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Protective effects of oxysophoridine on alcoholic hepatic injury in mice

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The aim of study is to detect the effects and relevant mechanisms of oxysophoridine on alcoholic hepatic injury in mice. Sixty male Institute of Cancer Research (ICR) mice were randomly divided into a normal control group, an alcoholic liver injury model group, a positive control (tiopronin) group and an oxysophoridine (250, 125, 62.5 mg/kg) group. Consecutive interventions were conducted on each group for 10 days; specimens were obtained according to requirements of the testing indicators 16 h after the last drug administration. The protective effects were evaluated by biochemical parameters including serum aspartate transaminase (AST), alanine transferase (ALT), reduced glutathione (GPx), liver malondialdehyde (MDA) and superoxide dismutase (SOD). The pathological changes of the liver in microstructure and ultrastructure were observed. A decreased level of serum ALT, AST activity and liver MDA content with increased liver SOD, GPx activity ($P<0.05$) were observed ($P<0.05$) in the oxysophoridine group compared with the alcoholic liver injury model group, in which elevated serum ALT and AST activity were recorded, along with a marked increase of liver MDA ($P<0.05$), decrease of liver SOD and GPx ($P<0.05$). The following changes of the liver were observed in the model group: Blurred contour of the hepatic lobule with punctated or focal necrosis in partial liver cells, multiple intracellular microvesicular steatosis, with lipid droplets formed in the cytoplasm, marked swelling of hepatocytes and disarrangement of hepatic cords, swelling of mitochondria, with disappeared or broken cristae, enlarged endoplasmic reticulum and condensed chromatin. Compared with the model group, a decrease in pathological changes of various degrees was observed in the oxysophoridine group at various doses. The result indicates that oxysophoridine prevents alcoholic liver damage in mice and the protective effect may be associated with anti-oxidative stress.

Key words: Alcohol, oxysophoridine, oxidative stress, antioxidants.

INTRODUCTION

Alcohol-related liver disease (ALD) is a major public health hazard in developed as well as in developing world

(Abdul et al., 2010). ALD is a multifactorial disease that progresses through a set of distinct stages. ALD involves hepatocellular injury induced by consumption of ethanol. ALD is considered to be progressive and associated with duration and quantity of alcohol consumed (Lucey et al., 2009). Upwards to 90% of individuals consuming alcohol on a daily basis develop fatty liver (steatosis) which can resolve upon cessation of alcohol consumption (Smathers et al., 2011). Despite extensive research, alcohol abuse remains one of the most common causes of acute and chronic liver diseases in the world. Despite our best efforts, alcohol remains one of the most common causes of both acute and chronic liver disease in the United States (Sofair et al., 2010). In Western countries, up to 50% of cases of end-stage liver disease have alcohol as a major etiologic factor (Orholm et al., 1985). Excessive alcohol consumption is the third leading preventable cause of death in the United States. Alcohol-related deaths, excluding accidents/homicides, accounted for 22,073 deaths in the United States in 2006 with 13,000 of those specifically attributed to ALD (Heron et al., 2009). Alcohol represents a major financial burden on the overall economy as well, with an estimated cost of US$185 billion annually lost productivity, motor vehicle accidents among others (Kim et al., 2002). In China, ALD incidence increased year by year. Due to increased frequency of drinking and change of diet construction, such as the increase of fat content, the incidence of alcoholic liver disease has increased in China, becoming another important risk factor for morbility and mortality in addition to viral hepatitis (Jing et al., 2008). Alcoholic liver disease is found to be a common disease in Zhejiang Province, indicating an urgent need for the Public education on alcohol abuse and the treatment on related health problems (Youming et al., 2003). Drugs including bifendate, tiopronin and bicyclol have been reported to have protective effect against ALD (Hirayama et al., 1983; Peng et al., 2003; Zhao et al., 2008); however, there is no satisfactory therapy for alcoholic liver disease at present. Except for the combination of abstinence from alcohol and supportive care (Bouneva et al., 2003), however, definite treatment strategies for ALD remain undefined. Developing agents possessing hepatoprotective effects from natural products and traditional Chinese formula has become the focus of research in recent years (Leeetal, 2007; Tsenget al., 2007; Chandan et al., 2008). Moreover, ALD imposes a significant economic burden from the lost wages family, high health care costs people, and lost productivity human. Chinese herbal medicine has the advantage in terms of multi-targeting efficacy, lower toxicity, as well as lower cost. In recent years, more and more researchers have begun to focus on herbs and their constituents in liver injury prevention and treatment.

Natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases.(Samy et al., 2013). The use of medicinal plants forms the basis of the traditional healing system in many developing countries (Steenkamp et al., 2013). The world health organization (WHO) highly appreciated the conventional medical practices for treatment and precautionary measure of diarrheal diseases (Barakat et al., 2013).

The plants of the genus *Sophora* (subfamily Papilionaceae in the family Leguminosae) range from the temperate to the tropic areas in the world. Some of the *Sophora* plants are important sources of Chinese drugs, such as ku-shen (roots of *Sophora flavescens* Ait.), Shan-dou-gen (roots of *Sophora tonkinensis* Gagnep), and ku-dou-zi (roots of *Sophora alopecuroides* L.). Phytochemical investigations show that there exist more than twenty chemical compounds, belonging to alkaloids, flavonoids, volatile oil, organic acid, amino acid, protein, saccharide among others (Hong et al., 2011). Alkaloids from *S. alopecuroides* L. show strong medicinal functions when used for treatment of many diseases such as depressant, analgesic, hypothermic, antipyretic, cardiotonic activities, virus, liver fibrosis, tumors and improves immunity (Xuegong et al., 2007; Hong et al., 2011).

Oxysophoridine (OSR) is a major alkaloid extracted from *S. alopecuroides* L. Previous studies have determined its pharmacological activities in terms of anti-inflammation, immune regulation, growth suppression of hepatocellular carcinoma (Jianqiang et al., 2001; Yao et al., 2012). Basic research has confirmed the hepatic protection effect of its structural analogue oxymatrine (Jue and Guojun, 2000); however, there has been no published account to demonstrate that OSR is beneficial in the treatment of ALD. This experiment was designed to observe the protective effect of OSR on alcoholic liver injury in mice, and to study its oxidation mechanism.

MATERIALS AND METHODS

Drugs

Oxysophoridine was provided by the Ningxia Institute of Materia Medica. It is a white powder with molecular weight (Mw) of 264, melting point (mp) of l62°C, purity of 99% with batch number, 991230. Tiopronin was obtained from the Henan Xinyi pharmaceutical Co., Ltd with batch number, 100909. Alanine transferase (ALT), aspartate transaminase (AST), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) kits were purchased from Jiancheng Bioengineering Institute (Nanjing). All other reagents used were of highest purity and were commercially available; the 50% ethanol solution was diluted from anhydrous ethanol, with a dose of 5 g/kg body weight.

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#These authors equally to this study, therefore they are both first authors.

Animals

Sixty male, healthy adult Institute of Cancer Research (ICR) mice weighing 18-22 g were provided by the Laboratory Animals Center of Ningxia Medical University (Yinchuan, China). The mice were housed in an animal care facility at room temperature of 25±1°C with a 12 h light/dark cycle, and were given free access to standard pellet diet and tap water. The mice were left for three days to acclimatize before the treatment. This study was approved by Institutional Animal Ethics Committee, at Ningxia Medical University. The study was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (1985).

Treatment

The mice were randomly divided in to six groups with ten in each to evaluate the protective effect of OSR pretreatment on ethanol induced acute liver toxicity. The normal control (NC) group received only distilled water. The alcohol control (AC) group received only ethanol (5 mg/kg body weight). Group OSR I received ethanol and oxysophoridine (250 mg/kg body weight). Group OSR II received ethanol and oxysophoridine (125 mg/kg body weight). Group OSR III received ethanol and oxysophoridine (62.5mg/kg body weight). The positive control (PC) group received ethanol and tiopronin.

Experimental design

The experiment was carried out for 10 days. All mice underwent administration by gavage. At 7:00-8:00, saline was given to the NC and AC groups, oxysophoridine was given to all the experimental groups (OSR I – OSR III), the PC group mice were fed with tiopronin (30 mg/kg body weight). At 19:00-20:00, the 50% ethanol was given at 5 g/kg body weight to all groups except the NC group.

Preparation of serum and tissue homogenate

Preparation of serum and tissue homogenate were determined by the method of Leelavinothan and Arumugam (2008). At the end of the experimental period, the animals were sacrificed by cervical dislocation under mild anesthesia. Blood was collected and centrifuged for serum separation. The tissues were dissected out, weighed and washed using ice cold saline solution. A part of tissues were minced and homogenized (10% w/v) in 0.9% physiological saline buffer and centrifuged at 10000 r/min at 4°C. The resulting supernatant was used for various biochemical assays.

Light microscopic examination

Liver tissues were cut into ~3 mm-thick slices and fixed with 10% neutral formalin. The tissue slices were embedded in paraplast. Tissue sections of 5 µm were stained by hematoxylin and eosin (H and E) and observed with a Nikon Eclipse E400 light microscope.

Electron microscopic examination

To observe ethanol-induced ultrastructural changes by conventional electron microscopy, livers were fixed *in situ* by vascular perfusion with Karnovsky's fixative (2% para-formaldehyde and 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4) and post-fixed in 1% osmium tetraoxide. Ultra-thin sections were stained by uranyl acetate and lead citrate and observed with a Philips transmission electron microscope.

Assay for serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

The activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed spectrophotometrically according to the standard procedures, using commercially available diagnostic kits (Jiancheng).

AST and ALT activities were determined by the method of Reitman and Frankel (1957). Each substrate, 0.5 ml (2 mMα-ketoglutarate and 200 mM L-alanine or L-aspartate) was incubated for 5 min at 37°C in a water bath. 0.1 ml serum was then added and the volume was adjusted to 1 ml with 0.1 M, pH 7.4 phosphate buffer. The reaction mixture was incubated for exactly 30 min at 37°C for ALT and AST, respectively. Then to the reaction mixture, 0.5 ml of 1 mM 2,4-dinitro-phenylhydrazine (DNPH) was added,after another 30 min at 37°C; the color was developed by addition of 5 ml of 0.4 N NaOH and the product read at 505 nm.

Hepatic malondialdehyde (MDA) determination

MDA was assayed by determining the rate of production of thiobarbituric acid-reactive components of Ohkawa et al. (1979). One gram of the liver was homogenized in 1.15% KCl buffer on ice. An aliquot of 0.2 mL was mixed with solution containing 20% acetic acid, 0.8% thiobarbituric acid and 8.1% sodium dodecyl sulfate, heated in water bath at 95°C for 60 min. The solution was centrifuged for 10 min at 4000 rpm, and the absorbance of the supernatant fraction was determined at a wavelength of 532 nm. The content of MDA was expressed in terms of nmol/mg protein.

Hepatic superoxide dismutase (SOD) Activity.

SOD was determined using the method of Winterbourn (1975), in which the light-triggered release of superoxide radicals from riboflavin leads to the formation of a blue complex through reaction with nitroblue tetrazolium. 1 g of the liver was homogenized in 0.1 M phosphate buffer pH 7.4 on ice and cleared by centrifugation at 3000 rpm at 4°C for 15 min. The supernatant fraction was incubated in solution containing 0.067 M phosphate buffer pH 7.8, 0.1 M ethlenediaminetetracetic acid (EDTA), 1.5 mM nitroblue tetrazolium (NBT) and 0.12 mM riboflavin for 10 min in an illuminated chamber with an 18 W fluorescent lamp. Absorbance was recorded at 560 nm, and SOD activity was expressed as units/mg protein.

Hepatic glutathione peroxidase (GPx) activity

GPx activity was measured by the method described by Ellman (1959). Briefly, reaction mixture containing 0.2 ml of 0.4 M phosphate buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of tissue homogenate (homogenate on 0.4 M phosphate buffer, pH 7.0), and 0.2 ml glutathione, 0.1 of 0.2 mM H_2O_2 was used. The content was incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% trichloroacetic acid (TCA) and centrifuged. Absorbance was recorded at 412 nm, and GPX activity was expressed as units/mg protein.

Statistical analysis

The data for various biochemical parameters were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using the statistical statistics software package (SPSS for Windows, V. 13.0). *P* values less than 0.05 were considered as statistically significant.

Table 1. Effect of OSR on hepatic markers (serum ALT and AST) in liver injury mice.

Group	ALT (IU/L)	AST (IU/L)
Control（NC）	12.51±5.02	22.97±2.89
50%Ethanol(AC)	88.31±7.88*	96.56±7.27*
Tiopronin(PC)	27.07±8.29*▲	42.06±4.20*▲
OSR250mg/kg	17.38±4.79▲	37.53±5.62*▲
OSR125 mg/kg	53.87±7.21*▲	42.98±3.20*▲
OSR 62.5 mg/kg	38.07±5.46*▲	45.36±4.94*▲

Table 2. Changes of activities of antioxidant enzymes in injury mice.

Group	MDA (nmol/mgprot)	SOD (U/ mgprot)	GPx (U/ mgprot)
Control(NC)	14.00±3.85	70.94±9.58	30.28±1.37
50%Ethanal(AC)	64.07±2.07*	47.97±3.67*	12.06±1.21*
Tiopronin(PC)	17.72±4.57▲	45.92±2.11*	22.71±2.87*▲
OSR250mg/kg	17.98±6.62▲	74.28±5.45▲	26.17±2.12*▲
OSR125mg/kg	25.46±3.50*▲	58.82±2.12*	21.63±2.03*▲
OSR62.5mg/kg	21.26±4.05*▲	31.45±3.77*	21.22±3.31*▲

Data were presented as mean (S.E.M). Number=10 per group. *$p < 0.05$ vs control group. ▲$p < 0.05$ vs AC group.

RESULTS

Effects of OSR on the hepatic enzymes

Results of the effect of OSR on ALT and AST are presented in Table 1. Protective effect of OSR on serum ALT and AST level were observed. Significance change in these parameters were found in the ethanol treated group as compared to control(p<0.05). The PC group was found significantly (p<0.05) effective in the normalization of these markers when compared to ethanol treated group. Pretreatment with OSR was found significantly (p<0.05) effective in the normalization of these markers when compared to ethanol treated group. The OSR groups show difference as compared to control. The activities of antioxidant enzymes SOD and GPx in liver are given in Table 2. A significant (P < 0.05) decrease in the activities of enzymatic antioxidants were observed in alcohol treated mice when compared with control mice. Administration of OSR to alcohol treated mice significantly (P < 0.05) increased the activities of enzymatic antioxidants when compared to alcohol treated mice.

MDA formation was measured to demonstrate the oxidative damage on LPO of ethanol induced liver injury in mice. A significant (p<0.05) increase of the MDA formation was found in the ethanol treated group when compared with control. We have observed that pretreatment with OSR at OSR I – OSR III leads to the significant (p<0.05) prevention of membrane damage when compared to ethanol treated group (Table 2).

Changes under light microscope

In the control group, light microscopy revealed clear hepatic lobules, orderly arranged hepatic plates, radial arrangement of hepatic cords, normal hepatic sinusoids, rich and pink cell plasma and clear structure of the nucleus, and a centered central vein (Figure 1A). However, hepatic changes were observed in the alcohol model group, including a blurred contour of the hepatic lobule, with punctate or focal necrosis in partial liver cells, as well as alcoholic foamy degeneration. The histological features of true microvesicular steatosis included hepatocyte enlargement, flocculent alteration of the cytoplasm as well as deep stained nucleus (Figure 1B). In the OSR groups, the structure of the lobules remained clear, hepatic cords were arranged radially and roughly in order, the hepatic sinusoids were relatively normal, and the cellular swelling was obviously alleviated with a few lipid droplets observed (Figure 1C).

Changes observed under the electron microscope

The hepatocytes of the control group were normally polygonal with oval-shaped nuclei and one or two nucleoli; the cytoplasm was filled with organelles, particularly rough endoplasmic reticulum, smooth endoplasmic reticulum, golgi apparatus, ribosomes, mitochondria and glycogen particles (Figure 2A). In contrast, the heaptocytes of the ethanol model group showed marked pathological alterations, which were represented by large

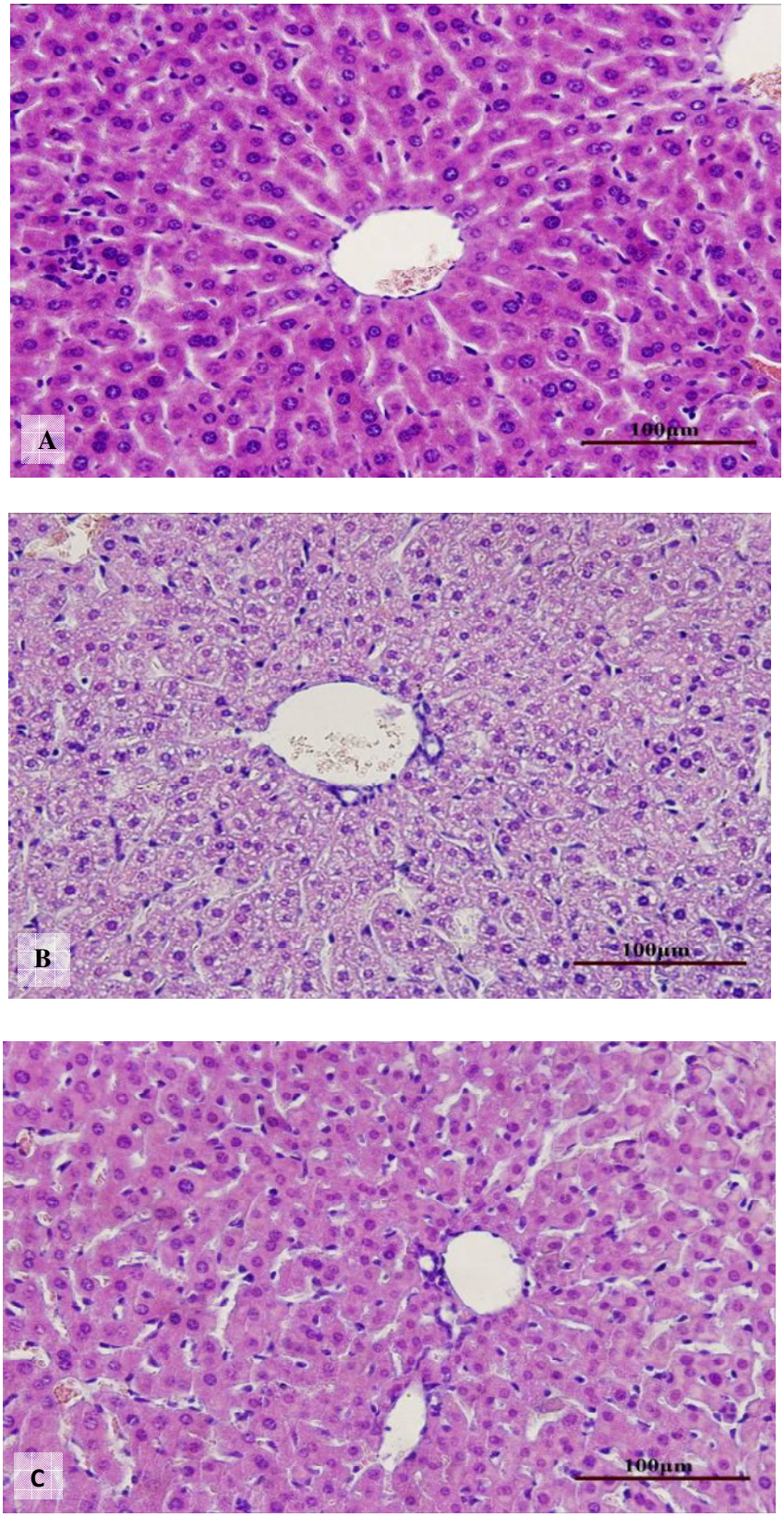

Figure 1. Hematoxylin-eosin-stained liver sections (400x). A, Control group showed normal liver histopathology; B, ethanol-treated group showed steatosis; C, OSR125mg/kg treatment showed examples of alleviation of steatosis and inflammation.

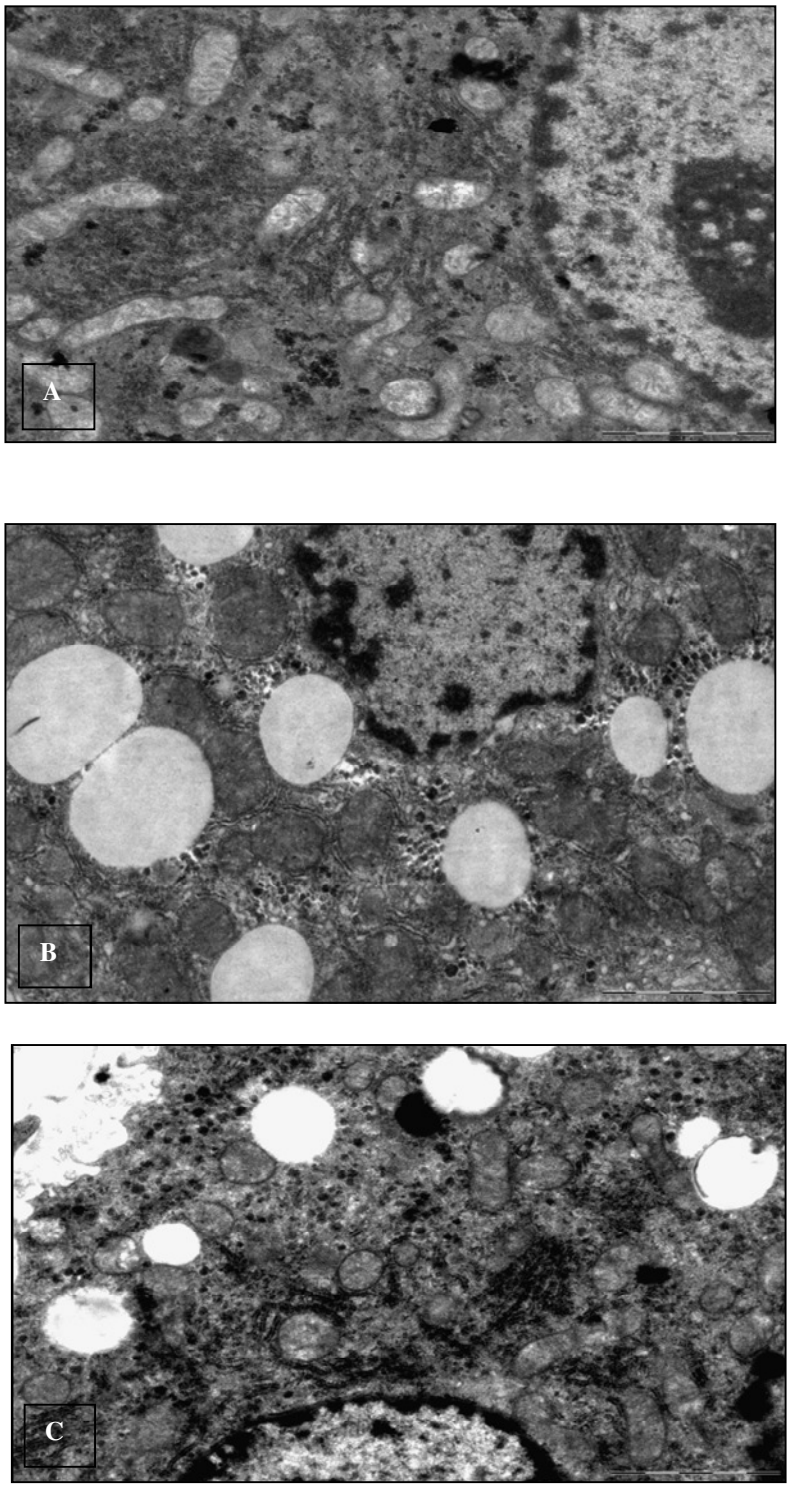

Figure 2. Electron micrograph of the liver (3000x). A, Control group showed normal liver histopathology; B, ethanol-treated group showed steatosis; C, ethanol + OSR 125 mg/kg treatment showed examples of alleviation in steatosis and inflammation.

lipid droplets in the hepatocytes, along with introcession of nuclear membrane, enlargement of mitochondria, disappeared mitochondrial crista, and dilated alteration of the rough endoplasmic reticulum (Figure 2B). The pathological changes were improved in OSR pretreated mice, which exhibited shape of normal liver ultrastructure and

organelle quantity (Figure 2C).

DISCUSSION

Alcohol abuse is a public health issue which has received wide attention. An abrupt increase of alcohol consumption in China was found in an epidemiological survey since the 1980s, accompanied by increasing problems due to alcohol abuse (Min et al., 2010). An accelerated tendency is observed in both morbidity and mortality of alcoholic liver disease, which was second compared to viral hepatitis.

To examine ethanol effects on the liver, most studies applied gastric intubation or intraperitoneal injection in rodents (Siegmund et al., 2003). Interestingly, the most commonly used amount of ethanol to mimic so called human binge drinking was 5–6 g/kg body weight, which approximately resembles 0.75 l of whiskey (40% v/v) in a 75 kg human (Sören et al., 2005). Our experiment chose to dilute the 50% ethanol solution from anhydrous ethanol every day, with a dose of 5g/kg body weight for 10 days. It caused alterations in liver morphology such as hepatocyte swelling and a decrease in sinusoidal fenestrae as seen by electron microscopy in mice.

The liver contains many kinds of enzymes. ALT is a useful screening factor for the detection of liver disease. AST and ALT are reliable markers of a liver function. Increased level of serum enzymes such as AST and ALT indicate increased permeability as well as damage and/or necrosis of hepatocytes (Hou et al., 2010). In our study, we have found that ethanol consumption caused a marked increase in the activities of ALT and AST due to severe damage of the tissue membrane. Decreased activities of these enzymes indicate the hepatoprotective effect of OSR.

The histological features of true microvesicular steatosis include hepatocyte enlargement, flocculent alteration of the cytoplasm and deep stained nucleus (Matthew and Elizabeth, 2008). In our study, the AC group cell showed alcoholic foamy degeneration and signs of hepatocyte ballooning. These results are consistent with several previous reports showing increased lipid peroxidation in other experiments (Min et al., 2010). Changes were improved in OSR and tiopronin pretreated mice, which exhibited areas of normal liver architecture and patches of necrotic hepatocytes, which indicates that pretreatment of OSR may protect liver cells from ethanol damage.

These changes were confirmed at the ultrastructural level. The hepatocytes from the ethanol model group showed large lipid droplets, along with introcession of nuclear membrane, enlargement of mitochondria, disappearance of mitochondrial crista, and dilated alteration of the rough endoplasmic reticulum. Compared with the AC group, pathological changes showed a noticeable improvement in the oxysophoridine group at varied degrees accordingly, which indicates that OSR may improve pathological changes of ultrastructure in hepatocytes, such as decrease of lipid droplets and recovery of the nuclei shape.

Many pathways are thought to be involved in ALD, including oxidative stress and mitochondrial damage (Stewart et al., 2001). Ethanol administration has been shown to induce oxidative stress (Dupont et al., 2000). Ethanol metabolism generates free radicals that result in degeneration of hepatic cells, due to alcohol-induced lipid peroxidation (Lieber, 1997). Lipid peroxidation serves as a marker of cellular oxidative stress and has long been recognized as a major causative factor of oxidative damage in chronic diseases (Son et al., 2007). Lipid peroxidation causes apoptosis and necrosis of hepatocytes, with the release of substances as MDA that triggers inflammatory and immune-mediated mechanisms of hepatocyte injury (Chikako et al., 2004). The elevation of MDA indicates increased production of lipid peroxide, suggesting that the content of MDA may reflect the extent of the cell being attacked by free radicals (Li et al., 2004). In our study, we found significant increase in serum MDA concentration in the AC group. Therefore, low levels of MDA in the experimental groups are indicative that OSR scavenges free radical.

Free radical scavenging enzymes, such as SOD and GPx, are the first line of defense against oxidative injury. The inhibition of antioxidant system may cause the accumulation of H_2O_2 or its decomposition products (Halliwell, 1994). Consistent with these reports, our results also showed decreased activities of SOD and GPx in tissues in the ethanol model group. Therefore, high level of these enzymes in the experimental groups suggests that the OSR helps eliminate free radicals in liver tissue. Administration of OSR restored the activities of enzymatic antioxidant in liver.

Conclusion

In conclusion, the results of functional tests together with histological observations suggest that OSR can play a significant role in preventing lipid peroxidation, and can be protective against alcohol-induced hepatotoxicity and liver damage. Furthermore, it enhances the antioxidant system of the body by elevating the activity of SOD and GPx.

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to development and implementation of this study.

ABBREVIATIONS

ALD, alcohol-related liver disease; **OSR,** oxysophoridine; **ALT,** alanine transferase; **AST,** aspartate transaminase; **MDA,** malondialdehyde; **SOD,** superoxide dismutase; **GPx,** glutathione peroxidase ; **NC,** normal control; **AC,** alcohol control; **PC,** positive control; H and E, hematoxylin and eosin; **TCA,** trichloroacetic acid; **EDTA,** ,ethlenediaminetetracetic acid; **NBT,**nitroblue tetrazolium; **DNPH,** 2,4-dinitro-phenylhydrazine .

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