in NASH pathogenesis, particularly via a component of the gram-negative bacterial population, through the production of LPS[29-33]. Among those patients who underwent PD, bacterial overgrowth may have occurred due to dissection around the SMA, leading to intestinal motor dysfunction and stasis, decreased secretion of gastric juices or blind loops.

The fatty liver is vulnerable to additional inflammatory insults, such as oxidative stress and gut-derived bacterial endotoxins, both of which can trigger hepatocellular inflammation and fibrosis[34,35]. Accumulation of FFA in the liver due to malabsorption of essential amino acids such as choline and gut-derived LPS perhaps from intestinal bacterial overgrowth are important in the pathogenesis of NAFLD after PD.

In conclusion, NAFLD after PD is characterized, not only by malnutrition, but also by up-regulation of CD14 on Kupffer cells with hepatic steatosis. Our results suggest that gut-derived endotoxin contributes to the development of NAFLD after PD.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD), which sometimes develops after pancreatoduodenectomy (PD), is characterized by non-obesity and a lack of both hyperlipidemia and insulin resistance. The pathogenesis of NAFLD after PD may thus differ from the pathogenesis of NAFLD associated with metabolic syndrome.

Research frontiers

It was reported that the clinical features of the patients with NAFLD after PD are similar to those found in the murine non-alcoholic steatohepatitis model induced by a methionine-choline-deficient (MCD) diet. In mice fed the MCD diet, portal endotoxemia was observed. Thus, gut-derived factors such as lipopolysaccharide (LPS) appear to be related with NAFLD after PD. The authors hypothesized that gut-derived factors such as LPS might trigger the release of inflammatory cytokines from Kupffer cells, in turn mediating severe hepatic steatosis and liver injury after PD. The process by which LPS activates Kupffer cells seems to be mediated by LPS-binding protein, CD14 and toll-like receptor 4. The authors therefore focused on CD14 expression on Kupffer cells in liver specimens from cases of NAFLD after PD.

Innovations and breakthroughs

Unlike the patients with NAFLD associated with metabolic syndrome, the patients who developed NAFLD after PD showed significantly decreased levels of serum albumin, total protein, cholesterol and triglycerides, but no glucose intolerance or insulin resistance. Furthermore, the present study demonstrated that Kupffer cells were significantly more common and LPS-induced CD14+ Kupffer cells were upregulated in specimens of NAFLD after PD compared to specimens of NAFLD associated with metabolic syndrome. These findings indicate that LPS plays a significant role in the occurrence of NAFLD after PD. NAFLD after PD is characterized by both malnutrition and up-regulation of CD14 on Kupffer cells. Gut-derived endotoxin appears central to the development of NAFLD after PD.

Applications

Elucidation of the pathogenesis of NAFLD after PD may serve to prevent the development of NAFLD after PD.

Terminology

CD14: CD14 is the main LPS-receptor that can activate monocytes in conjunction with serum LPS-binding protein. CD14 expression on Kupffer cells is low in the healthy human liver but increases in the presence of inflammatory liver disease. Expression of CD14 on Kupffer cells can be upregulated with LPS.

Peer review

The authors investigated the pathogenesis of NAFLD after PD. The paper is well written overall.

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BRIEF ARTICLE

Epidemiological profiles of human immunodeficiency virus and hepatitis C virus infections in Malian women: Risk factors and relevance of disparities

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Abstract

AIM: To document the epidemiologic patterns and risk factors of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections in Mali in order to develop prevention means for both diseases.

METHODS: Two prospective studies were conducted in Bamako in 2009 among 1000 pregnant women (*i.e.*, young women) who consulted six reference health centers, and in 2010, among 231 older women who attended general practice in two hospitals. Antibody tests and molecular analysis (performed only for HCV) were used to quantify the frequencies of both infections. The data were collected from patients recruited through a questionnaire. Transmission risk factors of both diseases were identified by univariate and multivariate analysis.

RESULTS: HCV seroprevalence was 0.2% for young and 6.5% for older women. HIV prevalence was similar in both populations (4.1% *vs* 6.1%). In older women, the analysis of risk factors highlighted an association between HCV infection and episodes of hospitalization ($P < 0.01$). The study did not show an association between HIV infection and the variables such as hospitalization, transfusion, tattoo, dental care, and endoscopy. A significant decrease of HIV seroprevalence was detected in young women who used condoms for contraception more than for other purposes ($P < 0.01$). By contrast, HIV seroprevalence was significantly increased in young women using condoms mainly to prevent sexual infections rather than for contraception ($P < 0.01$). No HCV/HIV coinfection was detected in our study.

CONCLUSION: Risk factors and epidemiologic data of HIV and HCV as well as the absence of co-infection strongly suggest epidemiological disparities between these diseases.

Key words: Hepatitis C virus; Human immunodeficiency virus; Epidemiology; Risk factors; Women; Mali; Bamako

Core tip: In Mali, hepatitis C virus (HCV) studies have been mostly conducted among specific populations such as blood donors, patients suffering from chronic hepatitis or hemodialysis patients. Studies on the extent and epidemiology of HCV infection in the general Malian population are not abundant. The present study demonstrates that the risk factors classically associated to HCV infection, such as transfusion, are not dominant in this African population. The data presented in this paper have important implications in designing effective prevention strategies for human immunodeficiency virus and HCV infections.

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INTRODUCTION

Human immunodeficiency virus (HIV) infection is propagated mainly through blood transfusion (before 1985), intravenous drug use, professional exposure in medical personnel, sexual transmission and mother-to-child transmission. Hepatitis C virus (HCV) contamination is also caused by intravenous drug use and blood transfusion (before 1991), while vertical transmission accounts for less than 5% of the cases and sexual transmission is exceptional[1]. Historically, HCV infection has developed in three waves: *via* medical care through needles and syringes reused without sterilization, *via* blood transfusion before 1991, and finally through intravenous drug injection or the sharing of straws for cocaine inhalation[2]. In industrialized countries, the risk of HCV transmission by blood transfusion is currently controlled by the measures taken to secure blood donation: clinical selection, screening of infectious markers among blood donors and application of viral inactivation processes. Drug addiction is now the major mode for transmission of HCV infection in industrialized countries[2]. The geographical distribution of HCV is variable, with areas of high prevalence such as Africa and Asia where the prevalence may exceed 10%, and low endemic regions such as North America or Western Europe with prevalence around 1%. The high prevalence of HCV infection in developing countries is mainly due to iatrogenic transmission occurred during injections in campaigns of treatment or mass vaccination. A well-known case is Egypt where campaigns of anti-schistosomiasis were undertaken from 1920 to 1980. HCV propagation still represents a major public health problem in developing countries in which high HCV prevalence is associated with suboptimal blood safety[2].

In countries where historically HCV epidemy has emerged early, there is already a notable increase of the incidence of hepatic complications. In Mali, HCV studies have been mostly conducted among specific populations such as blood donors, patients suffering from chronic hepatitis or hemodialysis patients[3-5]. Studies on the extent and epidemiology of HCV infection in the general Malian population are not abundant. The present study aims to document epidemiologic patterns, risk factors and modes of transmission shared by both diseases in Mali using the epidemiologic data previously published by our group[6].

MATERIALS AND METHODS

Patients

Two populations of Malian women were analyzed to characterize their serological status toward both HIV (screening and confirmation tests) and HCV [screening, serological confirmation, search for HCV-RNA by polymerase chain reaction (PCR) and genotyping]. Samples of serum and plasma were prepared from venous whole blood.

The first series included 1000 young women who attended antenatal clinics in the 6 health reference centers (named I to VI) located in Bamako. The second set consisted in 231 older women (over 50 years) attending the consultations of general medicine in two hospitals (Centre Hospitalier Mère-Enfant and CHU Gabriel Touré) located in Bamako. Patients with physical or mental condition precluding investigation as well as those treated with heparin (inhibitor of PCR) were excluded from the study. This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically under the reference number 08-0006/INRSP-CE (Ethical Committee of National Institute of Public Health) of Mali. All participating subjects remained anonymous and gave voluntarily informed consent.

Questionnaire on risk factors of HIV and HCV infections

A questionnaire was used to collect information about behaviours at risk or potentially at risk among all participants and the data were analyzed in order to identify risk factors for the transmission of both infections. Since Trypanosomiasis may interfere with HCV serology, it was searched for through specific questions allowing presumptive diagnosis[7]. To this end, the participants were asked whether they presented symptoms such as sleep problems, anorexia or skin rash.

The residence of the subjects was defined as follows: urban (municipalities of Bamako district or chief town of administrative region) or rural (areas outside both Bamako district and chief town of administrative region). Mali includes 8 administrative regions in addition to the Bamako district. The variables studied in the questionnaire were divided into two groups: (1) The sociodemographic and professional characteristics: age, parity, gravidity, marital status, occupation of the woman and the husband or partner, extent of knowledge of both HCV and HIV infections, use of condom (yes-no) and

Table 1 Demographic characteristics and clinical history among young and old women

Descriptive characteristics	Young women (≤ 50 yr)		Older women (> 50 yr)	
	n (%)	mean ± SD	*n* (%)	mean ± SD
Age (yr)	999	25.2 ± 6.3	231	62.1 ± 8.6
Sex (women)	1000 (100)		231 (100)	
Rural residence area	26 (2.6)		67 (29.1)	
Urban residence area	964 (97.4)		163 (70.9)	
Transfusion history	21 (2.1)		16 (7)	
Hospitalization history	137 (13.8)		76 (33.2)	
Knowledge of HIV status	577 (58.8)		35 (15.3)	
Knowledge of HCV status	16 (1.6)		11 (4.8)	
Condom use	217[1] (21.9)		N/I	
Condom use for ITS/HIV prevention	113 (52.1)			
Condom use for contraception	117 (53.9)			
Marital status				
Single	77 (7.7)		N/I	
Divorced	2 (0.2)		6 (2.6)	
Married	916 (91.9)		108 (47)	
Widowed	2 (0.2)		116 (50.4)	

[1]217 young women reported condom use; among them, 21 did not explain their motivation and 34 used it for contraception as well as for infections transmitted by sexual relation (ITS)/human immunodeficiency virus (HIV) prevention. N/I: Not implemented in old women (cultural context); HCV: Hepatitis C virus.

reason to use it, education (instruction level), residence (urban, rural), frequency of travel for husband or partner, frequency of travel for woman; and (2) Clinical history and clinical informations: knowledge of their HIV and/or HCV status before entering the study, parasitosis (Trypanosomiasis)[7], clinical history and medical treatments: heparin (PCR inhibitor), infibulation, hospitalization, transfusion, dialysis, dental care, fibroscopy, proctoscopy; socio-cultural practices: tattooing/scarification/piercing, excision, haircut visit of the husband or partner.

Screening and confirmation of infection with HIV and HCV

The HIV third generation rapid testing VIKIA 1/2 (Bio-Mérieux) was used for HIV screening. Positive screened samples were confirmed by using the INNO-LIA HIV I / II Score (Innogenetics)[6].

HCV mixed MONOLISA Ab/Ag Ultra test was used for both antibody and viral antigen detection in the serum or plasma of recruited patients. The results were analyzed in triplicates and samples found positive two or three times [*i.e.*, with test ratio (TR) ≥ 1] were confirmed by LIA HCV Score III v2 (Innogenetics). The results were interpreted according to the instructions given by the manufacturers. HCV samples confirmed positive by LIA were analyzed by molecular biology (HCV Cobas Monitor, Roche or Abbott Real Time HCV). Samples tested positive by Monolisa but negative by LIA and thus suspect of early infection were also analyzed by molecular methods. HCV genotyping was determined using Versant HCV Genotype Assay LiPA test from Siemens Healthcare Diagnostics[6].

Statistical analysis

Results are presented as mean ± SD (range) for continuous variables and as frequency (%) for categorical variables. Comparisons of categorical variables were assessed between groups using the χ^2 test. With HCV and HIV as dependent variables, risk factors were identified by univariate logistic regression. In the young women subpopulation (in which all variables were studied without restriction linked to gravidity status or cultural context), multivariate logistic regression was used. Results were considered significant at the 5% level of significance ($P < 0.05$). Calculations were performed using SAS version 9.3 for Windows (SAS Institute, Cary, NC, United States) and Statistica version 10 (Statsoft, Tulsa, OK, United States).

RESULTS

Demographic characteristics and clinical history of women enrolled in this study

Descriptive data for essential demographic characteristics and the clinical history of women enrolled in this study are summarized in Table 1. Young women averaged 25.2 ± 6.3 years (range: 14-50 years) and older women were 62.1 ± 8.6 years old (range: 51-89 years). The vast majority of young women came from urban areas (97.4% *vs* 2.6% from rural areas). Among older women, urban residence was also predominant (70.9% *vs* 29.1%). Transfusion history was more frequent in old women (7%) than in younger ones (2.1%), ($P < 0.01$), as well as hospitalization history (33.2% *vs* 13.8%), ($P < 0.01$). A minority of old women (15.3%) had an accurate knowledge of their HIV serological status, either positive or negative. This proportion was higher among young women (58.8%). By contrast, only 4.8% of old women and 1.6% of young women were aware of their HCV serological status. As for condom use, there was a low proportion of young women (21.9%) who claimed to use

Table 2 Risk factors according to both hepatitis C virus seropositivity and active infection in older women

Variables	Anti-HCV					HCV-RNA				
	Negative	Ind	Positive	OR (95%CI)	*P* value	Negative	Ind	Positive	OR (95%CI)	*P* value
Hospitalization										
No	146	4	3			151	0	2		
Yes	64	0	12	4.13 (1.60-10.97)	< 0.01	65	0	11	12.8 (2.75-59.30)	< 0.01
Transfusion										
No	196	4	14			202	0	12		
Yes	15	0	1	0.74 (0.09-5.83)	0.77	15	0	1	1.12 (0.14-9.22)	0.91

Ind: Indeterminate result of the INNO-LIA hepatitis C virus (HCV) confirmation test.

it quite often. Among them, 53.9% claimed to use it for contraception and 52.1% for the prevention of HIV and infections transmitted by sexual relation (HIV-ITS). Regarding marital status, 47% of older women were married, 50.4% were widowed and 2.6% were divorced. For young women, 91.9% were married, 7.7% were single, 0.2% were widowed and 0.2% were divorced.

Prevalence of HIV and HCV infections

HCV: Our study shows that HCV antibody prevalence was 0.2% (2/1000) among young women and that HCV-RNA prevalence in that subpopulation was 0.1% (1/1000). Among older women, prevalence was measured at 6.5% (15/231) for HCV antibodies and 5.6% (13/231) for viral HCV-RNA. HCV prevalence was significantly higher in old women compared to younger ones ($P < 0.01$). In both series HCV genotypes 1 and 2 were detected with a clear predominance of genotype 2 (84.6%). Interestingly, the INNO-LIA HCV confirmation test performed on positive MONOLISA HCV Ab/Ag samples allowed us to determine a rate of 42.9% and 9.5% of false positive results for young and older women respectively[6].

HIV: HIV screening of both young and old women allowed us to determine an HIV seroprevalence of 4.1% and 6.1% respectively in these subpopulations[6].

Knowledge of the HCV or HIV serological status among seropositive patients

HCV: Among HCV infected patients, respectively 93.3% (14/15) of older women and 100% (2/2) of young women were unaware of their serological status prior to the survey.

HIV: While 25% (10/40) of infected young women were unaware of their HIV status, 57.1% (8/14) of infected older women had no knowledge of their HIV status prior to the survey. Infected old women were significantly less informed about HIV than younger ones ($P < 0.05$).

Risk factors of HCV infection

Series of young women: Univariate analysis of the data collected in the young women cohort revealed a significant correlation between HCV infection and age: the median age for HCV seronegative women was 25 years while the median age for HCV seropositive women was 44.5 years OR = 1.6; 95%CI: 1.2-2.6; $P < 0.01$. Similarly, young rural women had a higher risk of HCV infection than those from urban areas (OR = 0.03; 95%CI: 0.002-0.43; $P < 0.05$). Of note, the very low prevalence of HCV infection among young women did not allow for a detailed analysis of variables in relation to HCV status.

Series of women > 50 years old: In older women, the univariate analysis (Table 2) showed a significant correlation between hospitalization history and both HCV seropositivity and active infection. Indeed, a significant proportion of older women infected with HCV (80% for antibodies and 84.6% for viraemia) had at least one hospitalization history.

In contrast to young women, HCV seropositivity in older women was not associated with age. Likewise, HCV seropositivity was not associated with urban or rural residence in older women (OR = 0.69; 95%CI: 0.26-1.84; $P > 0.05$). Furthermore, the logistic regression analysis of the HCV seropositivity according to both residence (rural or urban) and hospitalization history (yes or no), showed that the proportion of infected old women was 5 times higher in cases with hospitalization history (OR = 13.4; $P = 0.0002$), while the residence variable had no predictive value (OR = 2.7; $P = 0.13$).

Interestingly, our study did not show a correlation between HCV infection and blood transfusion in older women.

Comparison of HCV infectious risk in young and old women

Univariate analysis showed that prevalence of HCV infection was higher in older women compared to young ones. Furthermore, these older women were more exposed than younger ones to risk factors of HCV infection such as dental care (OR = 45; 95%CI: 10-193), fibroscopy (OR = 38; 95%CI: 8.7-166) and tattooing (OR = 37; 95%CI: 8.5-165), and these differences were highly significant ($P < 0.01$). As explained above, prevalence of HCV infection in young women was higher in rural areas while there was no difference for HCV seropositivity in older women with regard to rural or urban residence. Overall, the proportion of HCV seropositive women was significantly higher in rural areas (7%) than in urban areas (1%), ($P < 0.01$).

Table 3 Human immunodeficiency virus status - multivariate logistic model

Variables	Estimation	SE	OR	*P* value
Marital status (monogamous)	0.29	0.16	1.35	0.06
Travel (husband/partner)	0.55	0.17	1.73	0.002
Condom use (HIV or ITS prevention)	- 0.78	0.18	0.46	< 0.01
HIV knowledge (sexual routes)	- 0.43	0.26	0.65	0.09

HIV: Human immunodeficiency virus; ITS: Infections transmitted by sexual relation.

Risk factors for HIV infection

Series of young women: The median age of HIV seronegative women was 25 years, which was similar to the median age of 26 years observed in the seropositive cohort.

Univariate analysis showed an association between HIV infection and 9 explicative variables such as locality (higher prevalence of women from Municipality Ⅱ, $P = 0.03$); monogamy (low frequency of infection, $P = 0.075$); knowledge of sexual transmission (higher frequency of infection, $P < 0.01$); absence of knowledge on transmission modes (low frequency of infection, $P = 0.052$); condom use (very low frequency of infection, $P < 0.01$); condom use for ITS/HIV prevention (higher frequency of infection, $P < 0.01$); superior instruction level (very low frequency of infection, $P < 0.01$); travel of husband/partner (very low frequency of infection, $P < 0.01$); knowledge of the infectious status (high frequency of infection, $P = 0.059$).

Two additional variables (infibulation and anorexia/skin rash) were included because HIV seroprevalence was twice higher among subjects who reported infibulation history or presented such symptoms.

Multivariate analysis of these 11 variables (Table 3) showed (with inclusion $P = 0.05$ and exclusion $P = 0.10$): (1) a decrease of the HIV infection risk among monogamous married women; (2) a decrease of the HIV infection risk among women whose husbands or partners travel; (3) an increase of the HIV infection risk among women who used condoms for infectious diseases prevention; and (4) an increase of the risk of HIV infection among women who knew that acquired immunodeficiency syndrome is transmitted by sexual routes.

Series of women > 50 years old: The median age for HIV seronegative women was 60 years while the median age for HIV seropositive women was 52 years, but the difference was not significant. The HIV seroprevalence did not change significantly with either age or locality of sample collection. Likewise, HIV infection was not associated with the type of residence. Married and widowed older women were significantly less affected by HIV infection than divorced ones ($P < 0.05$).

Comparison of HIV infectious risk in young and old women: Univariate analysis showed that young women, who knew that HIV is spread through sexual relation (OR = 2.6; 95%CI: 1.3-5.3; $P < 0.01$), blood (OR = 2.5; 95%CI: 1.2-5.2; $P < 0.05$) or from mother to child (OR = 2; 95%CI: 1.0-3.95; $P < 0.05$), were more frequently infected with HIV than older women who knew these modes of transmission, respectively. Regarding subjects whose travel time exceeded 6 mo, old women were more frequently infected with HIV than the younger ones (11% *vs* 2%), (OR = 2.9; 95%CI: 1.04-7.9; $P < 0.05$).

In contrast to what has been described for HCV, the proportion of HIV infected women was not associated to rural or urban residence (6/92 *vs* 48/1120) ($P > 0.05$).

Comparison of risk factors in the two subpopulations: Univariate analysis (Table 4) showed that HIV seropositivity of young women was associated with variables such as locality of sample collection, knowledge of sexual route and dialysis, unlike old women.

We have noted that among young women with HIV infection, 39% came from municipality number Ⅱ (OR = 3.79; 95%CI: 1.2-11.6; $P < 0.05$). Surprisingly, it was observed that HIV prevalence was higher in young women who knew that HIV is transmitted by sexual relation, compared to those who were less informed (OR = 7.5; 95%CI: 1-55; $P < 0.05$). Young women who had undergone dialysis were also at greater risk of HIV infection (OR = 24; 95%CI: 1.5-387; $P < 0.05$). Out of two women who had undergone dialysis, one was HIV-positive and was aware of her status. As expected, young women who used condoms were significantly less infected than those who did not use it (OR = 3.6; 95%CI: 2-7; $P < 0.01$). Moreover, young women who used condoms as a contraceptive method were significantly less infected (OR = 0.14; 95%CI: 0.04-0.48; $P < 0.01$) than those who claimed to use it for other purposes. On the other hand, those who used it to prevent HIV-ITS were significantly more infected than those who claimed not to use it for this reason (OR = 5.5; 95%CI: 1.5-19; $P < 0.01$). Young women who reported that their partner was traveling quite often were paradoxically significantly less affected by HIV infection than those whose partner did not travel frequently (1.93% *vs* 6.22%) (OR = 0.3; 95%CI: 0.14-0.63; $P < 0.01$).

In older women, divorced subjects were significantly more affected by HIV infection than married and widowed ones (OR = 0.01; 95%CI: 0.01-0.78; $P < 0.05$).

Tattooing, scarification and/or piercing were not significantly associated with HIV infection. Likewise, the variables such as transfusion, hospitalization, dental care, excision and residence (rural or urban), were not associated with HIV infection.

DISCUSSION

The aim of our study was to carry out a comparative epidemiological analysis of risk factors for HIV and HCV infections in order to develop prevention means in Mali for these diseases. This study is the first to make a comparative epidemiological analysis of risk factors linked to HIV and HCV in Mali.

In this regard, two cohorts of patients (young women and women over 50 years) were recruited in two consecutive periods over two years in the same geographical

Table 4 Comparison of risk factors for human immunodeficiency virus infection among young and older women

Variables	Anti-HIV									
	Young women (≤ 50 yr)					Older women (> 50 yr)				
	Negative	%	Positive	%	*P* value	Negative	%	Positive	%	*P* value
Locality										
Municipality Ⅱ (CSRef)/CHU-GT (hospital)	150	15.7	16	39		149	69.6	9	64.3	
Other municipalities/CH-ME	803	84.3	25	61	0.020	65	30.4	5	35.7	0.680
Marital status										
Single	71	7.5	5	12.2						
Divorced	2	0.2	0	0		4	1.9	2	14	
Married	877	92.2	35	85.4		101	47.4	6	43	0.027
Widowed	1	0.1	1	2.4	0.070	108	50.7	6	43	0.022
Knowledge of sexual routes										
No	147	16.1	1	2.5		106	63.1	9	64.3	
Yes	766	83.9	39	97.5	0.047	62	36.9	5	35.7	0.930
Condom use										
No	751	79.2	20	51.3				N/I		
Yes	197	20.8	19	48.7	< 0.01					
Condom use to prevent HIV/ITS										
No	100	50.8	3	15.8				N/I		
Yes	97	49.2	16	84.2	0.008					
Condom use for contraception										
No	83	42.1	16	84.2				N/I		
Yes	114	57.9	3	15.8	0.002					
Travel of husband/partner										
No	482	51.4	32	78.1				N/I		
Yes	456	48.6	9	21.9	0.002					
Knowledge of the infectious status (from questionary)										
No	392	41.9	10	25		184	86.4	8	57.1	
Yes	544	58.1	30	75	0.038	29	13.6	6	42.9	0.007
Dialysis										
No	950	99.9	40	97.6		211	99.5	14	100	
Yes	1	0.1	1	2.4	0.026	1	0.5	0	0	0.990
Tattooing/scarification/piercing										
No	291	30.9	14	35.9		112	54.1	4	28.6	
Yes	652	69.1	25	64.1	0.510	95	45.9	10	71.4	0.075
Transfusion										
No	925	97.9	39	97.5		197	92.5	14	100	
Yes	20	2.1	1	2.5	0.870	16	7.5	0	0	0.980
Hospitalization										
No	816	86.1	37	90.2		141	66.5	10	71.4	
Yes	132	13.9	4	9.8	0.450	71	33.5	4	28.6	0.710
Dental care										
No	574	60.9	21	52.5		128	60.7	8	57.1	
Yes	369	39.1	19	47.5	0.290	83	39.3	6	42.9	0.790
Excision										
No	92	9.7	4	9.8				N/I		
Yes	859	90.3	37	90.2	0.970					
Residence area										
Rural	25	2.7	1	2.4		61	28.5	5	38.5	
Urban	919	97.3	40	97.6	0.930	153	71.5	8	61.5	0.450

N/I: Not implemented in old women (cultural context); CSRef: Reference health center; CHU-GT: Hospital university Gabriel TOURE; CH-ME: Mother-Child hospital; HIV: Human immunodeficiency virus.

region. Of note, the target population was essentially urban, but the proportion of old women coming from rural areas was higher than that of younger ones (29.1% *vs* 2.6%) ($P < 0.01$). The seroprevalence of HCV among young women was very low (0.2%). By contrast it was significantly much higher in women > 50 years (6.5%), ($P < 0.01$). This trend with age was also observed for the HCV viraemia (5.6% *vs* 0.1%). Overall, the seroprevalence of the Malian women population is estimated at 1.3%. Interestingly, Diarra *et al*[3] reported a seroprevalence of 3.3% for HCV infection in Malian blood donors.

In this study, we show that the proportion of HCV seropositive women in Mali is significantly higher in rural areas (7%) than in urban regions (1%), suggesting that the rural residence area is a risk factor in Mali for HCV infection. This is confirmed in the young women subpopulation although the HCV seroprevalence was low in this group and those women came mostly from urban areas. By contrast, the seroprevalence reached a higher level for older women, but we could not see any association between HCV infection and the residence area

for this category. This discrepancy can be explained by a second risk factor, the hospitalization history. Indeed, we observed that the proportion of hospitalized women is higher in older women than in young ones (33.2% *vs* 13.9%). Furthermore, the logistic regression analysis combining residence and hospitalization risk factors showed that the proportion of infected old women is approximately 5 times higher in previously hospitalized subjects (OR = 13.4; $P = 0.0002$), while residence was not significantly associated with HCV infection (OR = 2.7; $P = 0.13$). This suggests that hygienic precautions in health care may not be well respected in rural areas.

We observed increased HCV prevalence with respect to age, similarly to other reports[8-10]. There was no association between HCV seroprevalence (or viraemia) and gravidity or parity among young women. Older women are more exposed than young women to risk factors for HCV infection such as dental care, endoscopy (fibroscopy) and percutaneous procedures (tattooing), and these differences are highly significant (OR > 1; $P < 0.01$). As usually described in the literature, the origin of HCV infection is unknown in about 20% of cases. A very careful and thorough investigation can sometimes reveal a past episode of drug addiction or a previous medical procedure possibly associated with transfusion[2]. Nosocomial risk by itself is difficult to assess[11]. History of surgery or medical invasive procedures does not necessarily mean that HCV infection is nosocomial. Indeed, transfusion of blood products during surgery or reanimation may be ignored by the patients[11]. Outside transfusion and organ transplants, nosocomial infection is essentially due to the use of poorly sterilized materials[11-20]. Estimations of HCV transmission through unsafe injections in developing countries indicate that 2.3 to 4.7 million infections should occur annually because of such practices[2]. Some rare cases of doctor-to-patient transmission have also been reported during cardiothoracic and gynecologic surgery[21,22]. In addition, an important cultural practice, excision, performed usually on several women at once in non-sterile conditions, is common in Malian rural areas. Moreover, the excision has been reported as a risk factor for HCV transmission in Burkina Faso, a neighboring country of Mali[23]. We note that approximately 90% of young women recruited in our study reported to have been excised. Only 2 of them were HCV seropositive. Excision as a risk factor was not studied in older women because excision is a cultural taboo of Mali, despite awareness campaigns and, paradoxically, the high frequency of this practice.

Our study did not show any significant association between HCV infection and transfusion history among older women whose HCV seroprevalence is high. Similar observations were also reported by Maiga Moussa *et al*[4] who did not observe a single case of transfusion history in their cohort. Indeed, in our study only one of the oldest women transfused was HCV seropositive (6.25%), while no transfused younger women was HCV seropositive. Blood transfusion has been cited as the most important risk factor of HCV infection among pregnant women in Burkina Faso[23]. Our study seems to indicate instead that nosocomial transmission is the highest risk factor of HCV infection in Mali. Of note, the proportion of transfused subjects is low in the two subpopulations, 2% and 7% for young and older women respectively. Therefore, our present results should be confirmed on a larger cohort of transfused patients.

Drug addiction is the major mode for transmission of HCV infection in industrialized countries[2]. This parameter is not investigated by our study. Of note Maiga Moussa *et al*[4] reported the absence of drug addiction history in their series.

Previous studies have highlighted a relationship between HCV seroprevalence and dialysis, independently of the use of blood products[24-27]. However, our study shows no association between HCV seropositivity and dialysis. Again, our study included very small numbers of dialyzed subjects, respectively 0.2% in young women and 0.4% in older ones, thus precluding any definitive conclusions. Baby *et al*[5] have reported an HCV seroprevalence of 19.7% (13/66) in chronic hemodialysis patients admitted to the nephrology department of the Hospital Point G in Bamako.

In our study conducted among old women (over 50 years), we evaluated the prevalence of HCV infection at a lower rate than that reported in previous studies undertaken among chronic hepatitis patients (25.3%) or hemodialysed patients (19.7%). In addition, serological screening assays were not confirmed by Western blot or PCR analysis in these studies. Indeed, the rate of false positive results of the screening assay (MONOLISA HCV Ab/Ag Ultra) is close to 10% in the old women subpopulation[6]. The poor performance of HCV immuno-assays due to false positive reactions in African samples was also reported in other studies[28-32].

Our study has evaluated the HIV seroprevalence rate at 4.1% and 6.1% respectively in young and older women which is higher than 1.3% reported in the Malian general population[33]. As expected, we observed the importance of condom use as a factor that decreases the risk of HIV transmission, in accordance with EDSM report (EDSM-Ⅳ 2006, final report 2007)[33]. We have demonstrated that HIV rate is higher in young women who knew that HIV is propagated by sexual relation compared to those who did not know it. We have also shown that HIV frequency is higher in young women who use condoms to prevent infection than those who use it for another reason. This is confirmed by the fact that HIV infection was significantly less prominent in young women who used condoms more as a means of contraception than for another reason. This raises the question whether recommendations related to prevention such as information, education and communication (IEC) are well understood, correctly followed by the public, and diffused efficiently in the Malian population. The study shows that the young women who have a superior instruction level were significantly less infected

with HIV, which stresses the critical importance of public information in limiting HIV propagation.

Young women, who reported that their partner was traveling quite often, were less affected by HIV infection compared to those whose partners did not travel frequently. This is quite paradoxical and unexpected. A high rate of HIV infections was observed in the municipality number Ⅱ among young women. Indeed, in 2007, a support program for seropositive women was initiated in this health center, which resulted in increased attendance by these patients.

HIV infection rate was higher in divorced women compared to married women and widows. Several hypotheses may explain this observation: women may have divorced because of the unfaithfulness of their partner or divorced women may have several sexual partners. Our observation is confirmed by EDSM report (EDSM-Ⅳ 2006, final report 2007), that has showed that divorce is a risk factor for transmission of HIV infection[33]. According to this study, the level of HIV seroprevalence increases with the number of sexual partners: from 1.1% in women who had only one sexual partner, the prevalence increases to 3.3% among women who had 3 or 4 partners. Likewise, Kirere Mathe *et al*[34] reported that divorce is a risk factor of HIV infection.

Our study showed no significant association between HIV infection and the excision or infibulation in young women. We did not observe any association between percutaneous procedures (tattooing, scarification or piercing) and HIV or HCV infection among women. However, these practices are not trivial and can be considered as risk factors for the transmission of viral infection if health and safety measures are not followed. Although our study focuses on women living mainly in urban areas (*i.e.*, 97.37% for young women and 70.87% for older women), the overall proportion of women living with HIV, unlike HCV, does not change significantly according to the type of residence.

Interestingly the present study did not show any HIV and HCV coinfection. Of note a rare HIV/HCV coinfection has been reported in the general population in southern Cameroon where both infections are endemic[35].

In conclusion, our study reveals that HCV propagation is achieved mainly through medical procedures with contaminated supplies rather than through blood transfusion while HIV transmission may be predominantly of sexual origin in Mali. Our data confirms that condom use significantly reduces the risk of HIV transmission. Furthermore, we have observed that HIV seropositive patients who are aware of their serological status are informed about the risk of transmission. This study supports the idea that efforts should be made to promote the early screening, to reinforce prevention campaigns (IEC) and that targeted strategies should be developed in order to strongly sensitize people with low instruction level as well as the general Malian population to the risk of sexual transmission of HIV. Among HCV infected patients, respectively 93.3% of older women and 100% of young women were unaware of their serological status. Similarly, an estimated 45% to 85% of United States adults are chronically infected with HCV, yet unaware of their condition[36]. Efforts must be focused on screening and treatment in general, and specifically on reinforcing hygienic measures in medical centers, and mainly in rural areas, in order to reduce the risk of nosocomial transmission of HCV.

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COMMENTS

Background

Human immunodeficiency virus (HIV) infection is propagated mainly through blood transfusion (before 1985), intravenous drug use, professional exposure in medical personnel, sexual transmission and mother-to-child transmission. Hepatitis C virus (HCV) contamination is also caused by intravenous drug use and blood transfusion (before 1991), while vertical transmission accounts for less than 5% of the cases and sexual transmission is exceptional. In Mali, HCV studies have been mostly conducted among specific populations such as blood donors, patients suffering from chronic hepatitis or hemodialysis patients. Studies on the extent and epidemiology of HCV infection in the general Malian population are not abundant. The present study aims to document epidemiologic patterns, risk factors and modes of transmission shared by both diseases in Mali.

Research frontiers

Similarly to this study, other authors reported age as risk factor of HCV infection. In this study conducted among old women (over 50 years), the authors evaluated the prevalence of HCV infection at a rate of 3-4 times lower than those described in two other studies undertaken in Mali. These differences may be explained by the fact that previous studies were conducted on patients with chronic hepatitis or hemodialysed patients. In addition, serological screening assays were not confirmed by Western blot or polymerase chain reaction analysis in these studies. Indeed, the rate of false positive results of the screening assay (MONOLISA HCV Ab/Ag Ultra) is close to 10% in the old women subpopulation, as shown here. Moreover, the excision has been reported as a risk factor for HCV transmission in Burkina Faso, a neighboring country of Mali. The authors note that approximately 90% of young women recruited in this study reported to have been excised. Out of them, only 2 were HCV seropositive. Blood transfusion has been cited as the most important risk factor of HCV infection among pregnant women in Burkina Faso. This study seems to indicate instead that nosocomial transmission is the highest risk factor of HCV infection in Mali. Concerning HIV, the authors have estimated seroprevalence at 4.1% and 6.1% respectively in young and older women, which is higher than 1.3% reported previously in the Malian general population.

Innovations and breakthroughs

This study presents for the first time in Mali a comparative epidemiological analysis of risk factors for HIV and HCV infections and the molecular epidemiological profile of HCV. The observed genotypes are HCV-1 and HCV-2 (with predominance of genotype 2). This data is also evidence of epidemiological disparities for HIV and HCV transmission in Mali (HIV essentially sexual and HCV mainly nosocomial).

Applications

The study supports the idea that efforts should be made to reinforce prevention campaigns and that targeted strategies should be developed in order to strongly sensitize people with low instruction level as well as the general Malian popula-

tion to the risk of sexual transmission of HIV. Among HCV infected patients, respectively 93.3% of older women and 100% of young women were unaware of their serological status. Similarly, an estimated 45% to 85% of United States adults are chronically infected with HCV, yet unaware of their condition. Efforts must be focused on screening and treatment in general, and specifically on reinforcing hygienic measures in medical centers, and mainly in rural areas, in order to reduce the risk of nosocomial transmission of hepatitis C virus.

Peer review

This is an interesting manuscript.

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BRIEF ARTICLE

Differential expression of hepatic apurinic/apyrimidinic endonuclease 1, a DNA repair enzyme, in chronic hepatitis

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Abstract

AIM: To determine hepatic expression of apurinic/apyrimidinic endonuclease 1 (APE-1) and 8-hydroxydeoxyguanosine (8-OHdG) in patients with chronic hepatitis B and C.

METHODS: Liver biopsies were obtained from 27 patients with chronic hepatitis B virus (HBV), 30 with chronic hepatitis C virus (HCV), 6 with autoimmune hepatitis (AIH), and 6 with primary biliary cirrhosis (PBC). Normal liver tissue was obtained from surgical resection specimens of four patients. Hepatic APE-1 protein and mRNA expression were assayed by Western blot and by real-time polymerase chain reaction, respectively. Hepatocellular APE-1 and 8-OHdG expression were determined by immunohistochemistry.

RESULTS: The staining intensity of hepatocellular nuclear APE-1 was lower in the HBV group than in the other groups ($P < 0.05$). Hepatic APE-1 protein levels were reduced in the HBV group relative to the other groups. Hepatic APE-1 mRNA levels were also lower in the HBV group. The proportion of hepatocytes with 8-OHdG-positive nuclei was increased in the HCV, AIH and PBC groups ($P < 0.05$), but not in the HBV group. Hepatocellular nuclear APE-1 levels were positively correlated with hepatocellular 8-OHdG levels in both the HBV and HCV groups (HBV, $r = 0.34$, $P < 0.05$; HCV, $r = 0.54$, $P < 0.01$).

CONCLUSION: An imbalance between oxidative DNA damage and APE-1 expression may contribute to hepatocarcinogenesis in chronic viral hepatitis.

Key words: Apurinic/apyrimidinic endonuclease 1; 8-hydroxydeoxyguanosine; Oxidative stress; Viral hepatitis

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INTRODUCTION

Reactive oxygen species (ROS) generated by chronic inflammation are closely linked to hepatocellular oxidative DNA damage and may be involved in hepatocarcinogenesis[1]. During oxidative stress, ROS attack DNA leading to oxidative DNA damage such as 8-hydroxydeoxyguanosine (8-OHdG). Cellular DNA damage can lead to mutation induction and subsequent carcinogenesis if DNA repair processes are not completely effective[2]. Recently, enhanced oxidative DNA damage has been reported in the livers of patients with chronic hepatitis B virus (HBV) or chronic hepatitis C virus (HCV) infection[3,4]. This raises the possibility that oxidative DNA damage participates in the pathogenesis of hepatocarcinogenesis during chronic HBV or HCV infection.

Oxidative DNA damage can generate apurinic/apyrimidinic (AP) sites resulting from loss of bases in DNA, either spontaneously through free radical attack or by the action of DNA glycosylases that remove bases modified by ROS. AP sites must be repaired efficiently because of their potential mutagenicity; AP sites result in base substitution mutations and loss of genetic integrity[5]. Human AP endonuclease 1 (APE-1) (also designated reduction-oxidation factor-1) is a key enzyme of DNA repair that is distributed in a wide range of normal tissues including the liver[4]. This enzyme catalyzes the initial step in AP site repair by rapidly introducing DNA strand breaks on the 5' side of the AP site. APE-1 is also known to be a potent reduction-oxidation (redox) factor, regulating the DNA-binding activity of several transcription factors involved in cell differentiation, proliferation and apoptosis [*e.g.* activator protein-1, nuclear factor-κB (NF-κB) and p53], independently of its DNA repair function[6]. ROS mediate and enhance APE-1 expression and activity, while APE-1 controls intracellular ROS production by negatively regulating the activity of the ROS-related guanosine triphosphate hydrolase.

In view of its role in DNA repair and redox regulation, APE-1 is likely to protect DNA and transcription factors from oxidative damage and to repair damaged DNA in hepatocytes under conditions of enhanced oxidative stress in chronic viral hepatitis. Thus APE-1 may play an important role in the prevention of hepatocarcinogenesis. The aim of the present study was to compare hepatic APE-1 and 8-OHdG expression among patients with chronic HBV or HCV infection, patients with autoimmune liver diseases and normal controls, in whom hepatocellular carcinoma (HCC) is rarely encountered. This is a first step toward understanding the possible role of APE-1 in the pathogenesis of hepatocarcinogenesis in chronic viral hepatitis.

MATERIALS AND METHODS

Patients

This study included 27 consecutive patients with chronic HBV infection, 30 with chronic HCV infection, 6 with autoimmune hepatitis (AIH), 6 with primary biliary cirrhosis (PBC), who were evaluated and underwent liver biopsies at the University Hospital of Hamamatsu University School of Medicine. Inclusion of the 69 patients in this study was dependent upon the availability of sufficient biopsy material for histological and immunohistochemical assessment. Diagnosis of chronic HBV or HCV infection was based on elevated serum transaminase levels over at least 6 mo in the presence of HBV surface antigen or anti-HCV antibodies in serum. The sera of all 27 HBV and 30 HCV patients were HBV DNA-positive or HCV RNA-positive by polymerase chain reaction (PCR), respectively. Diagnosis of AIH or PBC was based on clinical, laboratory and histological findings. The sera of all six AIH patients were positive for anti-nuclear antibodies, and all 6 PBC patients had anti-mitochondrial antibodies in their serum. HBV surface antigen or anti-HCV antibodies were not detected in the sera of AIH and PBC patients. None of the patients received any specific treatment prior to liver biopsy. Normal liver tissue was obtained from surgical resection specimens of four patients who underwent hepatectomy for liver metastasis of colon cancer. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a priori approval by the institution's human research committee.

Liver biopsies

Liver tissue was obtained by percutaneous needle biopsy in all 69 cases. All liver biopsy specimens were fixed in formalin and paraffin-embedded for hematoxylin and eosin, and Azan-Mallory staining, and for subsequent immunohistochemical analysis. A portion of each sample from 50 patients (HBV, 21; HCV, 23; AIH, 3; PBC, 3) was immediately frozen in liquid nitrogen and stored at -80 ℃ until use for protein or RNA extraction. Histological characteristics of chronic viral hepatitis and AIH were evaluated using the standard criteria proposed by Desmet[7]. PBC liver specimens were staged according to the method proposed by Ludwig *et al*[8].

Immunohistochemical analysis

Immunohistochemical detection of APE-1 protein and 8-OHdG were performed using a streptavidin-biotin complex peroxidase kit according to the manufacturer's instructions (Nichirei, Tokyo, Japan). Briefly, deparaffinized sections (4 mm thick) were subjected to autoclave heating treatment in 10 mmol/L citrate buffer (pH = 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. The sections were treated with 10% normal goat serum to block nonspecific binding of antibodies, and incubated with rabbit polyclonal anti-APE-1 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:100) or mouse monoclonal anti-8-OHdG (Santa Cruz Biotechnology; dilution 1:50) antibodies at 4 ℃ overnight. After the incubation, biotinylated secondary antibody, and streptavidin conjugated with peroxidase were added sequentially, followed by color development with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide. Nuclear staining was carried out with Mayer's hematoxylin. The specificity of APE-1 staining was confirmed by preincubating the antibody with an excess of specific antigen peptide. As shown in Figure 1, the intensity of hepatocytic nuclear APE-1 immunoreactivity was graded into four categories (none, faint, weak, or strong staining). This evaluation assessed the mean signal intensity of the entire slide.

For semi-quantitative assessment of 8-OHdG expression, the number of nuclei positive for 8-OHdG among 300 hepatocytes was counted in each section, and the percentage of positive cells was calculated.

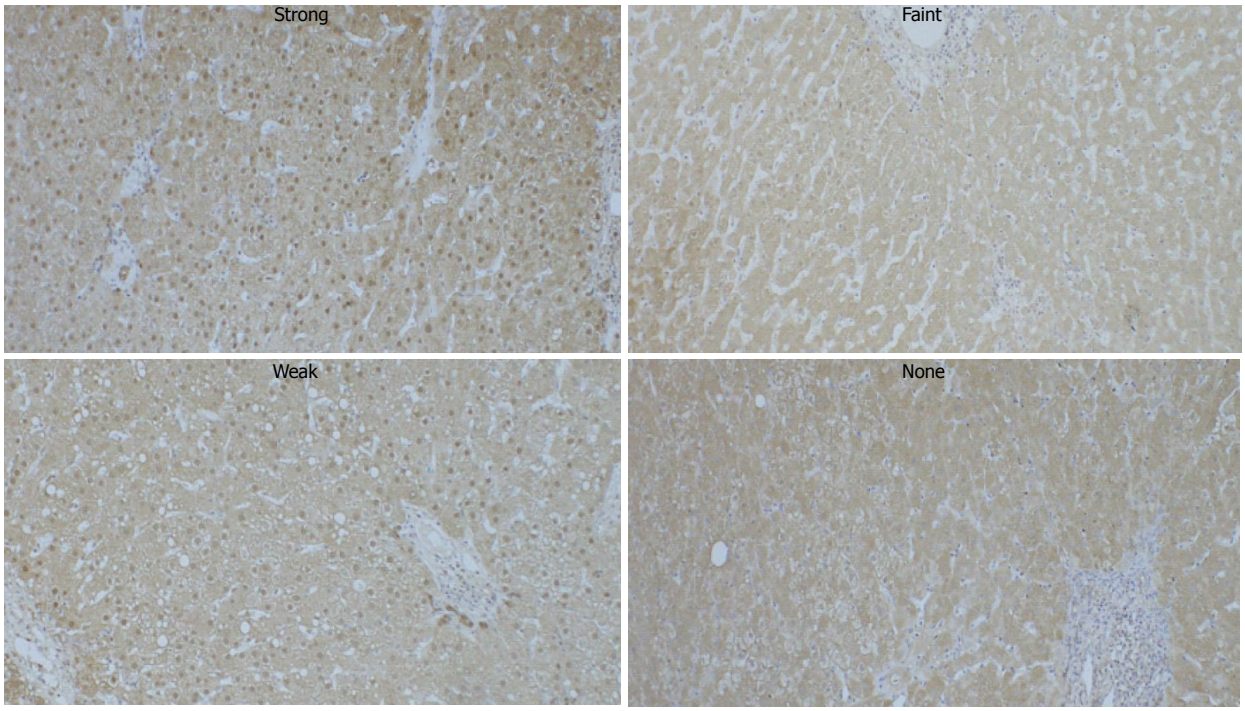

Figure 1 Immunohistochemical detection of hepatic apurinic/apyrimidinic endonuclease 1 protein in patients with chronic liver disease. The intensity of hepatocytic nuclear apurinic/apyrimidinic endonuclease 1 immunoreactivity was classified into four grades.

Western blot analysis

Frozen liver tissue from each patient (HBV, 16; HCV, 18; AIH, 3; PBC, 3; normal, 4) was homogenized in a radioimmunoreactive protein extraction assay lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, United States) containing a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The lysates were diluted 1:1 with ×2 Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Protein concentration of samples in SDS sample buffer was determined using Peterson's modification of the micro-Lowry method. The protein extracts were subjected to SDS-PAGE on 12.5% polyacrylamide gels. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes. The blots were blocked overnight at 4 ℃ with TBS-T buffer (20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, 0.5% Tween 20) containing 10% nonfat dry milk, and were probed with rabbit polyclonal anti-APE-1 (1:100 dilution) or anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) antibodies (Trevigen, Gaithersburg, MD, United States). Bound primary antibody was detected using anti-rabbit IgG horseradish peroxidase-conjugate (Santa Cruz Biotechnology; 1:5000 dilution) and the blots were visualized by chemiluminescence. Band signal intensities were determined with a densitometer. The levels of APE-1 were normalized to those of G3PDH.

Quantitative real-time PCR analysis

Total RNA was extracted from liver biopsy samples (HBV, 5; HCV, 5) using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), according to the manufacturer's instructions. A total of 1 μg of RNA was reverse-transcribed per reaction using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). SYBR green-based quantitative real-time PCR was performed in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) and the optimal reaction conditions were determined for each template. The cDNA for the human *APE-1* gene was amplified with the forward primer, 5'-AGTTTCTTACGGCATAGGCG-3' and the reverse primer 5'-ACTTCGAAAGGCTTCATCC-3' to generate a 161 bp product. β-actin cDNA was amplified with the forward primer 5'-CAGGGCGTGATGGTGGGCATG-3' and the reverse primer 5'-GGCGACGTAGCACAGCTTCTCC-3' (540 bp product) as an internal control.

Statistical analysis

Data are presented as mean ± SE unless otherwise stated. Comparisons of mean values between groups were performed using Mann-Whitney *U* tests. Comparisons between the frequencies of observations were performed using Fisher's exact test. The correlations between various parameters were calculated by univariate linear regression analysis and expressed as Pearson's correlation coefficients. A P value of < 0.05 was considered to indicate significance.

RESULTS

Clinical characteristics of patients

The clinical characteristics of 27 patients with chronic HBV infection, 30 with chronic HCV infection, 6 with

Table 1 Clinical characteristics in patients with chronic liver disease

Characteristics	HCV (n = 30)	HBV (n = 27)	AIH (n = 6)	PBC (n = 6)	HCV *vs* HBV
Gender (male/female)	18/12	18/9	0/6	1/5	NS
Age (yr)	56.8 ± 9.1 (39-73)	52.6 ± 12.9 (22-69)	58.5 ± 12.2 (47-74)	56.3 ± 7.0 (44-64)	NS
Platelet count (× 10^4/mm^3)	15.7 ± 5.9 (9.3-32.3)	14.6 ± 6.2 (8.2-28.4)	14.9 ± 7.6 (12.7-26.0)	16.0 ± 4.4 (13.4-23.6)	NS
AST (IU/L)	55 ± 36 (13-191)	135 ± 84 (59-341)	118 ± 32 (47-120)	32 ± 6 (21-35)	P < 0.001
ALT (IU/L)	71 ± 60 (16-307)	180 ± 153 (79-554)	138 ± 37 (54-144)	45 ± 18 (11-53)	P < 0.001
γGTP (IU/L)	45 ± 36 (14-175)	84 ± 43 (41-135)	147 ± 53 (35-228)	114 ± 63 (23-179)	P < 0.01
Viral load	> 500 KIU/mL (n = 16)				
	100-500 KIU/mL (n = 4)	> 7.6 log copies/mL (n = 11)			
	< 100 KIU/mL (n = 10)	⩽ 7.6 log copies/mL (n = 16)			
Liver histopathology				Ludwig's stage	
Necroinflammation (mild/moderate/severe)	5/20/5	7/15/5	2/3/1	Portal stage (n = 2) Periportal stage (n = 4)	NS
Fibrosis (none/mild/moderate/severe/cirrhosis)	0/17/8/4/1	3/10/8/4/2	1/4/1/0/0	Septal stage (n = 0) Cirrhotic stage (n = 0)	NS

Values represent mean ± SE unless otherwise stated. HCV: Hepatitis C virus; HBV: Hepatitis B virus; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGTP: γ glutamyl transpeptidase.

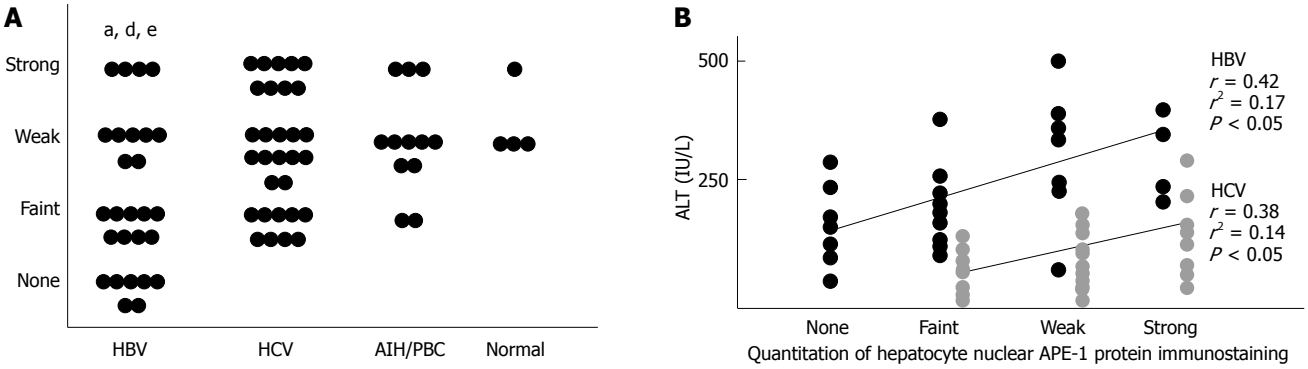

Figure 2 Quantitation of hepatocyte nuclear apurinic/apyrimidinic endonuclease 1 protein immunostaining. A: The percentage of positive hepatocellular nuclei throughout the slide was consistently 80%-100%, regardless of the etiology of chronic liver disease, while the staining intensity was lower in hepatitis B virus (HBV) group than in hepatitis C virus (HCV), autoimmune hepatitis (AIH)/primary biliary cirrhosis (PBC) and normal groups (^{a}P < 0.05 *vs* HCV, ^{d}P < 0.01 *vs* AIH/PBC, ^{e}P < 0.05 *vs* normal); B: The staining intensity of hepatocytic nuclear apurinic/apyrimidinic endonuclease 1 (APE-1) protein was positively correlated with serum alanine aminotransferase (ALT) levels in the HBV (r = 0.42, P < 0.05) and HCV groups (r = 0.38, P < 0.05).

AIH and 6 with PBC are shown in Table 1. There were no significant differences in gender or mean age among the chronic liver disease groups. Serum alanine aminotransferase (ALT), aspartate aminotransferase and γ glutamyl transpeptidase levels were significantly higher in the HBV group than in the HCV group (P < 0.001), but there was no significant difference in the degree of hepatic necroinflammation between these groups.

Hepatic APE-1 protein and mRNA expression

In liver specimens from patients with chronic liver disease, positive staining for APE-1 protein was detected preferentially in hepatocytes and bile duct cells, and occasionally in sinusoidal cells and portal inflammatory cells. In hepatocytes, the nucleus was preferentially stained in a mottled pattern. When nuclear staining was evaluated, the percentage of positive hepatocellular nuclei throughout the slide was fairly constant at 80%-100%, regardless of the etiology of chronic liver disease, while the staining intensity was lower in HBV than in HCV, AIH/PBC, or normal samples (Figure 2A). With regard to hepatocellular cytoplasmic staining, the staining pattern was uniform within individual cells, and homogeneous throughout slides in most specimens; no significant differences in staining intensity were observed among the four groups of patients. In addition, the staining intensity of hepatocytic nuclear APE-1 protein was positively correlated with serum ALT levels in HBV (r = 0.42, P < 0.05) and HCV (r = 0.38, P < 0.05) groups (Figure 2B); there were no other correlations between laboratory values and histological findings.

Western blot analysis showed that APE-1 protein levels were lower in HBV livers compared with normal livers, while there were no significant differences in APE-1 protein levels among HCV, PBC/AIH, and normal livers (Figure 3A and B). Hepatic APE-1 protein levels were reduced by 64% in the HBV group compared with the HCV group. There was a positive correlation between APE-1 protein levels by Western blot analysis and by immunohistochemistry in the livers of patients with chronic liver disease (Figure 3C).

Real-time PCR analysis showed that APE-1 mRNA

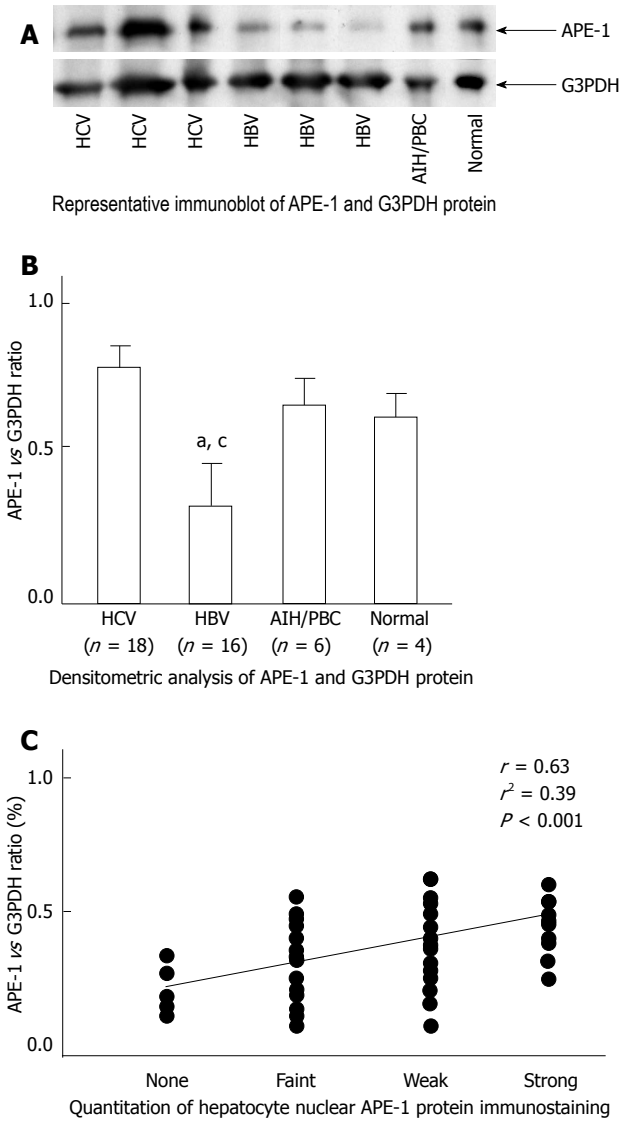

Figure 3 Hepatic apurinic/apyrimidinic endonuclease 1 protein expression in patients with chronic liver disease. A, B: Western blot analysis showed that apurinic/apyrimidinic endonuclease 1 (APE-1) protein levels were reduced in HBV livers compared with normal and HCV livers; HCV livers showed no significant difference in APE-1 protein level compared with normal livers ($^aP < 0.05$ *vs* HCV, $^cP < 0.05$ *vs* normal); C: Correlation between hepatocytic APE-1 protein determined by Western blot and immunohistochemistry in patients with chronic hepatitis. G3PDH: Anti-glyceraldehyde-3-phosphate dehydrogenase.

levels were 58% lower in HBV livers than in HCV livers (Figure 4).

Hepatic oxidative DNA damage in patients with chronic liver disease

In liver specimens from patients with chronic liver disease, positive staining for 8-OHdG was detected preferentially in the nuclei of hepatocytes, bile duct cells, and portal inflammatory cells, and occasionally in the nuclei of sinusoidal cells. As shown in Figure 5A, hepatocytic 8-OHdG labeling index was increased in HCV, AIH, and PBC groups (HCV, AIH or PBC *vs* normal; 64%, 64% or 68% *vs* 35%, respectively, $P < 0.05$). This was not seen in the HBV group, and the labeling index was

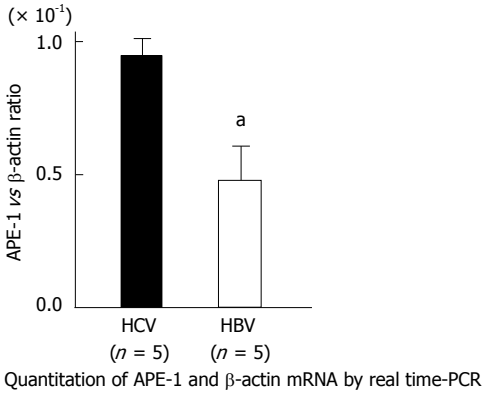

Figure 4 Hepatic apurinic/apyrimidinic endonuclease 1 mRNA expression in patients with chronic liver disease. Real-time polymerase chain reaction (PCR) analysis showed that apurinic/apyrimidinic endonuclease 1 (APE-1) mRNA levels were reduced by 58% in HBV livers compared with HCV livers ($^aP < 0.05$ *vs* HCV).

lower in HBV than in HCV samples (HBV *vs* HCV; 41% *vs* 64%, respectively, $P < 0.001$). In both the HBV and HCV groups, the labeling index was positively correlated with the staining intensity of hepatocytic nuclear APE-1 protein (HBV, $r = 0.34$, $P < 0.05$; HCV, $r = 0.54$, $P < 0.01$) (Figure 5B and C).

DISCUSSION

HCC is frequently encountered in chronic HBV or HCV infection. Hepatocarcinogenesis is most often a multistep process in which a number of genetic alterations accumulate in the hepatocyte[9]. In the case of HCC due to hepatitis virus infection, oncogenesis may be related to viral-induced chronic inflammation. Due to enhanced cell turnover, the repair of damaged DNA may be compromised, rendering the cells more susceptible to spontaneous or mutagen-induced alterations. In addition, with HBV, integration of viral DNA into the host cell genomic DNA may induce transformation. A third possible mechanism involves viral proteins [hepatitis B virus x (HBx) or HCV core] that may act as transactivators of cellular genes, or may induce cellular stress or oxidative stress leading to DNA damage. However, the mechanisms responsible for hepatocarcinogenesis have not been fully clarified. Therefore, we have focused on investigating hepatic expression of 8-OHdG, a useful marker of oxidative DNA damage, and APE-1, a rate-limiting enzyme in the repair of oxidative DNA damage[10], in patients with chronic HBV or HCV infection. Our findings can be summarized as follows: (1) hepatocellular nuclear APE-1 staining and hepatic APE-1 expression at the protein and mRNA levels are reduced in HBV patients, but not in HCV patients; and (2) the number of hepatocytes with 8-OHdG-positive nuclei is increased among HCV patients, but not HBV patients.

Our data suggest that hepatocellular nuclear expression of APE-1 protein was downregulated in patients with chronic HBV infection compared with normal controls. The downregulation of nuclear APE-1 may be

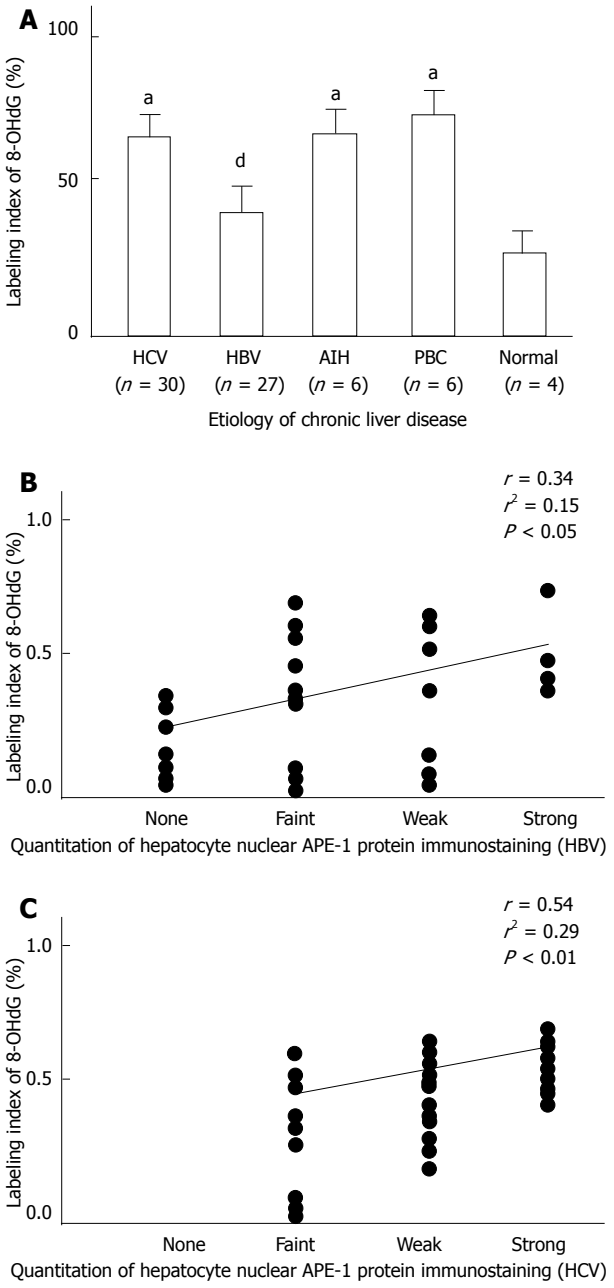

Figure 5 Hepatocytic 8-hydroxydeoxyguanosine expression in patients with chronic liver disease. A: Hepatocytic 8-hydroxydeoxyguanosine labeling index was increased in the hepatitis C virus (HCV), autoimmune hepatitis (AIH), and primary biliary cirrhosis (PBC) groups (HCV, AIH or PBC *vs* normal; 64%, 64% or 68% *vs* 35%, respectively, $^{a}P < 0.05$ *vs* normal), but not in the hepatitis B virus (HBV) group, and the labeling index was lower in the HBV than in the HCV group (HBV *vs* HCV; 41% *vs* 64%, respectively, $^{d}P < 0.001$ *vs* HCV); B, C: The labeling indices of HBV and HCV samples were positively correlated with the staining intensity of hepatocytic nuclear apurinic/apyrimidinic endonuclease 1 (APE-1) protein (HBV, $r = 0.34$, $P < 0.05$; HCV, $r = 0.54$, $P < 0.01$).

due to altered subcellular translocation, decreased protein synthesis, and/or increased protein degradation of APE-1. The nuclear import of APE-1 is mediated by its nuclear localization signal with the involvement of an importin system[11], while the nuclear export of APE-1 is controlled by S-nitrosation of its nuclear export signal[12]. In addition, the interaction with specific nuclear proteins such as GADD45a appears to maintain APE-1 within the nucleus[13]. Decreased nuclear import, increased nuclear export of APE-1 and/or reduced stability of nuclear APE-1 may downregulate nuclear expression of the protein in HBV-infected hepatocytes, given the interaction of HBx protein with the Crm1-dependent nuclear export pathway[14]. APE-1 induction can be reduced at the transcriptional level. The activation of negative calcium response elements in the promoter region of the *APE-1* gene represses APE-1 transcription[15]. In addition, activation of p53 downregulates APE-1 expression, interfering with Sp1 binding to the APE-1 promoter[16]. Similar mechanisms may be involved in the reduction of hepatic APE-1 mRNA levels in HBV-infected livers, since HBx can modify the transcriptional machinery[17]. The reduction of hepatocellular nuclear APE-1 expression may also be due to APE-1 degradation. APE-1 undergoes proteolysis by granzymes A and K, leading to enhanced cell death[18,19]. This may explain our observation that patients with chronic HBV infection had higher levels of serum transaminase and lower levels of hepatocellular nuclear APE-1 expression than those with chronic HCV infection. In addition, HBx may promote ubiquitin-mediated proteasomal degradation of APE-1, as it does of β-catenin[20], since ubiquitination of APE-1 by MDM2 leads to cytoplasmic translocation and proteasomal degradation of APE-1[21].

Our findings indicate that HCV patients had enhanced hepatic 8-OHdG accumulation compared with HBV patients. This confirms the findings of previous studies[22,23]. In addition, in both HBV and HCV patients, the staining intensity of hepatocytic nuclear APE-1 protein was found to be positively correlated with the proportion of 8-OHdG-positive hepatocytes. This suggests that hepatocellular nuclear APE1 expression depends on oxidative DNA damage in HBV- and HCV-infected livers, and reflects the adaptive response of APE-1 to oxidative stress[10]. Furthermore, our data suggest an imbalance between oxidative DNA damage and APE-1 expression. In HBV-infected livers, APE-1 expression is reduced without an increase in oxidatively damaged DNA. In HCV-infected livers, APE-1 expression is not sufficiently induced in proportion to enhanced oxidative DNA damage. These imbalances between oxidative DNA damage and APE-1 expression may lead to the accumulation of AP sites, causing base-substitution mutations and subsequent hepatocarcinogenesis in chronic HBV and HCV infection, as described in a study of ulcerative colitis[24]. Recently, accumulation of AP sites and reduced APE-1 expression were reported in the livers of mice deficient in methionine adenosyltransferase 1a, one of the experimental animal models of HCC[25]. Previous experimental studies showed enhanced spontaneous mutagenesis and cancer predisposition in APE-1 heterozygous mice[26,27].

In conclusion, hepatic APE-1 expression is reduced without enhanced accumulation of hepatic 8-OHdG in chronic HBV infection, while enhanced accumulation of

hepatic 8-OHdG exists without an increase in hepatic APE-1 expression in chronic HCV infection. The imbalance between oxidative DNA damage and APE-1 expression may contribute to hepatocarcinogenesis in chronic viral hepatitis.

COMMENTS

Background

Increased production of reactive oxygen species (ROS), which cause oxidative DNA damage, is considered to be related to hepatocarcinogenesis. Apurinic/apyrimidinic endonuclease-1 (APE-1) and 8-hydroxydeoxyguanosine (8-OHdG) are useful markers of DNA damage induced by oxidative stress. The aim of the present study was to examine hepatic expression of APE-1 and 8-OHdG in patients with chronic hepatitis.

Research frontiers

Hepatic APE-1 and 8-OHdG expression may undergo differential regulation in chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. Downregulation of hepatic APE-1 expression may contribute to hepatocarcinogenesis in chronic HBV infection.

Innovations and breakthroughs

Livers of patients with chronic HBV infection, but not chronic HCV infection, showed reduced expression of hepatic APE-1 compared with normal livers and other chronic liver diseases.

Applications

Future studies should evaluate the ROS increase generated by chronic inflammation, which is closely linked to the occurrence of hepatocellular oxidative damage and may be related to hepatocarcinogenesis.

Terminology

APE-1: APE-1 is one of the major DNA repair enzymes, and its altered expression is associated with tumorigenesis; 8-OHdG: 8-OHdG is a pro-mutagenic DNA lesion produced by oxygen radicals, and is recognized as a useful marker for estimation of DNA damage induced by oxidative stress.

Peer review

The authors have shown differential expression of hepatic APE-1, a DNA repair enzyme, in chronic hepatitis. By evaluating hepatic APE-1 and 8-OHdG expression in liver disease of various etiologies, they concluded that downregulation of hepatic expression may contribute to the development of hepatocellular carcinoma in chronic HBV infection. This study should be of value in elucidating the mechanisms that contribute to hepatic carcinogenesis.

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BRIEF ARTICLE

Hepatitis B infection among adults in the philippines: A national seroprevalence study

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Abstract

AIM: To determine the prevalence of hepatitis B surface antigen (HBsAg) seropositivity among adult Filipinos.

METHODS: Testing for HBsAg was performed on serum samples from persons aged ⩾ 20 years old who participated in the National Nutrition and Health Survey (NNHeS) conducted in 2003. Information on age, sex, marital status, educational attainment, employment status, and income were collected. For this study, marital status was classified as never married or otherwise (*i.e.*, married, divorced, separated, widowed); educational attainment was classified as high school graduate or below or at least some tertiary education; and employment status was classified as currently employed or currently unemployed. Annual income was divided into 4 quartiles in Philippine pesos (PhP): Q1, ⩽ PhP 53064; Q2, PhP 53065-92192; Q3, PhP 92193-173387; and Q4, ⩾ PhP 173388. Prevalence estimates were weighted so that they represented the general population. Social and demographic factors were correlated with HBsAg seropositivity. Multivariate analysis was used to determine independent predictors of HBsAg seropositivity.

RESULTS: A total of 2150 randomly selected adults, 20 years and over, out of the 4753 adult participants of NNHeS were tested for HBsAg. The HBsAg seroprevalence was 16.7% (95%CI: 14.3%-19.1%), which corresponded to an estimated 7278968 persons infected with hepatitis B. There was no significant difference between males and females (17.5% *vs* 16.0%; P = 0.555). This corresponded to an estimated 3721775 men and 3557193 women infected with hepatitis B. The HBsAg seroprevalence peaked at age 20-39 years old, with declining prevalence in the older age groups. The only independent predictor of HBsAg seropositivity was the annual income, with persons in the highest income quartile being less likely to be HBsAg positive (age-adjusted OR = 0.51; 95%CI: 0.30-0.86) compared to subjects in the lowest income quartile. Sex, marital status, educational attainment, and employment status were not found to be independent predictors of HBsAg seropositivity.

CONCLUSION: The high HBsAg seroprevalence among adults in the Philippines classifies the country as hyperendemic for HBV infection and appears unchanged over the last few decades.

Key words: Prevalence; Hepatitis B; Survey; Philippines; Asia

Core tip: Chronic hepatitis B has a significant public health impact in the Philippines because it is a common cause of end stage liver disease and is the leading cause of hepatocellular carcinoma, the latter being the fourth leading cancer and the second leading cause of cancer death in the country. However, an accurate estimate of the national prevalence of hepatitis B infection in the Philippines is lacking. To determine the national prevalence of hepatitis B infection in the Philippines, testing for hepatitis B surface antigen was undertaken on serum samples collected from subjects included in the National Nutrition and Health Survey.

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INTRODUCTION

Chronic hepatitis B (CHB) remains to be a significant public health burden affecting 400 million people worldwide, and is most prevalent in the Asia Pacific region[1]. The disease is estimated to account for 30% of cirrhosis and 53% of hepatocellular carcinoma (HCC) cases worldwide. As such, it is responsible for a staggering half a million deaths every year from hepatitis B virus (HBV)-related cirrhosis and HCC[2].

The Philippines is considered an endemic country for hepatitis B. Chronic hepatitis B has a significant public health impact in the Philippines because it is a common cause of end stage liver disease and is the leading cause of HCC[3,4], the latter being the fourth leading cancer and the second leading cause of cancer death in the country[5]. However, an accurate estimate of the national prevalence of hepatitis B infection in the Philippines is lacking. Although many prevalence studies have been done earlier, they either included small sample sizes, or were done only in select populations (*i.e.*, overseas employment applicants, certain ethnic groups, limited locality, institutional, *etc.*)[6-9]. Accurate prevalence estimates in the general population are important in the control of HBV infection and its complications, and may especially be instrumental in shaping health policies on primary and secondary prevention of this infection.

To determine the national prevalence of hepatitis B infection in the Philippines, testing for hepatitis B surface antigen (HBsAg) was undertaken on serum samples collected from subjects included in the National Nutrition and Health Survey (NNHeS).

MATERIALS AND METHODS

NNHeS and data collection

NNHeS was conducted in 2003 as a collaborative effort among the Food and Nutrition Research Institute of the Department of Science and Technology (FNRI-DOST), the department of health, and fourteen medical specialty associations in the country. The NNHeS was conducted in conjunction with the 6th National Nutrition Survey of the FNRI-DOST. The study protocol was approved by the Technical Committee and Ethical Review Board of the DOST. Studies on the prevalence and risk factors of atherosclerosis-related diseases and metabolic syndrome have been published using the data from the survey[10,11].

The survey utilized the National Statistics Office Master Sample with household listing taken from the Family Income and Expenditure Survey. A stratified multistage sampling design was employed to represent each of the 17 regions in the country. The sampling method utilized the "Barangay" which is the basic political unit in the Philippines that serves as the primary planning and implementing unit of government policies and programs and which is created out of a contiguous territory with at least 2000 inhabitants[10]. The first stage involved the selection of the Primary Sampling Units (PSUs) in a barangay or contiguous barangays with at least 500 households with probability proportional to the estimated number of households. The second stage involved selection of Enumeration Areas (EAs) within sampled PSUs with 150-200 households serving as the Secondary Sampling Units. The last stage was the selection of housing units within the sampled EAs which served as the ultimate sampling unit. As such, the household was considered as a cluster in which all the units became part of the survey. The clinical component of the NNHeS covered only one of the four replicates of the Master Sample and 25% of the households. A replicate was a sub-sample that possessed the properties of the full master sample such that each replicate was able to generate national level estimates of adequate precision. Adults 20 years and over served as participants of the study.

Information on age, sex, marital status, educational attainment, employment status, and income were collected. For this study, marital status was classified as never married or otherwise (*i.e.*, married, divorced, separated, widowed); educational attainment was classified as high school graduate or below or at least some tertiary education; and employment status was classified as currently employed or currently unemployed. Annual income was divided into 4 quartiles in Philippine pesos (PhP): Q1, ≤ PhP 53064; Q2, PhP 53065-92192; Q3, PhP 92193-173387; and Q4, ≥PhP 173388 according to categories of income used in a World Health Organization publication on non-communicable diseases and socioeconomic inequalities[12].

Laboratory methods

HBsAg testing was performed on serum samples col-

Table 1 Prevalence of hepatitis B surface antigen positivity according to demographic characteristics *n* (%)

	HBsAg positive	95%CI	*P* value
All adults	2150 (16.7)	14.3-19.1	
Sex			0.555
Females	1194 (16.0)	13.0-18.9	
Males	956 (17.5)	14.1-20.8	
Marital status[1]			0.177
Never married	261 (19.3)	13.6-25.0	
Otherwise	1883 (16.0)	13.5-18.4	
Educational attainment[1]			0.021
High school	1412 (18.6)	15.5-21.7	
At least some tertiary	720 (14.6)	11.3-18.0	
Employment status			0.189
Unemployed	1077 (15.4)	12.0-18.8	
Employed	1073 (17.5)	14.4-20.7	
Income[1]			0.003
Q1	484 (20.6)	13.2-18.4	
Q2	564 (19.2)	14.6-23.8	
Q3	566 (16.8)	12.1-21.5	
Q4	502 (11.3)	7.0-15.6	

[1]Missing data. HBsAg: Hepatitis B surface antigen.

lected from adults aged 20 years or over. Samples were tested using the AxSym HBsAg (V2) assay from Abbott Laboratories. Samples with values less than 2.00 were considered non-reactive by the AxSym HBsAg (V2) assay and those with values greater than or equal to 2.00 were considered reactive.

Statistical analysis

Data set was checked for inconsistencies and values were validated. Estimates of prevalence were weighted so that they represented the national population of the Philippines in 2003. Sampling weights were also applied to account for the selection of a subsample of subjects on whom HBsAg testing was performed. Weighted prevalence by age, sex and demographic variables were computed using Stata Release 11. Age-adjusted multivariate analysis was done to determine independent predictors of HBsAg positivity. A P value of < 0.05 was considered statistically significant.

RESULTS

A total of 2150 randomly selected adults, 20 years and over, out of the 4753 adult participants of NNHeS were tested for HBsAg. The prevalence of HBsAg seropositivity among adults in the Philippines was 16.7% (95%CI: 14.3%-19.1%), which corresponded to an estimated 7278968 persons infected with hepatitis B (Tables 1 and 2).

The prevalence of HBsAg seropositivity was higher among men compared to women but the difference was not statistically significant (17.5% *vs* 16.0%, $P = 0.555$) (Table 1). This corresponded to an estimated 3721775 men and 3557193 women infected with hepatitis B (Table 2). The prevalence of HBsAg seropositivity according to various age groups is shown on Table 2. HBsAg seropositivity was highest among the 20-39 age group and it was lower in the older age groups. Table 1 shows the prevalence of HBsAg seropositivity according to the other demographic variables. A significantly higher prevalence of HBsAg seropositivity was observed among those with an educational attainment of high school or less compared to those with at least some tertiary level of education (18.6% *vs* 14.6%, $P = 0.021$). The prevalence of HBsAg seropositivity was also statistically different among the different income groups with the highest prevalence of HBsAg seropositivity observed among those belonging to the lower annual income quartiles (Q1 = 20.6% *vs* Q2 = 19.2% *vs* Q3 = 16.8% *vs* Q4 = 11.3%, $P = 0.003$). While HBsAg seropositivity was more prevalent among those who were never married and those who were employed, the differences were not statistically significant. However, on multivariate analysis, only the annual income was found to be independently predictive of HBsAg seropositivity. Those belonging to the highest income quartile (Q4) were less likely to be HBsAg seropositive (age-adjusted OR = 0.51; 95%CI: 0.30-0.86) compared to those in the lowest income quartile (Q1) (Table 3).

DISCUSSION

This study shows that the prevalence of HBsAg seropositivity in the Philippines remains in the hyperendemic range at 16.7%, translating to an estimated 7.3 million adult Filipinos infected with HBV. Earlier studies have placed the prevalence of HBV infection to be between 4% to 18%[6-9]. A study done in 1986 from 4 rural villages in the country showed a prevalence of 9%-15%, with two peaks observed in the 3-4 and 30-40 year-old age groups[9]. More recent studies done almost 20 years later showed that HBsAg seroprevalence in healthy adults appeared to be lower at 5.9% in an urban area[6] and 8.4% in a rural area[7]. The study done in the urban setting tested archived samples and the study in the rural setting only evaluated persons in a single town within a province. Therefore, both studies do not represent the general population. A study on blood donors and applicants for overseas employment likewise showed a lower prevalence estimate at 4.2% for both groups[8]. It is interesting to note that studies that looked at archived serum from "healthy" volunteers and blood donors have shown much lower rates of HBV infection[6,8]. The fact that blood donors are pre-screened and excluded outright if they report risk factors for blood-borne diseases (*e.g.*, injection drug use, tattoos, *etc.*) and that so-called "healthy" volunteers are generally persons with access to health care and probably have self-selected based on prior knowledge of HBV infection status may account for the discrepancy compared with estimates done in other population groups. The current study was conducted on a large sample size that represented the general adult population in the Philippines and is therefore a more accurate estimate of the prevalence of hepatitis B infection in this population.

The high HBsAg seroprevalence in the Philippines in 2003 is in contrast to other countries in the Asia Pacific

Table 2 Prevalence of hepatitis B surface antigen seropositivity and population estimates according to age group and gender *n* (%)

Age group	Total			Males			Females		
		95%CI	Estimated number		95%CI	Estimated number		95%CI	Estimated number
20-29	329 (18.1)	13.6-22.6	2580340	170 (19.5)	13.1-25.9	1392647	159 (16.6)	10.5-22.8	1187693
30-39	314 (17.6)	13.2-22.0	1960314	163 (20.2)	13.6-26.8	1137688	151 (14.9)	9.0-20.7	822626
40-49	252 (16.0)	11.2-20.9	1314240	112 (13.4)	7.2-19.7	567308	140 (18.2)	11.4-25.1	746932
50-59	162 (14.3)	9.0-19.5	715781	66 (10.1)	3.2-16.9	265155	96 (17.2)	9.3-25.0	450626
60-69	638 (14.3)	11.4-17.2	443882	267 (17.6)	12.7-22.6	254760	371 (11.9)	8.3-15.5	189122
70 and over	455 (13.6)	10.0-17.3	264411	178 (12.4)	8.1-16.8	104217	277 (14.4)	9.5-19.2	160194

Table 3 Relative odds of hepatitis B surface antigen positivity among adults by selected demographic variables

	Adjusted OR	95%CI
Sex		
Females	1.00	
Males	1.06	0.76-1.45
Marital status		
Never married	1.00	
Otherwise	0.75	0.48-1.16
Educational attainment		
High school	1.00	
At least some tertiary	0.78	0.55-1.13
Employment status		
Unemployed	1.00	
Employed	1.18	0.85-1.71
Income		
Q1	1.00	-
Q2	0.94	0.59-1.50
Q3	0.82	0.51-1.31
Q4	0.51	0.30-0.86

region where the latest estimates show that the prevalence of HBV infection has already fallen below previous estimates. In Northeast China and South Korea, recent seroprevalence surveys indicate a two- to three-fold decrease in HBsAg seroprevalence in the last 1-2 decades[13,14]. Both countries adopted universal infant vaccination and attributed the declines in HBsAg seroprevalence to the effect of vaccination programs. The current study did not include adults younger than 20 years old and children. Thus, it is not possible to make a complete assessment of the effectiveness of the universal infant vaccination program that was introduced in 1992[15].

The highest prevalence of HBV infection is seen in the 20-39 year-old age group with decreasing HBsAg seroprevalence in the older age groups. This trend is similar to data from other countries in the Asia-Pacific region. While this may be due to older patients with HBV infection dying from cirrhosis and HCC, there is also evidence that even in countries where the predominant mode of HBV transmission is perinatal, HBsAg seroclearance can occur in up to 28% of CHB patients by the time they reach 60 years of age, with a median age of 48 years at the time of HBsAg seroclearance[16]. A lower annual income was the only independent predictor of HBsAg seropositivity in our study, and may reflect inequity in the access to health care, including preventive programs such as vaccination, among the different socioeconomic levels. This is not surprising since studies have shown that patients with lower socioeconomic status are more likely to have delayed and missed vaccinations[17], and are more likely to be ignorant of how HBV is transmitted[18]. Moreover, it is also important to note that a low educational status was associated with a higher HBsAg seroprevalence on univariate analysis. The results of the study highlights the importance of a government-subsidized immunization program and a grassroots HBV education program in a nation where the poverty level still hovers around 27%[19].

The continued high HBsAg seroprevalence probably explains why the yearly incidence of HCC for both males (20-21 per 100000) and females (7-8 per 100000) in the Philippines has not changed over the past 20 years[20]. The burden of CHB infection expectedly spills over into the public health arena because of the high cost of taking care of one patient with CHB. In Asia, the cost can range from United States Dollar 185-1321 per year for patients with compensated CHB-related cirrhosis, to United States Dollar 49000-66000 for every CHB patient needing a liver transplant[21,22]. In countries like the Philippines where there is very little government assistance for healthcare, the cost for CHB care falls squarely on the shoulders of the patients themselves. Therefore, the finding that persons in the lower income brackets are more likely to be infected with HBV makes the impact of this infection on the utilization of healthcare resources in the Philippines even more significant.

Our study has several limitations. We did not include adults younger than 20 years old and children. Thus, the current estimate may consequently not be reflective of the true national HBsAg seroprevalence. However, while universal infant HBV vaccination was first introduced into the national immunization program as early as 1992[15], due to insufficient funds, the program was never fully implemented until January 2007[23]. Therefore, since fully funded efforts to curb the perinatal transmission of HBV started only after the study was made, the HBsAg seroprevalence of adults younger than 20 years old and children is not expected to be very different from the estimates in this study. A study evaluating the seroprevalence of HBV in children is needed and is expected to be conducted in the immediate future as the Philippines together with countries in the World Health Organization Western Pacific Region have adopted a region-wide goal to reduce the seroprevalence of HBV in children 5 years

or younger to < 2% by 2012[24]. Although the result of this study does not reflect the effects of a successful vaccination program on HBV prevalence rates, it may later serve as a comparison group for adults born after its full implementation. Another study limitation is that testing of the serum antibody to hepatitis B surface antigen and antibody to hepatitis B core antigen were not included. This information could have helped elucidate on the level of HBV exposure and/or success of vaccination. There were missing data for some participants on marital status, educational attainment, and income. However, a reanalysis including only those with complete demographic data did not change the results. Other risk factors for HBV transmission like serology for human immunodeficiency virus, hepatitis C, herpes simplex, and syphilis, history of hemodialysis, of injection drug use, and of blood transfusion as well as sexual histories were not determined and would have helped expound on the epidemiology of HBV infection in the country.

We have shown that HBsAg seroprevalence among adults in the Philippines remains high and appears to be unchanged from previous estimates. That HBV infection is more common among those in the lower income brackets emphasizes the importance of prevention in the control of this infection, especially in a healthcare resource-challenged setting like in the Philippines. The recent gains with universal infant HBV vaccination are a significant step towards bringing down the prevalence of HBV infection. Universal infant HBV vaccination should be part of a government-led multi-sectorial national comprehensive hepatitis B control program that will bring together all the various initiatives on the prevention of HBV infection as well as the management of chronic HBV infection and its consequences.

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COMMENTS

Background

Hepatitis B virus (HBV) infection poses a significant health burden in the Philippines. However, an accurate estimate of its national prevalence is lacking. This study aims to determine the prevalence of hepatitis B surface antigen (HBsAg) seropositivity among adult Filipinos.

Research frontiers

The present study determined the seroprevalence of hepatitis B in the adult general population in the Philippines, which has not been previously performed.

Innovations and breakthroughs

The findings of the present study show that the seroprevalence of hepatitis B in the adult general population of the Philippines remains high at 16.7%.

Applications

The findings of high prevalence of hepatitis B infection in the Philippines as well as the association between income and HBsAg seropositivity can be used in the formulation of health policy and programs in hepatitis B control and prevention.

Peer review

The manuscript reports epidemiological data about HBV infection in Philippines, which could improve the health care in this endemic country. The content is of scientific interest and relevance.

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BRIEF ARTICLE

PPARγ agonist-induced alterations in Δ6-desaturase and stearoyl-CoA desaturase 1: Role of MEK/ERK1/2 pathway

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Abstract

AIM: To investigate the effect of MEK/ERK1/2 pathway on peroxisome proliferator-activated receptors (PPARγ) agonist-induced alterations in Δ6-desaturase (Δ6D) and stearoyl-CoA desaturase 1 (SCD1) in hepatocellular carcinoma cell line HepG2.

METHODS: HepG2 cells cultured in RPMI-1640 were exposed to the commonly used ERK1/2 pathway inhibitor PD98059 and PPARγ agonist, pioglitazone. Total RNA was isolated and reverse transcribed from treated cells. Changes in gene expression and metabolites ratio, as activity index for Δ6D and SCD1, were then determined using reverse transcription-polymerase chain reaction and gas liquid chromatography, respectively.

RESULTS: The expression of both Δ6D (P = 0.03) and SCD1 (P = 0.01) increased following PD98059 treatment, with a higher impact on SCD1 (24.5% *vs* 62.5%). Although pioglitazone increased the mRNA level (1.47 ± 0.10 *vs* 0.88 ± 0.02, P = 0.006) and activity index (1.40 ± 0.07 *vs* 0.79 ± 0.11, P < 0.001) of Δ6D, no such changes have been observed for SCD1 activity index in pioglitazone-treated cells. *SCD1* gene expression (+26.4%, P = 0.041) and activity index (+52.8%, P = 0.035) were significantly increased by MEK inhibition in the presence of pioglitazone, as compared with pioglitazone alone and control cells. However, the response of Δ6D expression and activity index to pioglitazone was unaffected by incubation with PD98059.

CONCLUSION: PPARγ and ERK1/2 signaling pathway affect differentially and may have inhibitory crosstalk effects on the genes expression of Δ6D and SCD1, and subsequently on their enzymatic activities.

Key words: Pioglitazone; PD98059; Δ6-desaturase; Stearoyl-CoA desaturase; HepG2 cells

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INTRODUCTION

Fatty acid desaturation is a lipid modification process that is critically important in multiple biological functions, such as cell membrane fluidity, signal transduction, differentiation, inflammatory responses and brain development[1,2]. Both the Δ6-desaturase (Δ6D) and stearoyl-CoA desaturase 1 (SCD1) are two important regulatory enzymes in hepatic *de novo* fatty acid synthesis. Activities of these enzymes provide essential precursors for structural cell components and bioactive metabolites such as prostaglandins[3]. Altered levels of both SCD1 activity[4-6] and Δ6D activity[7] have been reported in various human diseases.

The expressions of both enzymes are coordinately regulated and efficiently induced by the addition of thiazolidinediones (TZDs). TZDs are known as agonists of the gamma isoform of the peroxisome proliferator-activated receptors (PPARγ), a family of nuclear receptors regulating the expression of genes involved in fatty acid metabolism. Indeed, functional PPAR response elements in the promoter region of the Δ6D and SCD1 have been identified[8,9].

Pioglitazone, a member of the TZDs family, is widely used as an antidiabetic agent with glucose-lowering and lipid modifying effects in non-insulin-dependent diabetes mellitus[10]. Despite the increasing clinical use, the mechanisms by which pioglitazone exerts its effects are yet relatively unknown.

Alternatively, signaling pathways might modulate the activity of PPARγ to regulate cellular fatty acid desaturation events. We have recently shown that fatty acid content of HepG2 cells is susceptible to inhibition of MEK/ERK1/2 pathway[11]. Exposure of cells to the ERK1/2 pathway inhibitor induced an increase in monounsaturated fatty acids (MUFA) and the fatty acid desaturation index. Consistent with these findings, the data of Mauvoisin *et al*[12] show that SCD1 expression level is modulated *via* the ERK1/2 signaling. PPARγ agonists and ERK1/2 kinases may also interact in a complex manner with one another. It has been shown that pioglitazone activates ERK1/2 pathway[13]. ERK1/2, on the other hand, modulates PPARγ activation by altering protein phosphorylation and gene expression[14]. Thus, it is possible that ERK1/2 signaling affects Δ6D and SCD1 expression by altering PPARγ activity.

Based on the importance of PPARγ activity and ERK1/2 signaling in the regulation of cellular lipid, we tested the effects of pioglitazone and ERK1/2 signaling pathway blockade by PD98059 on Δ6D and SCD1 expression in HepG2 human hepatic cell line.

MATERIALS AND METHODS

Materials

Cell culture materials, media, FBS and standard fatty acid methyl esters were obtained from Sigma Chemical Company (St. Louis, MO, United States). Pioglitazone and PD98059 were purchased from Cayman Chemical (Ann Arbor, MI, United States). HepG2 cell line was obtained from the Pasteur Institute Culture Collection in Tehran. The TRIzol reagent for RNA isolation was purchased from Invitrogen (Carlsbad, CA, United States). AccuPower RT PreMix for the first-strand cDNA synthesis was purchased from Bioneer (Daejeon, South Korea). All other chemicals used were of analytical grade.

Cell culture

HepG2 cells were grown in RPMI1640 containing 10% FBS, L-glutamine (2 mmol/L), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 ℃, 5% CO_2. The cells were seeded at a density of 2.5×10^5/well in a 6-well plate. After allowing the cells to attach overnight, the medium was replaced with fresh medium containing ± pioglitazone (20 μmol/L) and PD98059 (20 μmol/L). Following 48 h incubation, culture medium was removed; the cell monolayer was washed, and collected for gene expression study and cellular fatty acid measurement.

RT/PCR analysis

Total RNA was purified with TRIzol reagent. One μg of RNA was reverse-transcribed using the AccuPower cDNA kit as per the manufacturer's instructions. Semiquantitation of the cDNA was performed by RT/PCR using the primer-dropping method according to a previous report[15]. Each reverse transcript (*i.e.*, Δ6D and SCD1 cDNA) was amplified with β-actin as an internal control. Under standard conditions of PCR, all the test transcripts were amplified within their log linear phase. The primers used were: D9D: F (5'-CATAATTCCCGACGTGGCTTT-3'), R (5'-AGGTTTGTAGTACCTCCTCTGGAACA-3') (150 bp); D6DF: (5'-CTGCCAACTGGTGGAATCATC-3'), R (5'-ACAAACACGTGCAGCATGTTC-3') (94 bp), β-actin: F (5'-TGGACTTCGAGCAAGAGATG-3'), R (5'-GAAGGAAGGCTGGAAGAGTG-3') (137 bp). For comparing the amount of PCR product between samples, a gel digitizing software, UVItec (version 11.01), was used for estimating the intensity of each band on the gel. Each experiment was repeated four times. The coefficients of variation (CV) were about 5%-8%.

Fatty acid analysis

Fatty acid methyl esters were extracted and analyzed for fatty acid composition, as described previously by us[16]. Briefly, fatty acid methyl ester derivatives formed from

Table 1 Effect of pioglitazone on the fatty acid composition of HepG2 human hepatic cells

	Control	Pioglitazone	Pioglitazone + PD98059
14:0 (myristic acid)	2.01 ± 0.42	2.03 ± 0.35	1.77 ± 0.17
16:0 (palmitic acid)	23.12 ± 1.24[a]	23.26 ± 1.11[a]	19.84 ± 1.21[b]
16:1 (palmitoleic acid)	7.44 ± 1.11	9.60 ± 1.55	8.52 ± 1.45
18:0 (stearic acid)	10.44 ± 1.29	11.12 ± 1.37	8.78 ± 1.14
18:1n 9 (oleic acid)	38.72 ± 1.43[a]	37.33 ± 0.80[a]	44.94 ± 1.08[b]
18:2n 6 (linoleic acid)	9.09 ± 0.27[a]	6.18 ± 0.68[b]	6.09 ± 0.36[b]
18:3n 6 (linolenic acid)	0.89 ± 0.13	0.72 ± 0.12	0.61 ± 0.12
20:4n 6 (arachidonic acid)	7.19 ± 0.79	8.62 ± 0.58	8.85 ± 0.85
20:5n 3 (eicosapentaenoic acid)	0.42 ± 0.13	0.47 ± 0.21	0.14 ± 0.10
22:6n 3 (docosahexaenoic acid)	0.68 ± 0.14	0.67 ± 0.10	0.46 ± 0.11
Saturated fatty acids	35.58 ± 1.29[a]	36.41 ± 2.18[a]	30.39 ± 2.05[b]
Monounsaturated fatty acids	46.16 ± 1.75[a]	46.93 ± 2.24[a]	53.46 ± 1.53[b]
Polyunsaturated fatty acids	18.27 ± 0.61	16.66 ± 1.44	16.15 ± 1.22

Cells were incubated with pioglitazone (20 μmol/L) and PD98059 (20 μmol/L) for 48 h. Lipid extracts were prepared and analyzed by gas liquid chromatography for a comprehensive fatty acid profile. The mean ± SD of 3 independent experiments done in duplicate are given. [a]$P < 0.05$ and [b]$P < 0.01$ (Tukey's test, [a]$\alpha = 0.05$). Detection limit was 0.05% of the total area.

isolated cellular lipids were separated on a 60 mm × 0.25 mm Teknokroma TR-CN100 column using a Buck Scientific model 610 gas chromatograph equipped with a split injector and a flame ionization detector. Helium was used as the carrier gas. The oven temperature program was 170-210 ℃, 1 ℃/min, and then isothermal for 45 min. Tridecanoic acid (13:0) was used as internal standard. Peak retention times were identified by injecting known standards.

Statistical analysis

Data presented are the mean ± SD of 3 or 4 separate experiments done in duplicate. Calculation of significance between groups was done according to analysis of variance (ANOVA) with *post hoc* Tukey's tests for multiple comparisons, and a $P < 0.05$ was considered statistically significant.

RESULTS

To determent the effect of ERK1/2 MAPK pathway on Δ6D and SCD1, HepG2 cells were treated with PD98059. PD98059 significantly increased the expression levels of both Δ6D ($P = 0.03$) and SCD1 ($P = 0.01$). Our data also revealed that ERK1/2 deprivation had a higher impact on SCD1 expression than on Δ6D expression (24.5% *vs* 62.5%; Figure 1).

To determent the effect of PPARγ stimulation on Δ6D and SCD1 expression, HepG2 cells were treated with pioglitazone, a PPARγ agonist. Δ6D showed significant increase in mRNA level (1.47 ± 0.10 *vs* 0.88 ± 0.02, $P = 0.006$), whereas SCD1 expression did not significantly change ($P = 0.47$; Figure 1). We next determined the effect of the PPARγ agonist on fatty acid composition of HepG2 cells (Table 1). The ratios of arachidonic acid (20:4n-6)/linoleic acid (18:2n-6) and oleic acid (18:1n-9)/stearic acid (18:0) were calculated as indices of Δ6 fatty acid desaturase and SCD1 activity, respectively. Incubation with pioglitazone reduced 18:2n-6 levels ($P = 0.001$) and increased Δ6D activity index (1.40 ± 0.07 *vs* 0.79 ± 0.11; $P < 0.001$). However, no such change has been observed for SCD1 activity index in pioglitazone-treated cells (Figure 2).

Comparison of control with the combined drug condition showed a significant increase in the expression of both Δ6D ($P = 0.02$) and SCD1 ($P = 0.04$). The expression of Δ6D increased in comparison to the condition which was just treated with PD98059 ($P = 0.032$), but comparable to pioglitazone alone. The expression of SCD1 was more than the situation treated with pioglitazone alone (1.15 ± 0.15 *vs* 0.91 ± 0.20, $P = 0.041$, Figure 1). Consistent with data from gene expression analyses, MEK inhibition induced a significant increase in SCD1 activity index (+52.82%, $P = 0.035$), compared with pioglitazone-treated and control cells. These changes were coupled with significant alteration in fatty acid composition, including increased percentage of MUFA ($P = 0.012$) and reduced saturated fatty acids (SFA; $P = 0.018$). In addition, the response of Δ6D activity index to pioglitazone was unaffected by incubation with PD98059 when compared to cells incubated with pioglitazone alone (Figure 2).

DISCUSSION

The expression of Δ6D and SCD1 is regulated by complex environmental and hormonal factors[17,18]. Activities of these enzymes can affect several hepatic metabolic processes, such as glucose metabolism and membrane permeability, through modulation of cellular fat content. On the other hand, altered lipid content of hepatic cells makes a major contribution in the rate of *de novo* lipogenesis and inducing steatosis[19]. Abnormal lipid uptake or *de novo* lipogenesis has been reported in various types of hepatic disorders, which is characterized by increased production of bioactive lipids and an inflammatory response[20-22]. In a previous study, we have demonstrated that the fat composition of hepatocellular carcinoma

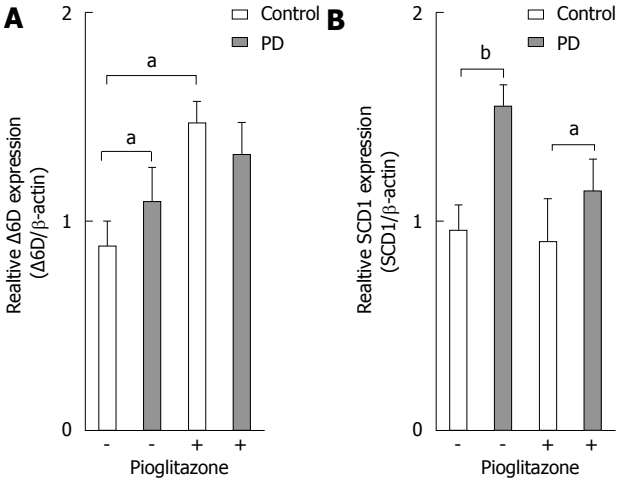

Figure 1 Effect of pioglitazone and MEK inhibition on mRNA expression of Δ6 desaturase and stearoyl-CoA desaturase. HepG2 cells were incubated for 48 h ± 20 μmol/L pioglitazone with or without 20 μmol/L PD98059 (PD) as indicated. Cell lysates were prepared and analyzed by reverse transcription-polymerase chain reaction for expression levels of Δ6 desaturase (Δ6D) and stearoyl-CoA desaturase (SCD1). Expression of Δ6D (A) and SCD1 (B) in each lysate were quantified and normalized to the amount of β-actin. The mean ± SD of four independent experiments are given. $^{a}P < 0.05$ and $^{b}P < 0.01$, Student's *t* test, respectively.

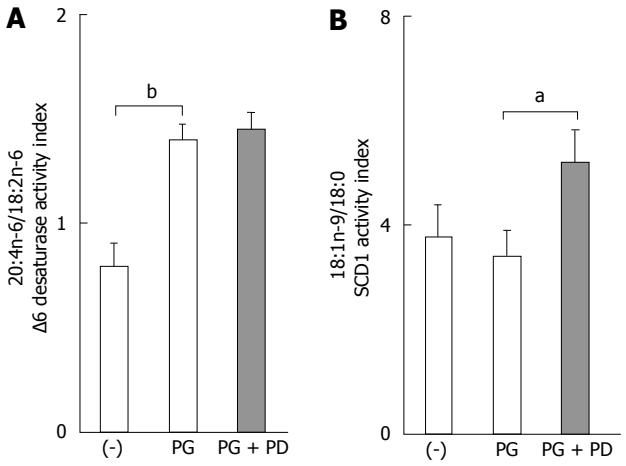

Figure 2 Effect of pioglitazone on derived fatty acid indices of HepG2 human hepatic cells. A: Δ6 desaturase activity index (20:4n 6/18:2n 6); B: Stearoyl-CoA desaturase 1 (SCD1) activity index (18:1n 9/18:0). Cells were incubated with pioglitazone (PG) (20 μmol/L) and PD98059 (PD; 20 μmol/L) for 48 h. Data are mean ± SD, $n = 3$. $^{a}P < 0.05$ and $^{b}P < 0.01$, Tukey's test, $\alpha = 0.05$. 18:0, stearic acid; 18:1n 9, oleic acid; 18:2n 6, linoleic acid; 20:4n 6, arachidonic acid.

cell line HepG2 was affected by MEK/ERK1/2 signaling. We concluded that MEK/ERK1/2 kinase signaling serves to coordinate fatty acid metabolism in HepG2 cells. In the current study, the expression levels of Δ6D and SCD1 were increased in the presence of MEK inhibitor. These findings are consistent with our earlier report indicating increased 18:1n-9/18:0 and 20:4n-6/18:2n-6 ratios as indices of desaturase activity after inhibition of ERK1/2 signaling. Thus, these enzymatic activities may be under inhibitory control of ERK1/2 signaling. Presumptively, ERK1/2 modulates desaturases expression by implying several molecular mechanisms, including enhanced affinity of transcription factors[12], gene suppression through direct interaction with DNA[23], modulation of transcription factors[24], and MAPK-dependent attenuation of PPARγ transcriptional activity[14,25]. Camp *et al*[14] reported that activation of ERK1/2 in adipocytes abrogates both ligand-independent and ligand-dependent activities of PPARγ. Taken together, the regulation of desaturases expression by key transcription factors such as PPARγ could be modulated by ERK1/2 cascade.

PPAR-γ has been shown to be critically important in multiple biological functions[26]. TZDs, high-affinity synthetic PPARγ agonists, mediate the transcription of PPARγ dependant genes by binding to PPARs as a ligand[27]. Herein, we have shown that treatment of HepG2 cells with pioglitazone, a PPARγ agonist, increased both Δ6D mRNA expression and Δ6D activity index whereas had no effect on SCD1. These results led us to speculate that an additional mechanism was at work. Remarkably, it has been illustrated that PPARγ agonists not only can function in a PPARγ dependent manner but also are capable of activating ERK1/2 pathway independently of PPARγ[28]. Accordingly, Kempná *et al*[13] demonstrated that pioglitazone activates ERK1/2 MAPK pathway in NCI-H295R cells. Presumably, no changes in SCD1 expression could be attributed to equal and opposite effects of pioglitazone *via* PPARγ dependent and PPARγ independent mechanisms. In accordance with our results, the administration of pioglitazone to Zucker obese rats did not affect the mRNA level of SCD1[29]. However, treatment of rats fed a high-sucrose diet with TZDs decreased significantly the hepatic SCD1 mRNA expression[30]. In this context, TZDs have also been reported to significantly reduce Δ6D mRNA level[31,32]. These contradictory findings might be due to differences in applying TZDs, types of cell lines, tissues and animal models[33,34].

Cotreatment of HepG2 cells with pioglitazone and ERK1/2 inhibitor PD98059 resulted in enhanced rather than additive or synergistic expression. It is of particular interest that activated ERK1/2 MAPK pathway *via* pioglitazone in a PPARγ independent manner could also occur without phosphorylation of upstream MEK[13]. So, this underscores pioglitazone ability in activation of ERK1/2 MAPK pathway through other additional pathways. Accordingly, in presence of both PD98059 and pioglitazone the ERK1/2, which may be activated independently of MEK, could prevent synergistic increase of SCD1 expression, and subsequently SCD1 activity. Studies in humans have reported that increased expression of SCD1 may protect against insulin resistance[35,36]. The fact that a combination treatment using the PPARγ agonist pioglitazone and the MEK/ERK1/2 inhibitor was more efficient at inducing SCD1 than pioglitazone alone suggests that TZDs along with MEK/ERK1/2 inhibition may be therapeutically beneficial for insulin

resistance related to type 2 diabetic patients.

To our knowledge, this study is the first study to examine the combined effect of PPARγ agonist and ERK1/2 blockade on the gene expression and derived activity index of fatty acid desaturases. The regulatory effects were simultaneously analyzed by studying the expression and endogenous activity index of both Δ6D and SCD1, which made it possible to identify similarities and differences. It remained to be clarified what mechanism is involved in PPARγ and ERK1/2 MAPK crosstalk in the regulation of fatty acid desaturases in the liver cells.

In conclusion, our study showed that PPARγ and ERK1/2 MAPK signaling pathway affect the gene expression and activity of Δ6D and SCD1 in hepatic HepG2 cells. Furthermore, a possible inhibitory crosstalk between PPARγ and ERK1/2 MAPK signaling pathway may have different affects on Δ6D and SCD1 genes expression, and subsequently on their enzymatic activities.

COMMENTS

Background

The Δ6-desaturase (Δ6D) and stearoyl-CoA desaturase 1 (SCD1) are two important regulatory enzymes in hepatic *de novo* fatty acid synthesis. Altered levels of desturases activity have been reported in various human diseases. Both enzymes are coordinately regulated by Pioglitazone, a drug that is widely used as an antidiabetic agent with glucose-lowering and lipid modifying effects. But up to now, the mechanisms by which pioglitazone exerts its effects are yet relatively unknown. Intracellular signaling pathways might modulate the effect of Pioglitazone on fatty acid metabolism.

Research frontiers

Pioglitazone specifically stimulates peroxisome proliferator-activated receptor gamma (PPARγ). In the area of hepatic lipid metabolism, the research hotspot was to clarify the integrated effect of pioglitazone and MEK/ERK1/2 pathway signaling in regulation of fatty acid desaturation.

Innovations and breakthroughs

Previously, it has been shown that PPARγ agonists not only can function in a PPARγ dependent manner but also are capable of activating ERK1/2 pathway independently of PPARγ. Based on the importance of PPARγ activity and ERK1/2 signaling in the regulation of cellular lipid, the authors tested the effects of pioglitazone and ERK1/2 signaling pathway on Δ6D and SCD1 activity in a human hepatic cell line. The regulatory effects were simultaneously analyzed by studying the expression and endogenous activity index of both Δ6D and SCD1, which made it possible to identify similarities and differences.

Applications

The study results suggest that a combination therapy using pioglitazone and MEK/ERK1/2 inhibition might improve the treatment efficiency of lipid disorders, in particular the changes in diabetes, obesity and atherosclerosis.

Terminology

Desaturase: A fatty acid desaturase is an enzyme that removes two hydrogen atoms from a fatty acid, creating a carbon/carbon double bond. Δ9 desaturase, also known as stearoyl-CoA desaturase-1, produces oleic acid by desaturating stearic acid. Δ6D synthesize highly unsaturated fatty acids such as eicosopentaenoic and arachidonic acid; MEK/ERK1/2 signaling pathway: The MEK/ERK1/2 pathway is a chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. The pathway includes many proteins, including ERK and MEK; PPARγ, also known as the glitazone receptor, is a nuclear receptor which regulates fatty acid storage and glucose metabolism in human.

Peer review

The present manuscript reports the modulatory effect of ERK/MEK1/2 pathway and PPARγ on Δ6D and SCD1 in hepatic HepG2 cells. The study is interesting and well written.

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CASE REPORT

Giant cell hepatitis with autoimmune hemolytic anemia in a nine month old infant

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Abstract

Giant cell hepatitis (GCH) with autoimmune hemolytic anemia is a rare entity, limited to young children, with an unknown pathogenesis. We report the case of 9-mo old who presented with fever, diarrhea and jaundice four days before hospitalization. Physical examination found pallor, jaundice and hepatosplenomegaly. The laboratory workup showed serum total bilirubin at 101 μmol/L, conjugated bilirubin at 84 μmol/L, hemolytic anemia, thrombocytopenia and immunoglobulin G (IgG) and anti-C3d positive direct Coombs' test. The antinuclear, anti-smooth muscle and liver kidney microsomes 1 non-organ specific autoantibodies, antiendomisium antibodies were negative. Serological assays for viral hepatitis B and C, cytomegalovirus, herpes simplex and Epstein Barr virus were negative. The association of acute liver failure, Evan's syndrome, positive direct Coomb's test of mixed type (IgG and C3) and the absence of organ and non-organ specific autoantibodies suggested the diagnosis of GCH. The diagnosis was confirmed by a needle liver biopsy. The patient was treated by corticosteroids, immunomodulatory therapy and azathioprine but died with septicemia.

Key words: Giant cell hepatitis; Anemia; Hemolytic; Autoimmune; Child

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INTRODUCTION

Giant cell hepatitis (GCH) associated with autoimmune hemolytic anemia (AHA) is a rare individualized entity, particularly affecting infants, with an unknown pathogenesis and poor outcome[1,2]. Only 27 cases have been reported in pediatric reviews[2-6]. It usually presents as a severe hepatitis, jaundice and fever that begins about 1 year of age and is associated with AHA with a positive direct Coombs' test[3]. In this article, we describe a new case in a nine month old infant who presented with pallor, fever and jaundice and whose outcome was severe in spite of early treatment. We also review literature data concerning clinical presentation and therapeutic modalities of this rare entity.

CASE REPORT

A 9-mo old infant with no pathological medical history presented with jaundice, fever and watery diarrhea for the last 4 d. Physical examination showed a eutrophic,

Table 1 Biological findings before and after treatment (prednisone + azathioprine)

	J1 of hospitalization	J10 of hospitalization	J17 treatment	J60 treatment
Leucocytes (elements/μL)	12400	19800	23300	27800
PNN (%)	77	42	60	57
Hemoglobin (g/dL)	9.3	6.8	11.4	10.4
MCV	79.9	86.6	85.4	96.6
Reticulocytes (elements/μL)	114000	17600	92300	184000
Platelets (elements/μL)	71000	7000	153000	152000
SAT/ALT (UI/L)	1020/810	1430/1886	368/600	1310/522
Total bilirubin	101	425	190	425
Direct bilirubin (μmol/L)	82.6	122	78	291
GGT (UI/L)	170		122	34
PT (%)	55	51	70	20
CRP (mg/L)	42	-	12	20
AFP (ng/mL)	179		29	29.63
LDH (UI/L)	2089	1092	400	-
Haptoglobin (g/L)	0.6	0	-	-
DCT	-	+ à IgG/C3d	-	+ à IgG/C3
Ferritinemia (ng/mL)	163	-	-	-
Fibrinogen (g/L)	3.3	-	-	-
Triglycerides (mmol/L)	3.99	-	-	-

PNN: Polynuclear neutrophils; MCV: Mean corpuscular volume; SAT: Serum aspartate transaminase; ALT: Serum alanine amino transferase; GGT: Gamma glutamyl transpeptidase; PT: Prothrombine time; CRP: C reactive protein; AFP: Alpha fetoprotein; LDH: Lactate deshydrogenase; DCT: Direct Coombs' test; IgG: Immunoglobulin G.

febrile (38.3 ℃) infant. Jaundice was obvious with pallor, hepatomegaly (liver span of 11 cm) and enlarged spleen. Laboratory features (Table 1) showed a bicytopenia: normochromic normocytic regenerative anemia and thrombocytopenia, associated with hepatic insufficiency, cytolysis and cholestasis with elevated conjugated bilirubin and gamma glutamyl transpeptidase. Alpha fetoprotein (AFP) was high as well.

With this association of a febrile jaundice with liver injury, infectious causes were first suspected. Serologies of hepatitis A, B and C, of cytomegalovirus, Epstein Barr virus, herpes simplex virus and human immunodeficiency virus were all negative. A metabolic cause, particularly tyrosinemia, was also evoked given the elevated levels of AFP and delta-aminolevulinic acid (12.08 mg/mL); this diagnosis was also ruled out since the amino and organic acids chromatographies were normal. We also sought for an autoimmune hepatitis, given the presence of bicytopenia, but anti-mitochondrial, anti-LKM1, anti-nuclear and anti smooth muscle were absent. Immune system screening revealed high immunoglobulin G (IgG) levels (11.4 g/L) and low complement fractions (C3 = 0.73 g/L and C4 = 0.04g/L). Cellular immunity was normal.

On the other hand, an *Escherichia Coli* (*E. Coli*) was detected in both urine and blood cultures. This infection was handled with adequate intravenous antibiotics. Abdominal ultrasonography showed an enlarged hyperechogenous liver, a homogenous splenomegaly, normal non dilated biliary ducts and the presence of a hyperechogenous cuff surrounding the hepatic pedicle.

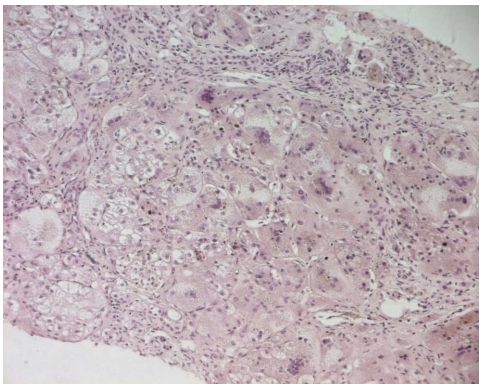

Figure 1 Liver biopsy showing a diffuse transformation of hepatocytes into giant cells with necrosis areas.

Initially, there was no positive progression as fever persisted and both jaundice and pallor worsened. At day 10 of hospitalization, hemoglobin decreased down to 6.8 g/dL (normocytic normochromic regenerative anemia) and platelets were at 6000 elements/μL. Haptoglobin was null and direct Coombs' test was positive (IgG and C3d).

The association of hepatic insufficiency and AHA in a young infant on one hand and the absence of auto-antibodies on the other hand, made us consider the diagnosis of GCH. Thus, a liver biopsy was performed after blood and platelet transfusions and the patient's stabilization.

Histological analysis confirmed the diagnosis; there was a diffuse transformation of hepatic cells into giant cells with areas of necrosis (Figure 1).

The patient first received an intravenous immunoglobulin course at the dose of 1g/kg per day during 2 d, then both steroids (prednisone: 2 mg/kg per day) and immunosuppressive [Azathioprine (Imurel®): 1 mg/kg per day] therapies were started.

The immediate course was favorable, with clinical as well as biological improvement since day 17 of steroid therapy (Table 1). At day 30 of hospitalization, the infant was discharged from hospital under the same treatment. He was seen 15 d later with stable biological data.

However, he was hospitalized again 60 d after the onset of the therapy, with fever, edema and ascites. We found a urinary tract infection with identification of a multiresistant *E. Coli*, hepatic insufficiency [prothrombin time (PT) = 20%] and aggravation of the cytolysis (Table 1). The patient received intravenous antibiotics. Liver insufficiency was handled with symptomatic measures. In spite of this management, fever persisted, renal failure appeared and liver function worsened (PT = 12%). The infant died with septic shock. Follow up had lasted 3 mo since the beginning of symptoms.

DISCUSSION

GCH associated with AHA was described in 1981 in young children with a severe presentation and high mortality (39% of reported cases)[4]. This pathological entity is rare[1,2]. 27 cases have been reported in pediatric reviews[2,4-6]. In 2011, an Italian pediatrician's team reported the biggest series of cases of this pathology. They described 16 children during a 28-year period[4].

The pathogenesis of this entity is still unknown[1]. Several authors have suggested an autoimmune origin, especially in the presence of AHA and hypergammaglobulin levels[1].

According to Maggiore *et al*[4], elements suggesting an autoimmune origin among their 16 patients were a positive family history of autoimmune conditions, such as type 1 diabetes, thyroiditis and psoriasis, positive findings for auto-antibodies in some patients, thrombocytopenia, the improvement under immunosuppressive therapy and the decline when tapering doses. However, this hypothesis is not approved by other authors since auto-antibodies are often absent and there is no typical histological evidence of autoimmune hepatitis[2]. Another hypothesis suggests that the underlying mechanism could be a non controlled release of cytokines by activated T lymphocytes as well as Küppfer cells[2]. In the present case, we found neither family nor personal history of autoimmune disease; however, there was an initial positive response to steroids as well as immunosuppressive therapy.

GCH associated with AHA specifically occurs in young infants; the first signs often begin between 2.5 and 24 mo of age[4,5]. In our patient, the first signs were seen at 9 mo. Hepatocyte transformations into giant cells can happen during the neonatal period and is considered a non specific reaction of immature hepatocytes to various aggressions[4].

Each time hepatitis is associated with AHA with positive Coombs' test, either with IgG or complement, in an infant, liver biopsy must be performed as soon as possible in order to confirm the diagnosis of GCH so that early treatment can be started[4]. The prognosis of this pathology is often poor[7]. Our patient had an early relapse and died of septic shock.

Treatment possibilities include steroids and/or azathioprine, cyclosporine, cyclophosphamide, tacrolimus, intravenous immunoglobulins, mercaptopurine, mycophenolate mofetil, vincristine, plasmapheresis and anti CD20 (Rituximab®)[1]. Splenectomy was also proposed in some patients as an alternative for AHA resistant to medical treatment[1,8].

In the biggest reported series in the literature, initial treatment was based on prednisone (2-3 mg/kg per day) and azathioprine (1-2 mg/kg per day) in 13 out of 16 cases. With the association of cyclosporine in the 3 remaining cases, the latter had a severe presentation[4]. Total remission with a normalized transaminases level was reached in 8 cases/16. Remission was partial in 6 cases/16 and absent in the 2 remaining cases[4]. Relapse occurred in 11 patients, 10 of whom presented an AHA resistant to medications. Anti CD20 were successfully used in 2 patients and splenectomy was performed in 5 cases/10; only 2 of them got positive results[4].

In this series, 4 patients died because of severe sepsis and post transplantation, respectively in 3 and 1 cases. The other patients (12 cases) are alive and one underwent liver transplantation[4]. This series illustrates the severity of this pathology and the difficulties of treatment, given the high risk of relapse as well as therapy resistant AHA after relapse. Liver transplantation was reported in 6 patients in the literature; 3 cases relapsed after transplantation[4].

In our patient, we started therapy by an intravenous course of immunoglobulins followed by the association of prednisone and azathioprine with partial response (improvement without normalization of transaminases). Relapse was rapid (less than 4 mo) with severe hepatic insufficiency but without recurrence of hematological disorders. Our patient died due to septic shock.

In conclusion, GCH associated with AHA is a severe pathological entity. This diagnosis should be evoked when hepatitis of unknown origin occurs in an infant and will be confirmed by liver biopsy. Early treatment associating corticotherapy and immunosuppressive drugs with sufficient doses is essential to reach total remission with normal transaminases. Treatment must be maintained as long as possible in order to avoid relapses which are more resistant to therapies.

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CASE REPORT

Dermatomyositis associated with gallbladder carcinoma: A case report

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Abstract

Patients with gallbladder carcinoma can present with a variety of paraneoplastic syndromes, including Cushing's syndrome, hypercalcemia, acanthosis nigricans, bullous pemphigoid, dermatomyositis and the sign of Leser-Trélat. Surgical resection of the primary tumor results in resolution of these paraneoplastic syndromes. We present a 67-year old female with facial and cervical erythema who was initially diagnosed with dermatomyositis. However, an abdominal computed tomography (CT) and positron emission tomography-CT scan was suspicious for gallbladder carcinoma with lymph node metastasis. After surgical resection, her dermatomyositis was resolved. This case demonstrates that dermatomyositis may be a manifestation of preexisting gallbladder carcinoma.

Key words: Dermatomyositis; Paraneoplastic syndromes; Gallbladder carcinoma

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INTRODUCTION

Prior research has shown that paraneoplastic syndromes can be eliminated by surgical resection of the primary tumor. We present a case of a 67-year old female with dermatomyositis who underwent radical resection of a gallbladder carcinoma. After surgery, her dermatomyositis completely disappeared.

CASE REPORT

In February of 2012, a 67-year old woman, who presented with a two month history of facial and cervical erythema accompanied by pruritis and 20 d of arthralgia of the hands and morning stiffness, was admitted to our institution's Department of Dermatology[1]. She had a past medical history of hypertension for about 5 years. She denied prolonged fever, malaise, anorexia, weight loss, recent vaccination, Raynaud's phenomenon, cough, hemoptysis, hematemesis, melena, hematochezia, oral ulcer, alopecia, jaundice, drug intake (*e.g.*, penicillamine, clofibrate, statins, corticosteroids, emetine, colchicine, chloroquine or zidovudine), breast lumps or abdominal pain. She was a nonsmoker, non-diabetic and without any history of exposure. There was no history of loss of consciousness, headache, vomiting, seizures, sensory dysfunction or involvement of distal limb muscles, bladder or bowels[2].

Physical examination revealed an elderly woman without pyrexia, jaundice, clubbing or lymphadenopathy[2]. Her pulse was 80/min, regular rate and rhythm, and her blood pressure was 130/86 mmHg, both supine and standing. Her temperature was 36.6 ℃ and respira-

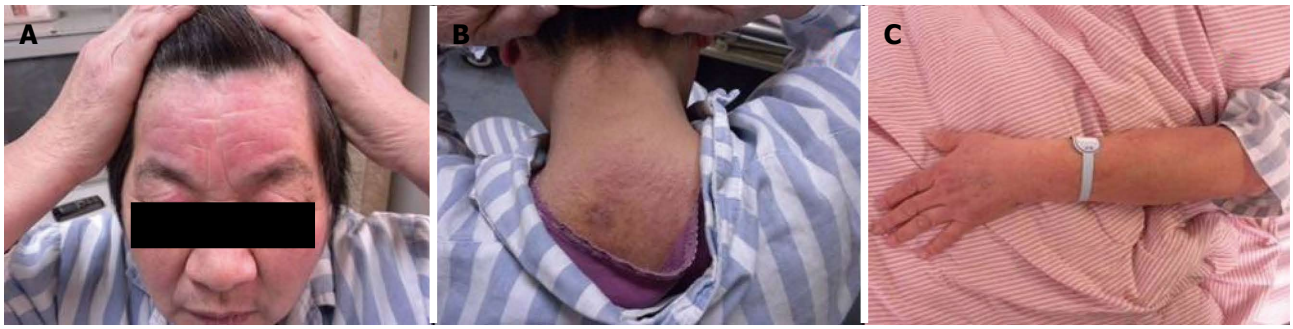

Figure 1 Physical examination. A: The skin lesions in the forehead and upper eyelids; B: The skin lesions in the neck; C: Erythema to the nail fold.

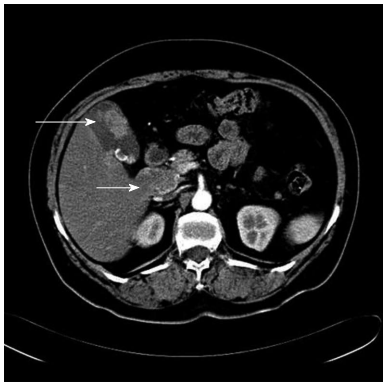

Figure 2 Abdominal computed tomography. Gallbladder carcinoma (long arrow) with lymph node metastasis (short arrow).

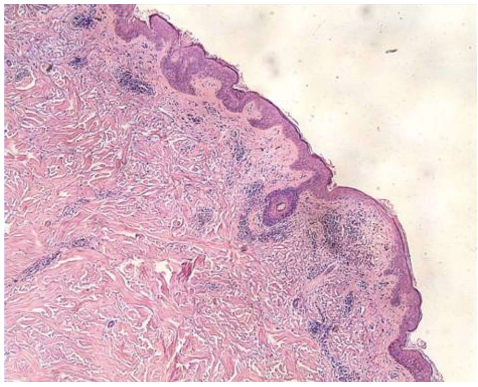

Figure 3 Biopsy of skin. Mild degree of hyperkeratosis, hair follicle angle plug, epidermal atrophy, basal cell liquefaction degeneration, dermal papilla edema, agglomerate lymphocytic infiltrate around vessels, and dermal adnexa.

tory rate 18/min. Her forehead, neck and upper eyelids were erythematous and moderately edematous. The skin lesions were distributed over the extensor side of the metacarpophalangeal joints of the hand. Erythema to the nail fold was seen (Figure 1). These lesions were of mixed character, like maculopapular eruptions. She did not have weakness of the forearms, arms, shoulder or pelvic girdle muscles.

Laboratory examinations demonstrated a hemoglobin of 118 g/L, a total leucocyte count of 10200/mm^3 (differential count N 88.6; L 6.4; M 4.9; E 0) and an erythrocyte sedimentation rate of 10 mm. Her serum bilirubin, urea and creatinine levels were 18.8 μmol/L, 6.31 mmol/L and 49.1 μmol/L, respectively. Her tests of synthetic liver function tests revealed an alkaline phosphatase of 51.1 U/L, aspartate aminotransferase of 58.0 U/L, alanine aminotransferase of 66.1 U/L, gamma-glutamyltransferase of 19.3 U/L, total protein of 58.8 g/dL, serum albumin of 35.1 g/dL and globulin of 23.7 g/dL. Chest X-ray, electrocardiogram and serum electrolytes (sodium, potassium and magnesium) were within normal limits. Her C-reactive protein was 14.1 mg/L, serum creatine kinase 61.7 U/L and lactate dehydrogenase 213 U/L. Her serum antinuclear antibody was positive at a titer of 1:320 with a speckled pattern. Serum tumor markers included cytokeratin-19 4.71 ng/mL and neuron-specific enolase 19.08 μg/L. Carcinoembryonic antigen, alpha-fetoprotein, carcinoembryonic antigen (CA)-125, CA-19-9 and CA-724 did not reveal any abnormalities.

Computed tomography of the abdomen and positron emission tomography-computed tomography scans were performed, which were suspicious for gallbladder calculi with associated soft tissue density and peripancreatic lymphadenopathy (Figure 2).

A biopsy of the skin was performed, which showed a mild degree of hyperkeratosis, hair follicle angle plug, epidermal atrophy, basal cell liquefaction and degeneration with more bite pigment cells, dermal papillary edema, agglomerate lymphocytic infiltration around the vessels and dermal adnexa, and a small amount of mucin between collagen fibers (Figure 3).

She was treated with loratadine, cetirizine and hydroxychloroquine to reduce the sensitivity to ultraviolet rays and magnesium isoglycyrrhizinate as an anti-inflammatory. Vitamin C, calcium gluconate and coenzyme complex was used to relieve symptoms.

Radical cholecystectomy was performed on February 27, 2012. The gallbladder measured 8 cm × 6 cm × 4 cm and a hard, whitish mass on the fundus measured 4 cm, without clear borders. A hard, pale lump was seen on the liver surface, between the gallbladder and the inferior vena cava, with a clear boundary and a diameter of 2 cm. In addition, encapsulated and demarcated lumps were seen between the hepatoduodenal ligament and the inferior vena cava and duodenum, with a diameter of 6 cm; the tumor-node-metastasis classification for this patient is T4N2M0 (Figure 4). The gallbladder and tumor on the

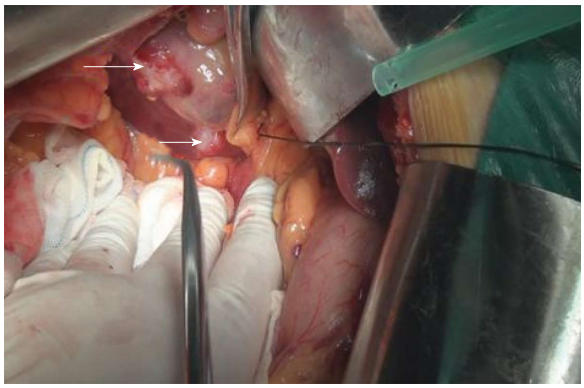

Figure 4 Radical resection of the gallbladder was performed. A hard, whitish mass located at the fundus of gallbladder (long arrow) and a hard, pale lump was seen in the liver surface (short arrow), between the gallbladder and the inferior vena cava.

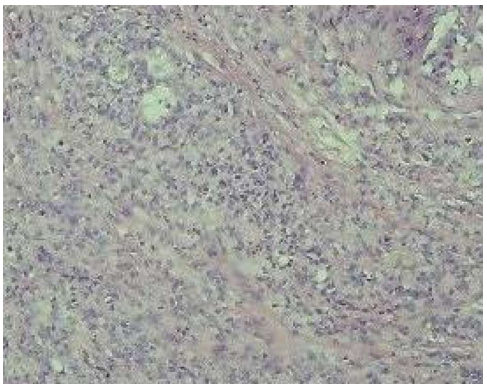

Figure 5 Histopathology of tumor. Low differentiated gallbladder adenocarcinoma, involving all layers of the gallbladder and infringing upon nerves.

liver surface, with an additional 2 cm margin of normal liver parenchyma, were completely resected.

On pathology, a well-differentiated gallbladder adenocarcinoma measuring 3 cm × 2 cm × 1.5 cm was found, involving all layers of the gallbladder wall, with perineural invasion. Mucosal glands showed moderate and high-grade dysplasia. The liver lesion was consistent with a 2 cm × 2 cm × 1 cm well-differentiated metastatic deposit. One lymph node was excised and was positive for adenocarcinoma (Figure 5). Immunohistochemical staining of the gallbladder tumor was negative for synaptophysin, chromogranin A and CD56, but immunoreactive for e-cadherin and vimentin. Some tumor cells were positive for cytokeratin 7. Liver tumor cells were negative for hepatocyte and cytokeratin 7.

The patient had an uncomplicated hospital course and was discharged on postoperative day 14. The skin lesions gradually abated. As of July 2012, there has been no evidence of cancer recurrence and the skin lesions have completely disappeared.

DISCUSSION

Dermatomyositis is an uncommon idiopathic inflammatory disorder of the skin and skeletal muscle that affects 10 adults per million and 3.2 children per million worldwide[3]. Cutaneous manifestations are classified as pathognomonic, characteristic or compatible[4]. Pathognomonic features, as in our patient, include small purple or red flat papules on the extensor surfaces, particularly the joints of the hand (Gottron papules). A skin or muscle biopsy is helpful in differentiating this condition from other papulosquamous diseases or contact or atopic dermatitis. Changes to the nail fold and a heliotropic rash are highly characteristic manifestations. For up to 40% of patients, skin findings may be the only manifestation[5].

Paraneoplastic syndromes are defined as a constellation of systemic symptoms in cancer patients remote from the tumor, either due to metastasis or caused by treatment[6]. Most of the symptoms are caused by a release of a constellation of tumor-related proteins, which can either be produced directly by the tumor itself or alternatively be secreted by the immune system[7].

A number of studies have suggested an association between inflammatory myopathy and malignancy, which appears stronger with dermatomyositis and weaker with polymyositis[8-11]. The cause of malignancy in patients with dermatomyositis is not clear, but compromised immunity or immunosuppressive or cytotoxic therapy may be involved. Secretion of hormones, such as adrenocorticotropic hormone, growth hormone and serotonin, has been previously described. With the exception of gastric and lung carcinoma, which seem to more commonly associated with dermatomyositis, the spectrum of cancers in patients with inflammatory myopathy is similar to that of the general population[10]. However, the association of dermatomyositis with gallbladder carcinoma is extremely rare. In this case of dermatomyositis associated with gallbladder carcinoma, her skin lesions completely disappeared after radical gallbladder cancer surgery. In conclusion, dermatomyositis can be considered as a paraneoplastic syndrome in gallbladder carcinoma, especially in adults over the age of 50 years. Abdominal ultrasound or computed tomography is necessary to make the diagnosis. Prognosis will be improved significantly through appropriate treatment of the primary tumor in the early stage.

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Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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