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The anti-fatty liver effects of garlic oil on acute ethanol-exposed mice

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ABSTRACT

The protective effects of single dose of garlic oil (GO) on acute ethanol-induced fatty liver were investigated. Mice were treated with ethanol (4.8 g/kg bw) to induce acute fatty liver. The liver index, the serum and hepatic triglyceride (TG) levels and the histological changes were examined to evaluate the protective effects. Hepatic malondialdehyde (MDA), glutathione (GSH) levels and superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GSH-Px), glutathione-*S*-transferase (GST) activities were determined for the antioxidant capacity assay. Acute ethanol exposure resulted in the enlargement of the liver index and the increase of the serum and hepatic TG levels ($P < 0.01$), which were dramatically attenuated by GO pretreatment in a dose-dependent manner ($P < 0.01$). GO treatment (simultaneously with ethanol exposure) exhibited similar effects to those of pretreatment, while no obviously protective effects were displayed when it was used at 2 h after ethanol intake. Histological changes were paralleled to these indices. Beside this, GO dramatically prolonged the drunken time and shortened the waking time, and these effects were superior to those of silymarin and tea polyphenol. In addition, GO dose-dependently suppressed the elevation of MDA levels, restored the GSH levels and enhanced the SOD, GR and GST activities. Compared with the ethanol group, the MDA levels decreased by 14.2% ($P < 0.05$), 29.9% and 32.8% ($P < 0.01$) in GO groups 50, 100 and 200 mg/kg, respectively. The GST activity increased by 9.97%, 19.94% ($P < 0.05$) and 42.12% ($P < 0.01$) of the ethanol group in GO groups 50, 100 and 200 mg/kg, respectively, while the GR activity increased by 28.57% ($P < 0.05$), 37.97% ($P < 0.01$), 50.45% ($P < 0.01$) of the ethanol group in GO groups 50, 100 and 200 mg/kg, respectively. These data indicated that single dose of GO possessed ability to prevent acute ethanol-induced fatty liver, but may lose its capacity when used after ethanol exposure. The protective effects should be associated with its antioxidative activities.

1. Introduction

Alcoholic liver diseases (ALD) attract attentions of the researchers all over the world. Although the mechanisms underlying are still not well understood, increasing evidence indicates the involvement of oxidative stress in the development of the ALD [1–4]. Previous studies have demonstrated that ethanol-induced liver injury was associated with enhanced lipid peroxidation, protein carbonyl formation, formation of the 1-hydroxyl ethyl radical, formation of lipid radicals and decreases in hepatic antioxidant defense, especially GSH [3]. Pretreatment with antioxidants such as vitamin E, vitamin C, and other agents that

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enhance antioxidant capacity have been tried to deal with the ethanol-induced liver injury [5–8]. Alcoholic fatty liver is one of the important and earliest pathological changes caused by the ethanol abuse, and there is still lack of effective therapies to prevent or reverse this disease. Along with the resurgence of the medical herbs in the world, some natural compounds isolated from plants or fruits have been used to cope with alcoholic fatty liver, and these protective effects were believed to be associated with the antioxidant activity [9–11].

Garlic (*Allium sativum*) has been widely used as a foodstuff and also a traditional medicine for many centuries throughout the world. Garlic oil (GO), one of the products of garlic, is usually prepared by steam distillation and has been demonstrated to contain more than 30 organosulfur compounds including diallyl trisulfide (DATS), diallyl disulfide (DADS) and diallyl sulfide (DAS), which have been considered to be the major biological agents [12–14]. A series of biological benefits (such as antiatherosclerotic, antihypertensive, antimicrobial, anticancer, immunomodulation, and radioprotection) have been reported [15], and these benefits have been mainly attributed to the antioxidative activity [16]. GO and its organosulfur components have been shown to increase the activity of GSH-related antioxidant system in rat liver [17], and have been demonstrated to provide protective effects against oxidative stress by chemicals [18–21].

Searching the published papers, most of the previous studies about garlic focus on the single component (e.g. DAS, DADS, DATS), and no reports about the protective effects of GO on acute ethanol-induced fatty liver were found. Since GO is composed of many organosulfur compounds which may work synergistically, it may exhibit some biological activities which single component may not possess. Beside this, as the distillate, the effective dose can be more easily achieved by consumption with GO than that with garlic. In addition, GO is now commercially available and can be taken as a functional food easily. Furthermore, GO pretreatment for 30 days was found to markedly prevent acute ethanol-induced increase of the activity of the serum aminotransferase and the hepatic lipid peroxidation in our previous studies [22]. In view of these, chemoprotective effects of GO on acute ethanol-induced liver injury were worthwhile to be further studied.

The current experiment was designed to investigate the protective effects of single dose of GO on acute ethanol-induced fatty liver. We aimed (i) to investigate the dose-dependent effects and the possible mechanisms; (ii) to explore the anti-fatty liver activities when used after ethanol exposure; (iii) to compare with some other useful agents (tea polyphenol and silymarin).

2. Material and methods

2.1. Materials

GO was purchased from Xuchang Yuanhua Biotechnology, Inc. (Xuchang, CN). Tea polyphenol (TP) was obtained from Tea Research Institute of China Academy of Agricultural Sciences (Hangzhou, CN). Silymarin was provided by Kangwei pharmacy (Wendeng, CN). Triglyceride (TG) assay kit was purchased from Zhongsheng Beikong Bioengineering Institute (BJ, CN). Malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GSH-Px) and glutathione-*S*-transferase (GST) assay kits were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, CN). BCA™ protein assay kits were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). All other chemicals were of the highest quality commercially available.

2.2. Animals

Male Kun-Ming mice, weighing 18–22 g, were provided by the Laboratory Animal Center of Shandong University. The mice were maintained at approximately 22 °C with a 12-h light cycle, and had free access to commercial food and tap water.

2.3. Experimental procedure

2.3.1. Dose-dependent effects of GO on acute ethanol-induced fatty liver

60 mice were used for the dose-dependent studies. After acclimated to the standard laboratory conditions for 3 days, the mice were divided into 5 groups randomly, including one control group, one ethanol group, and three GO groups (50, 100 and 200 mg/kg bw). The animals were pretreated by gavage with different dose of GO or corn oil before receiving ethanol (4.8 g/kg bw, i.g.), and sacrificed after 16 h of ethanol exposure. The serum was obtained for the TG assay. The liver was dissected, weighed, frozen in liquid nitrogen, and then stored at −80 °C for the hepatic biochemical examination and histological assay.

2.3.2. The protective effects of GO applied at different time

60 mice were used to evaluate the anti-fatty liver activities of single dose of GO administrated at different time. The mice were randomized into 5 groups with 12 in each, including one control group, one ethanol group and 3 GO groups. Mice in GO groups were administrated with GO (2 h before, simultaneously with, or 2 h after ethanol exposure, respectively) by gavage at the same doses of 100 mg/kg bw. The animals were sacrificed at 16 h after acute ethanol exposure (4.8 g/kg bw, i.g.). The serum and hepatic TG levels were determined. The liver sections were stained by Sudan III for histological assay.

2.3.3. The comparison of the protective effects of GO, TP and silymarin

60 mice were used to compare the protective effects of GO, TP and silymarin. The mice were pretreated with GO (100 mg/kg bw), TP (100 mg/kg bw), silymarin (150 mg/kg bw) or saline orally, before exposed to ethanol (4.8 g/kg bw, i.g.), and were sacrificed at 16 h after ethanol treatment. The drunken test, the serum and hepatic TG levels, and the histological changes were used for the comparative study.

Table 1
Effects of GO on the liver index, serum and hepatic TG levels (mean ± S.D., $n = 12$)

Groups	Body weights (g)	Liver weights (g)	Liver index (%)	Serum TG (mmol/L)	Hepatic TG (mg/g liver)
Control	25.71 ± 2.31	1.218 ± 0.130	4.70 ± 0.33	0.93 ± 0.27	8.9 ± 1.6
Ethanol	23.05 ± 1.83**	1.292 ± 0.152	5.61 ± 0.63**	2.54 ± 0.54**	20.4 ± 5.0**
GO (50 mg/kg)	23.64 ± 1.77	1.243 ± 0.115	5.31 ± 0.41	1.53 ± 0.31##	16.6 ± 2.6#
GO (100 mg/kg)	23.40 ± 2.29	1.227 ± 0.209	5.23 ± 0.58#	1.41 ± 0.35##	14.6 ± 2.2##
GO (200 mg/kg)	23.27 ± 1.40	1.185 ± 0.103	5.10 ± 0.39##	1.40 ± 0.27##	10.7 ± 3.1##

The mice were pretreated with GO (50, 100 and 200 mg/kg bw) or corn oil, and then were exposed to ethanol (4.8 g/kg). Compared with the control group, $^{**}P < 0.01$; compared with the ethanol group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

2.4. Calculation of the liver index

$$\text{Liver index}\,(\%) = \frac{\text{liver weight}}{\text{body weight}} \times 100$$

2.5. Determination of the serum and hepatic TG levels

Serum TG levels were determined using Automatic Biochemical Analyzer (BTS 370, Spanish) according to the methods provided by the commercial assay kits. The liver tissues were powdered under liquid nitrogen in a mortar firstly, and the hepatic TG was extracted for 48 h using chloroform/methanol (2:1, v/v) at 4 °C [23]. Then the hepatic TG content was quantified using Spectrophotometer (UV/visible-120-2, SHIMADZU Corporation) as the method described by the commercial assay kit. The concentration was expressed as mmol/L and mg/g liver, respectively.

2.6. Preparation of the 10% liver homogenate

Liver tissue was homogenized in 9 volume of cold buffer (0.01 mol/L Tris–HCl, 0.0001 mol/L EDTA-2Na, 0.01 mol/L saccharose, and 0.8% saline, pH 7.4) at 4 °C. Then the homogenates were centrifuged at 4 °C (3000 rpm/min, 15 min) and the supernatant was stored for determination. Protein content was determined using BCA™ protein assay kit.

2.7. The hepatic lipid peroxidation assay

The lipid peroxidation (LPO) was evaluated by the thiobarbituric acid reactive substances method (TBARS) and was expressed as MDA level, which was assayed with a commercial kit according to the manufacturer's instruction. The method was used to obtain a spectrophotometric measurement of the color produced during the reaction of TBA with MDA at 535 nm. The MDA level was expressed as nmol/mg protein.

2.8. Hepatic antioxidant system examination

The levels of GSH and activities of SOD, GR, GSH-Px, GST were measured using commercial assay kits (Nanjing Jiancheng Institute, China) according to the manufacturer' instructions.

In brief, the GSH level was determined at 412 nm with a spectrophotometer following the reaction with 5,5-dithiobes-(2-ni-trobenzoic acid) (DTNB), and GSH level was expressed as mg/g protein. SOD activity was measured following the reduction of nitrite by a xanthine–xanthine oxidase system which is a superoxide anion generator. The activity was expressed as U/mg protein. GR activity was quantified by the decrease of the NADPH, which can be measured at 340 nm, and GR activity was expressed as U/g protein. GSH-Px and GST activities were assayed by the decrease of the GSH levels, which can be determined at 412 nm. The activities were expressed as U/mg protein and U/mg protein, respectively.

2.9. Histological assessment

Sudan III staining was used for the assessment of the fat accumulation in the liver. Briefly, 5 μm sections were cut from frozen samples, affixed to microscope slide, and allowed to air-dry at room temperature. The liver sections were firstly stained in Sudan III for 2 min, and then counterstained with hematoxylin. The hepatic fat accumulation was observed under light microscope, and each liver section was assigned a score from 0 to 3, where 0 = no steatosis; 1 = slight steatosis; 2 = moderate steatosis; 3 = severe steatosis.

2.10. Statistics

All data were expressed as mean ± S.D. SPSS 13.0 statistical software was used for the statistical analysis. One-way analysis of variance (ANOVA) was performed for the analysis of the biochemical indices. Nonparametric Test (Rank sum test) was used for the histological examination comparison. Differences were considered significantly at $P < 0.05$ level.

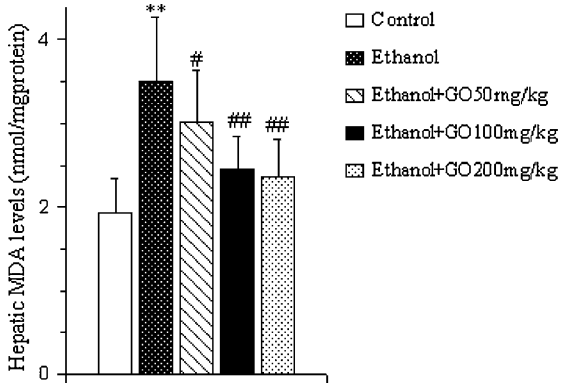

Fig. 1. Effects of GO on the hepatic MDA levels. Data were shown as mean ± S.D. ($n = 12$). Compared with the control group, $^{**}P < 0.01$; compared with the ethanol group, $^{\#}P < 0.01$, $^{\#\#}P < 0.01$.

Table 2
Effects of GO on the hepatic antioxidant system (mean ± S.D., $n = 12$)

Groups	GSH (mg/g protein)	SOD (U/mg protein)	GR (U/g protein)	GSH-Px (U/mg protein)	GST (U/mg protein)
Control	32.00 ± 2.14	253.40 ± 15.25	7.72 ± 2.02	126.80 ± 39.20	26.81 ± 3.18
Ethanol	28.67 ± 2.22**	241.20 ± 20.23	5.53 ± 1.41**	119.53 ± 27.23	25.38 ± 5.04
GO (50)	28.74 ± 1.74	261.21 ± 32.51	7.11 ± 1.16#	121.76 ± 24.09	27.91 ± 4.67
GO (100)	30.57 ± 2.40#	276.12 ± 15.58##	7.63 ± 1.48##	118.27 ± 18.97	30.44 ± 7.16#
GO (200)	31.26 ± 3.35##	282.20 ± 31.95##	8.32 ± 1.62##	125.70 ± 21.41	36.07 ± 7.15##

The mice were pretreated with GO (50, 100 and 200 mg/kg bw) or corn oil, and then were exposed to ethanol (4.8 g/kg). Compared with the control group, **$P < 0.01$; compared with the ethanol group, #$P < 0.05$, ##$P < 0.01$.

3. Results

3.1. Dose-dependent effects of GO on acute ethanol-induced fatty liver

3.1.1. Effects of GO on the liver index, serum and hepatic TG levels

As shown in Table 1, acute ethanol intake led to the marked increase of the liver index, and the serum and hepatic TG levels ($P < 0.01$). Single dosage of GO dose-dependently suppressed these adverse effects. Compared with the ethanol group, the serum TG levels decreased by 39.8%, 44.5% and 44.9% ($P < 0.01$) in GO groups 50, 100, 200 mg/kg, respectively, while the hepatic TG levels decreased by 18.6%, 28.4% and 47.5% ($P < 0.01$), respectively.

3.1.2. Effects of GO on the hepatic lipid peroxidation

The lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substrates (TBARS) in homogenate and expressed as the MDA levels. Acute ethanol exposure induced the significant increase of the MDA level ($P < 0.01$), which was dramatically attenuated by GO pretreatment. Compared with the ethanol group, the MDA levels decreased by 14.2% ($P < 0.05$), 29.9% and 32.8% ($P < 0.01$) in GO groups 50, 100 and 200 mg/kg, respectively (Fig. 1).

3.1.3. Effects of GO on the hepatic GSH level and the activities of GR, SOD, GSH-Px and GST

The hepatic GSH level decreased slightly but significantly after acute ethanol exposure ($P < 0.01$). GO restored the GSH levels in a dose-dependent manner. The activities of GST and GR were dramatically enhanced in GO-pretreated groups' mice. The GST activity increased by 9.97%, 19.94% ($P < 0.05$) and 42.12% ($P < 0.01$) of the ethanol group in GO groups 50, 100 and 200 mg/kg, respectively, while the GR activity increased by 28.57% ($P < 0.05$), 37.97% ($P < 0.01$), 50.45% ($P < 0.01$) of the ethanol group, respectively (Table 2).

3.1.4. Histological examination

Acute ethanol exposure (4.8 g/kg bw) induced typical hepatosteatosis with higher scores, while GO-pretreatment markedly inhibited the accumulation of fat droplets (Table 3 and Fig. 2). 6 mice (50%) in GO group (200 mg/kg bw) did not exhibit fat droplets in liver sections and the morphology of the liver was similar to that of the mice in control group.

3.2. The protective effects of GO applied at different time

3.2.1. Effects of GO on the liver index, serum and hepatic TG levels

GO pretreatment significantly inhibited acute ethanol-induced increase of the TG level and the enlargement of liver index. GO treatment (simultaneously with ethanol) exhibited similar effects to those of GO pretreatment, while GO post-treatment did not show effective protection as the pretreatment (Table 4).

3.2.2. Histological assay

As shown in Table 5 and Fig. 3, GO treatment (2 h before or simultaneously with ethanol intake) significantly attenuated acute ethanol-induced accumulation of fat in the mice liver. However, many droplets were observed when it was used at 2 h after ethanol exposure.

3.3. The comparison of the protective effects of GO, TP and silymarin

3.3.1. Drunken test examination

9 mice in ethanol group exhibited disappearance of body-righting reflex, which was markedly reduced by GO (or silymarin, TP) pretreatment. However, the drunken time (the time from the ethanol exposure to the disappearance of the righting reflex) was prolonged only in GO-pretreated group, while that of other reagents was reduced. The waking time (time from the disappearance of the righting reflex to the reoccurrence) was all reduced in GO (silymarin and TP) pretreated-groups, and GO displayed the most potent activity (Table 6).

Table 3
Effects of GO on ethanol-induced fat accumulation in the mice liver

Groups	The scores of the hepatosteatosis				Mean rank
	0	1	2	3	
Control	12	0	0	0	12.50
Ethanol	0	1	3	8	51.08**
GO (50 mg/kg)	2	5	3	2	36.25#
GO (100 mg/kg)	4	6	2	0	28.00##
GO (200 mg/kg)	6	4	2	0	24.67##

The mice were pretreated with GO (50, 100 and 200 mg/kg bw) or corn oil, and then were exposed to ethanol (4.8 g/kg). 0: no fat droplet; 1: slight steatosis; 2: moderate steatosis; 3: severe steatosis; Nonparametric statistics (Rank sum test) was used for the data analysis. **$P < 0.01$, compared with control group; #$P < 0.05$, ##$P < 0.01$, compared with the ethanol group.

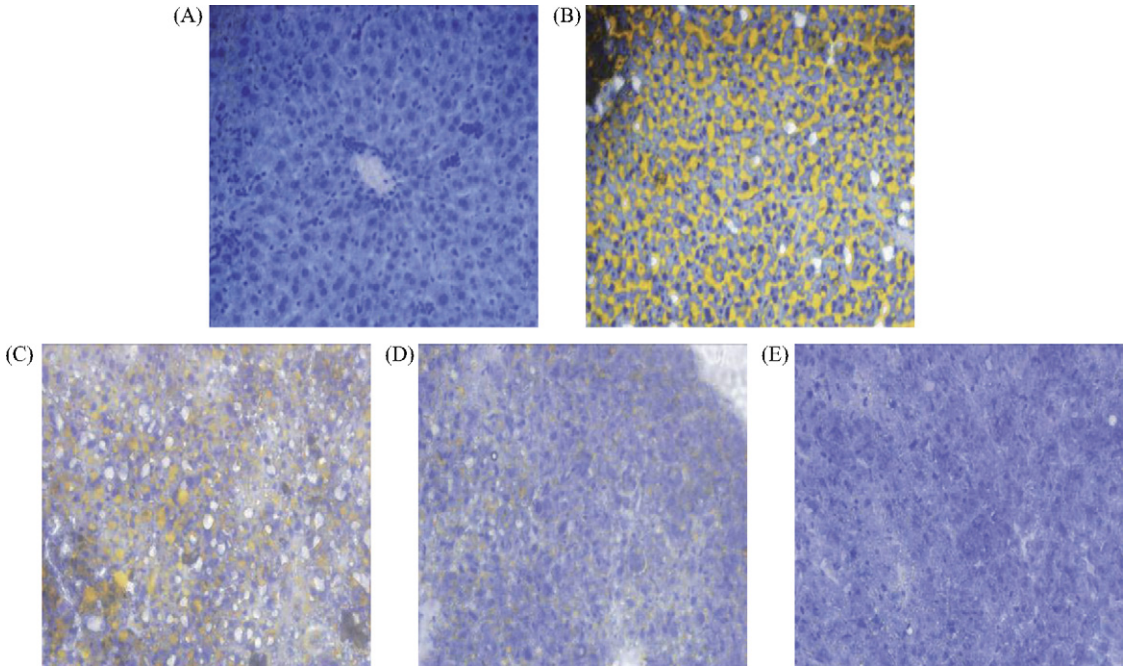

Fig. 2. Dose-dependent effects of GO on ethanol-induced fat accumulation in mice liver (200×). (A) Control group. No fat droplets were observed. (B) Ethanol group. Severe hepatic steatosis with many fat droplets was observed. (C) GO group (50 mg/kg). Moderate hepatic steatosis was observed. (D) GO group (100 mg/kg). Slight hepatic steatosis with several fat droplets was observed. (E) GO group (200 mg/kg). No obvious droplets were observed.

3.3.2. The liver index, the serum and hepatic TG levels determination

GO (silymarin and TP) pretreatment all obviously inhibited the increase of the serum and hepatic TG levels, and the effects of GO are comparable to those of silymarin and TP (Table 7).

3.3.3. Histological examination

All the three reagents dramatically attenuated the fat accumulation in the mice liver (Table 8 and Fig. 4).

4. Discussion

Hepatic steatosis has been defined as either more than 5% of hepatocytes containing fat droplets or total lipid exceeding 5% of liver weight [24]. Accumulation of fat is the earliest and most common response to heavy alcohol intake. Alcoholic fatty liver is usually characterized by the enlargement of the liver, the increase of the serum and hepatic TG levels, together with a lot of fat droplets in the liver sections. In the current study, acute ethanol (4.8 g/kg bw) administration resulted in the considerable increase of the liver index, the elevation of the serum and hepatic TG levels, suggesting that acute ethanol administration induced typical fatty liver. However, dose-dependent studies showed that single dose of GO effectively suppressed these adverse effects (Table 1). Paralleled to these changes, histological examination showed few droplets in GO-pretreated mice liver (100, 200 mg/kg bw). These data strongly indicated that pretreatment with single dose of GO can effectively prevent fatty liver induced by acute ethanol exposure.

Recently, the chemoprotective effects of GO have been extensively studied, and these benefits are mainly attributed to the antioxidative activities [20,21,25]. Numerous studies have illustrated the key role of ROS in the development of ALD. Oxidative stress was originally defined as the disequilibrium between prooxidants and antioxidants in biological systems. Once this imbalance appears, lipid peroxidation (LPO) occurs. LPO is a free radical-related process that may occur under enzymatic control, e.g. for the generation of lipid-derived inflammatory mediators, or nonenzymatically [26]. The level

Table 4
Effects of GO on the liver index, serum and hepatic TG levels

Groups	Liver index	Serum TG (mmol/L)	Hepatic TG (mg/g liver)
Control	4.72 ± 0.30	1.04 ± 0.30	10.38 ± 1.83
Ethanol	5.71 ± 0.57**	2.19 ± 0.47**	26.93 ± 7.70**
Pretreatment	4.94 ± 0.25##	1.24 ± 0.24##	16.65 ± 5.61##
Simultaneous treatment	5.07 ± 0.23##	1.42 ± 0.32##	18.70 ± 6.21##
Post-treatment	5.48 ± 0.39▲▲	1.98 ± 0.46▲▲	21.27 ± 6.06#▲

The mice were treated with GO (2 h before, simultaneously with, or 2 h after ethanol exposure) at the doses of 100 mg/kg bw. Compared with control group, $^{**}P < 0.01$; compared with ethanol group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; compared with pretreatment group, $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$.

Table 5
Effects of GO on the ethanol-induced fat accumulation in mice liver

Groups	The scores of the hepatosteatosis				Mean rank
	0	1	2	3	
Control	12	0	0	0	9.5
Ethanol	0	0	4	8	46.50**
Pretreatment	3	5	3	1	25.17##
Simultaneous treatment	3	2	4	3	31.04#
Post-treatment	0	2	5	5	40.29

The GO group mice were treated with GO (2 h before, simultaneously with, or 2 h after ethanol exposure) at the doses of 100 mg/kg bw. 0: no fat droplet; 1: slight steatosis; 2: moderate steatosis; 3: severe steatosis; Nonparametric statistics (Rank sum test) was used for the data analysis. $^{**}P < 0.01$, compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the ethanol group.

of malondialdehyde (MDA) has been widely used as a biomarker of LPO for many years [27]. In the current study, GO was found to dramatically inhibit the elevation of MDA levels caused by ethanol in a dose-dependent manner (Fig. 1), indicating that the anti-fatty liver effects of GO may be associated with antioxidant activities.

GSH is the most abundant low-molecular-weight thiol, and GSH/glutathione disulfide is the major redox couple in animal cells. GSH plays an important role in the antioxidant effects, nutrient metabolism, and regulation of cellular events [28]. As shown in Table 2, hepatic GSH level was slightly but significantly decreased after ethanol treatment, which was consistent with other studies [6,8,29]. The decrease of GSH reduces the antioxidative capacity and increases the sensibility to oxidative stress. GSH effectively scavenges free radicals and other oxygen species (e.g. hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2) through nonenzymatic and enzymatic process. In such reaction, GSH is oxidized into GSSG which can be reduced to GSH by GSH reductase (GR) with the consumption of NADPH [30]. Beside this, glutathione peroxidase (GSH-Px, a selenium-containing enzyme) catalyzes the GSH-dependent reduction of H_2O_2 and other peroxides. In addition, GSH can also react with various electrophiles, physiological metabolites (e.g. estrogen, melanins, prostaglandins, and leukotrienes), and xenobiotics (e.g. bromobenzene and acetaminophen) to form mercapturates, which were catalyzed by glutathione-*S*-transferase (GST) [31]. In addition to the GSH-related antioxidant system, SOD is also known to take important part in the protection against oxidative damage. SOD can catalyze the clearance of the superoxide anion radicals, preventing the formation of H_2O_2. Wu et al. showed that DADS and DATS (two major components of GO) significantly increased the activities of GST and GR [17]. Fukao et al. illustrated that DATS (10 μmol/kg) and DADS (100 μmol/kg) significantly increased the activities of GST, quinone reductase and GSH-Px, and dramatically reduced CCl_4-induced liver injury in rats [32]. In the current study (Table 2), single dose of GO was found to markedly enhance the activities of SOD, GR and GST, indicating that the antioxidant capacity was elevated. The restoration of the GSH level and the enhancement of the GR, SOD, and GST activities may account for the preventive effects.Fat acids are transported to the mitochondrial matrix through fat acid-carnitine complex and then are decomposed into CO_2 and H_2O via β-oxidation. As mitochondrion contains relatively

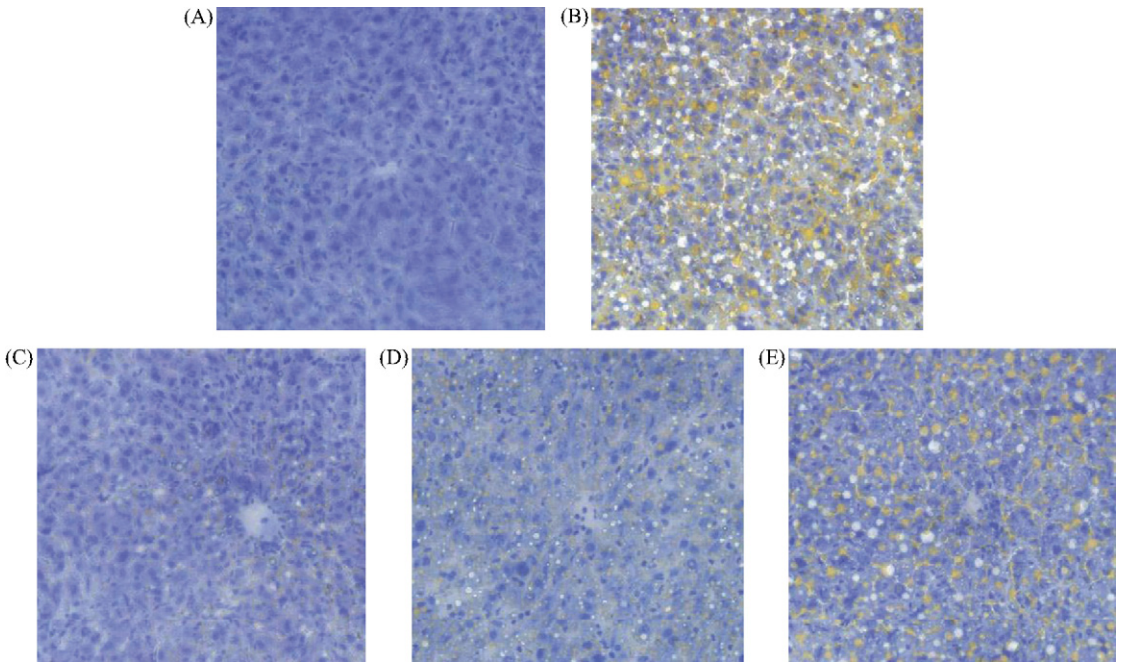

Fig. 3. Effects of GO (administrated 2 h before, simultaneously with, or 2 h after ethanol exposure) on acute ethanol-induced fat accumulation in mice liver (200×). (A) Control group. No fat droplets were observed. (B) Ethanol group. Severe hepatic steatosis with many fat droplets was observed. (C) GO pretreated group. Slightly hepatic steatosis with several fat droplets was observed. (D) GO and ethanol simultaneously treated group. The morphology of the liver is similar to the pretreatment group. (E) GO post-treated group. Severe hepatic steatosis with many fat droplets was observed.

Table 6
Drunken test examination of the mice after acute ethanol treatment (mean ± S.D., $n = 12$)

Groups	Drunken mice	Drunken time (min)	Waking time (min)	Drunken ratio (%)
Control	0	–	–	–
Ethanol	9	39 ± 19	125 ± 44	75
GO	1	80	45	8.3
Silymarin	6	35 ± 15	93 ± 34	50
TP	4	18 ± 12	90 ± 23	33.3

The mice were pretreated with GO (or silymarin, TP), and then exposed to ethanol (4.8 g/kg bw). Drunken status was defined as the disappearance of the body-righting reflex. The time from the ethanol exposure to the disappearance of the righting reflex was recorded as the drunken time. The time from the disappearance of the righting reflex to the reoccurrence was recorded as the waking time.

large amounts of polyunsaturated fatty acids in phospholipids, it should be more sensitive to the ethanol-induced oxidative stress [33]. Convincing evidences demonstrated that acute/chronic ethanol-associated hepatic damage was accompanied by the dysfunction of the mitochondria [34–36]. Oxidative stress can lead to the open state of PT pore, resulting in the rapid ion movement, followed by extensive mitochondrial swelling and the loss of the mitochondrial membrane potential. Thus, the metabolism of the fat acid should be impaired due to the mitochondria damage. Beside this, the mitochondria dysfunction may also lead to the depletion of ATP, resulting in the decrease of the synthesis of apoprotein in hepatocytes. In addition, the acetaldehyde produced from ethanol oxidation can adduct tubulin and inhibit microtubule polymerization, which can impair the trafficking of VLDL-containing vesicles [37,38]. In our study, GO restored the hepatic GSH level, boosted the activities of the antioxidant enzymes and prevented the enhancement of the ethanol-induced LPO. Therefore, the mitochondria should be protected by GO treatment. Moreover, GSH can react with acetaldehyde, preventing the impairment of the microtubules, which may also be helpful for the metabolism of the fat acids.

In order to elucidate whether GO possess ability to reverse ethanol-induced fatty liver when used after acute ethanol exposure, the mice were treated with GO 2 h before ethanol, simultaneously with ethanol, or 2 h after ethanol administration, respectively. As shown in Table 4 and Fig. 3, when mice were post-treated with single dose of GO (100 mg/kg bw), no obvious protective effects exhibited. Time-dependent studies showed that plasma MDA levels significantly increased at 2 h after ethanol intake (4 g/kg bw) [39], indicating oxidative stress has occurred and LPO has been initiated at 2 h after ethanol exposure. Under such circumstance, GO may lose its power to effectively abrogate ethanol-induced fatty liver. The dosage of GO is another key factor we must take into account. GO may display more potent protective effects at higher doses, e.g. 200 mg/kg bw, as the antioxidative ability can be elevated following the increase of the doses. However, we can still conclude that the protective effects of GO used before ethanol intake should be more potent than that used after ethanol exposure.

Silymarin, derived from *Silybium marianum*, has been used in traditional medicine as a remedy for diseases of the liver and biliary tract. Silymarin has been found to effectively attenuate acute ethanol-induced liver injury in previous studies [7,40]. TP acts as the antioxidants in vitro by scavenging reactive oxygen and has also been showed to attenuate ethanol-induced liver injury [41,42]. Silymarin, TP and GO, were all considered to exhibit biological activities via antioxidative characters. The comparative study showed that the protective effects of GO were comparable to those of silymarin and TP (Table 7 and Fig. 4). Interestingly, the drunken test showed that the drunken time was prolonged by GO pretreatment, accompanied by the reduction of the waking time. We hypothesized that the ethanol metabolism was controlled by some components of GO. Ethanol is mainly catalyzed into acetaldehyde by alcoholic dehydrogenase (ADH) and cytochrome P4502E1, and the acetaldehyde was then catalyzed to acetate by acetaldehyde dehydrogenase (ALDH) [43]. Previous studies have demonstrated that DAS and DADS were potent inhibitor of CYP2E1 [44,45]. Therefore, GO should inhibit the production of acetaldehyde via suppressing the CYP2E1 activity. The other two important enzymes, ADH and ALDH, may also be affected. ALDH was likely to be boosted and ADH might be inhibited. The measurements of the serum alcohol, acetaldehyde, acetate levels and the activities of the ADH, ALDH, and CYP2E1 will help to explain this interesting phenomenon.

In summary, GO dramatically prevented acute ethanol-induced hepatosteatosis when used before or simultaneously with ethanol intake, and its protective effects were comparable to those of silymarin and tea polyphenol. The preventive effects, at least partly, should be attributed to

Table 7
Effects of GO (silymarin, TP) on the liver index, the serum and hepatic TG levels

Groups	Liver index (%)	Serum TG (mmol/L)	Hepatic TG (mg/g liver)
Control	4.79 ± 0.25	0.94 ± 0.27	13.62 ± 2.76
Ethanol	5.62 ± 0.35**	1.93 ± 0.44**	48.84 ± 17.28**
GO	5.30 ± 0.35$^{\#}$	0.95 ± 0.37$^{\#\#}$	20.32 ± 4.06$^{\#\#}$
Silymarin	5.35 ± 0.20	1.01 ± 0.35$^{\#\#}$	24.83 ± 7.17$^{\#\#}$
TP	5.57 ± 0.56	0.93 ± 0.49$^{\#\#}$	23.08 ± 5.49$^{\#\#}$

The mice were pretreated with GO (or silymarin, TP), and then exposed to ethanol (4.8 g/kg bw). Compared with control group, $^{**}P < 0.01$; compared with ethanol group, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$, $P < 0.01$.

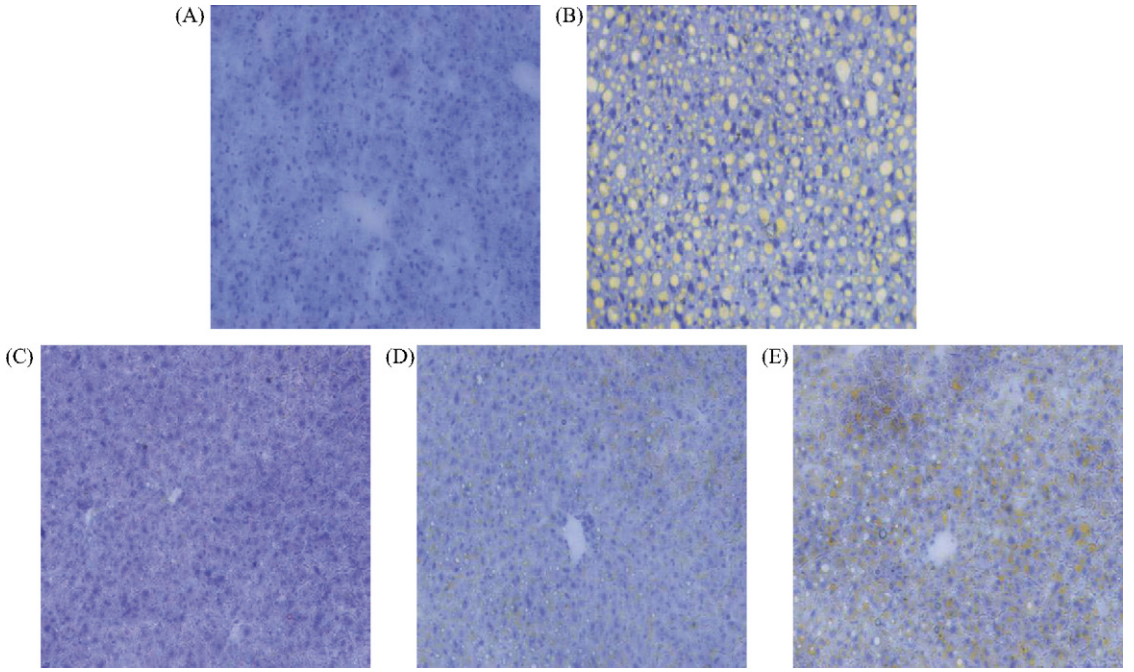

Fig. 4. Comparative study of the effects of GO, TP and silymarin on the fat accumulation in mice liver (200×). (A) Control group. (B) Ethanol group. Severe hepatic steatosis with many fat droplets was observed. (C) GO pretreated group. The fat droplets were obviously decreased. (D) Silymarin pretreated group. The fat droplets were obviously decreased. (E) TP pretreated group. The fat droplets were slightly decreased.

Table 8
Effects of GO (silymarin, TP) on ethanol-induced fat accumulation in mice liver

Groups	The scores of the hepatosteatosis				Mean rank
	0	1	2	3	
Control	12	0	0	0	12.00
Ethanol	0	0	4	8	50.17**
GO	4	4	3	1	28.58##
Silymarin	3	4	3	2	32.13##
TP	4	3	4	1	29.63##

The mice were pretreated with GO (or silymarin, TP), and then exposed to ethanol (4.8 g/kg bw). 0: no fat droplet; 1: slight steatosis; 2: moderate steatosis; 3: severe steatosis; Nonparametric statistics (Rank sum test) was used for the data analysis. $^{**}P < 0.01$, compared with control group; $^{\#\#}P < 0.01$, compared with the ethanol group.

the antioxidant activities. No obvious protective effects exhibited when it was administrated after ethanol exposure.

Conflict of interest

None.

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