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Effects of glutamine administration on inflammatory responses in chronic ethanol-fed rats

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Abstract

The purpose of this study was to investigate the effects of glutamine supplementation on inflammatory responses in chronic ethanol-fed rats. Male Wistar rats weighing about 160 g were divided into five groups. Two groups were fed a normal liquid diet and three groups were fed a glutamine-containing liquid diet. After 1 week, one of the normal liquid diet groups was fed an ethanol-containing liquid diet (CE), and the other group served as the control (CC) group. At the same time, one of the glutamine-containing liquid diet groups was continually fed the same diet (GCG), but the other two groups were fed ethanol-containing diet supplemented with glutamine (GEG) or without glutamine (GE). The following items were analyzed: (1) liver function, (2) cytokine contents, and (3) hepatic oxidative stress. The activities of aspartate transaminase (AST) and alanine transaminase (ALT) and levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in the CE group had significantly increased. In addition, hepatic cytochrome P450 2E1 (CYP2E1) expression had significantly increased in the CE, GE and GEG groups. However, the activities of AST and ALT and levels of TNF-α and IL-1β in the GE group were significantly lower than those of the CE group. The results suggest that the plasma inflammatory responses of rats fed an ethanol-containing liquid diet for 7 weeks significantly increased. However, pretreatment with glutamine improved the plasma inflammatory responses induced by ethanol.

Keywords: Alcoholic liver disease; Glutamine; Rats

1. Introduction

Long-term excessive alcohol consumption induces alcoholic liver disease (ALD). However, ALD is a result of complex pathophysiological mechanisms. These mechanisms include oxidative stress and lipid peroxidation [1], immunological responses initiated by the formation of protein adducts [2], and activation of Kupffer cells by endotoxin and the subsequent release of cytokines, chemokines, and adhesion molecules [3,4]. Among the mechanisms implicated in alcohol-dependent liver disease, an increase in intestinal permeability caused by alcohol that leads to endotoxemia appears to play an important role.

The biological activity of endotoxin is associated with lipopolysaccharide (LPS), a component of the outer wall of gram-negative bacteria. LPS is released when Gram-negative bacteria break down. LPS activates Kupffer cells by interacting with CD14 and Toll-like receptor 4 molecules on the surface of Kupffer cells when they enter the bloodstream and move to the liver [5,6]. Activation of Kupffer cells results in the release of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 [7–9]. These cytokines act to mediate the innate immune response. It was reported that endotoxin plays an important role in alcohol-induced liver injury. The reasons for alcohol-induced endotoxemia include bacterial overgrowth in the small intestine, mucosal injury in the upper gastrointestinal tract and increased gut permeability [10]. Therefore, preventing endotoxin from entering the blood circulation by decreasing the alcohol-induced intestinal permeability or by maintaining the intestine mucosal function may diminish alcohol-induced liver injury.

Glutamine is the most abundant amino acid in the circulation and in the intracellular amino acid pool. It was reported that glutamine is important for maintaining intestinal mucosal metabolic function [11], and it has immunomodulatory properties [12]. Glutamine stabilizes the intestinal barrier by preventing the translocation of bacteria or other toxins and providing an energy source for cell proliferation. Furthermore, glutamine also prevents oxidative damage via the production of glutathione [13]. In addition, it was reported that glutamine supplementation attenuates proinflammatory cytokine

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release and protects against organ damage in a rat model of endotoxemia [14]. Previous studies pointed out that glutamine and alanine administration prevented the inhibition of liver regeneration caused by alcohol [15], and allowed the recovery of body weight and spontaneous motor activity to normal levels in ethanol-treated rats [16]. In addition, it was also reported that glutamine prevents the acetaldehyde-induced redistribution of intercellular junctions in the human colonic mucosa [17]. But few studies have shown the effects of glutamine on the innate immune responses in alcohol-administrated rats. Therefore, the purpose of this study was to investigate the effects of glutamine supplementation on inflammatory responses in chronic ethanol-treated rats.

2. Methods and materials

2.1. Animals

Male Wistar rats (National Taiwan University, College of Medicine, Laboratory Animal Center) weighing about 160 g were used in this experiment. All rats were housed in individual stainless steel cages in an animal room maintained at 22±2°C with 50–70% humidity and a 12-h light-dark cycle. All rats were allowed free access to a standard rodent diet (LabDiet 5001 Rodent Diet; PMI Nutrition International, St. Louis, MO, USA) and water for 1 week during acclimation before the study. All procedures were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

2.2. Study protocol

After 1 week of acclimation, the rats were divided into five groups according to the same aspartate transaminase (AST) and alanine transaminase (ALT) activities in each group ($n = 10$). In the first week, two groups were fed a control liquid diet, and three groups were fed a glutamine-containing liquid diet before treating the ethanol (equal to the pre-administration of glutamine). During the next 7 weeks, one of the two groups fed the control diet was treated with ethanol by feeding an ethanol-containing liquid diet (CE), while the other group was used as the control (CC) group. One of the three groups fed the glutamine-containing liquid diet, was continually fed the same diet (GCG), but the other two groups were fed an ethanol-containing diet, which was supplemented with glutamine (GEG) or without glutamine (GE). The diets were modified from Lieber and DeCarli [18]. Following the study of Yeh et al. [19], part of the casein was replaced by glutamine, which provided 25% N of the total amino acid in glutamine-containing liquid diet (0.84% glutamine). All diets were isonitrogenous and identical in energy and nutrient distributions (Table 1). At the end of the experimental period, the rats were anesthetized and sacrificed. Blood samples were collected in tubes containing heparin and were centrifuged to separate the plasma (1200×*g* for 15 min at 4°C). All plasma samples were stored at −80°C until being assayed. Liver tissues were rapidly excised and stored at −80°C for further analysis.

Table 1
Composition of the experiment diets in each group[a]

Ingredients[b]	Concentration [g/L (1000 kcal)]			
	Control	Control+glutamine	Ethanol	Ethanol+glutamine
Casein	41.4	31.03	41.4	31.03
L-Cystine	0.5	0.5	0.5	0.5
DL-Methionine	0.3	0.3	0.3	0.3
Corn oil	8.5	8.5	8.5	8.5
Olive oil	28.4	28.4	28.4	28.4
Safflower oil	2.7	2.7	2.7	2.7
Choline bitartrate	0.53	0.53	0.53	0.53
Fiber	10	10	10	10
Xanthan gum	4	4	4	4
ICN: AIN-76 vitamins	2.5	2.5	2.5	2.5
ICN: AIN-76 minerals	2.6	2.6	2.6	2.6
Maltodextrin	115.2	117.1	25.6	27.5
Ethanol	–	–	50	50
Glutamine[c]	–	8.437	–	8.437
Total nitrogen	6.47	6.47	6.47	6.47

[a] Group CC is control diet; group GCG is control diet with glutamine; group CE is control diet in the first week, and ethanol diet during the second to eighth weeks; group GE is control diet with glutamine in the first week, and ethanol diet during the second to eighth weeks; group GEG is control diet with glutamine in the first week, and an ethanol diet with glutamine during the second to eighth weeks.

[b] L-cystine, DL-methionine, choline bitartrate, Fiber, AIN-76 vitamins, AIN-76 minerals, and maltodextrin were purchased from the ICN Biochemicals (Costa Mesa, CA, USA). Xanthan gum, ethanol, and glutamine were purchased from the Sigma-Aldrich (St. Louis, MO, USA). Corn oil and olive oil were purchased from the God Bene Enterprise (Yunlin, Taiwan). Safflower oil was purchased from the Taiwan Sugar Corporation (Taipei, Taiwan).

[c] It contains 0.84% glutamine in the control diet with glutamine and in the ethanol diet with glutamine.

2.3. Measurements and analytical procedures

2.3.1. Liver function

To investigate the liver function during the experimental period, plasma AST and ALT activities were measured with a Beckman Synchron LXTM system at 340 nm.

2.3.2. Histological examinations

Liver tissue was fixed in 10% formaldehyde and embedded in paraffin. Paraffin sections were cut and processed for histological examination according to three kinds of histopathological stain including hematoxylin-eosin (H&E), Masson and silver stains. A semiquantitative histological evaluation was carried out by a pathologist blinded to the treatment groups. The grading of H&E stain ranged from 0 to 4 where 0 is absent, 1, trace, 2, mild, 3, moderate and 4, severe. The scale for semiquantitation of hepatic tissue fibrosis (Masson and silver stains) was as follows: 0 means no collagen; 1 means the existence of collagen but no septal formation; 2, the existence of collagen and septum, but no connective tissue; 3, the existence of collagen with a few thin connective tissue septa and 4, the existence of collagen with thick connective tissue septa.

2.3.3. Measurement of hepatic triglyceride and cholesterol levels

Liver lipids were extracted by the method of Folch et al. [20]. Hepatic triglyceride (TG) and cholesterol (TC) concentrations in the liver were determined with diagnostic kits (Randox Laboratories, Antrim, UK) with triglyceride and cholesterol as standards, respectively.

2.3.4. Inflammatory responses and cytokine contents

Liver tissue (0.5 g) was homogenized in 1.5 ml ice-cold buffer [50 mM Tris (pH 7.2), 150 mM NaCl and 1% Triton-X] plus 0.1% protease inhibitor. The homogenate was then shaken on ice for 90 min. After shaking, the homogenate solution was centrifuged at 3000×*g* at 4°C for 15 min [21]. The supernatants were collected for subsequent analysis.

To evaluate the inflammatory response, plasma and hepatic cytokine concentrations were measured using enzyme-linked immunosorbent assay kits. The TNF-α concentration was determined using a rat TNF-α/TNFSF1A kit (R&D Systems, Minneapolis, MN, USA). The IL-1β concentration was determined using a rat IL-1β/IL-1F2 kit (R&D Systems). The IL-6 concentration was determined using a rat IL-6 kit (R&D Systems). Assays of samples and standards were simultaneously conducted according to the assay kit instructions. The optical density was read at 450 nm for all cytokines using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.3.5. Hepatic oxidative stress analysis

To investigate the oxidative status of the liver, the reduced glutathione (GSH) and oxidative glutathione (GSSG) ratio, lipid peroxidation and CYP2E1 expression were measured. One gram of liver tissue was homogenized in 4-ml buffer (0.25 mM sucrose, 10 mM Tris-HCl, and 0.25 mM phenylmethylsulfonyl fluoride; pH 7.4). The homogenate was then centrifuged at 10,000×*g* for 15 min at 4°C. The supernatants were analyzed for the GSH/GSSG ratio and lipid peroxidation.

2.3.5.1. GSH/GSSG ratio. The GSH concentration were measured spectrophotometrically at 405 nm according to the method of Tietze [22], and the GSSG concentration was measured spectrophotometrically at 405 nm according to the method of Griffith [23]. Finally, the ratio of GSH/GSSG was calculated as (GSH−2GSSG)/(GSSG).

2.3.5.2. Lipid peroxidation. Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in the liver with minor modifications [24]. Briefly, 20 μl of liver homogenates or 1,1,3,3-tetramethoxypropane (as the standard) were shaken with 800 μl of 0.22% H_2SO_4 in a 2-ml centrifuge tube. Then, 100 μl of 10% phosphotungstic acid and 200 μl 0.67% TBA (in H_2O: glacial acetic acid=1: 1, v/v) were added to the mixture, mixed well, and warmed for 60 min in a boiling-water bath followed by rapid cooling. It was shaken into 600 μl of a n-butyl-alcohol layer in a separation tube, and the MDA content was measured fluorometrically (at excitation and emission wavelengths of 531 and 590 nm, respectively) using a Wallac Victor-2 1420 Multilabel Counter (Perkin-Elmer, Waltham, MA, USA).

2.3.5.3. Hepatic microsomal CYP2E1 protein expression. Liver tissues were homogenized in 10 volumes of ice-cold buffer (0.25 M sucrose, 10 mM Tris–HCl, and 0.25 mM phenylmethylsulfonyl fluoride; pH 7.4). The homogenates were centrifuged at 17,000×*g* for 20 min at 4°C. The supernatant was transferred to a new tube and centrifuged at 105,000×*g* for 60 min at 4°C to separate the microsomes. The microsomal pellet was dissolved in 50-mM potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT (pH 7.4). Microsomal protein (30 μg) was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After separation, the proteins were electroblotted onto a polyvinylidene difluoride transfer membrane (Amersham Biosciences, Little Chalfont, Bucks, UK). These blots were then incubated with mouse monoclonal anti-rat CYP2E1 (Oxford Biomedical Research, Oxford, MI, USA) and mouse anti-actin monoclonal (Chemicon International, Temecula, CA, USA) antibodies. Finally, the blot was treated with goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Chemicon International) or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and specific bindings of antibodies detected using a Western Lightning kit (PerkinElmer Lifesciences, Boston, MA, USA). The bands were quantified using Image-Pro Plus 4.5 software analysis.

2.3.6. Measurement of total protein concentration

The total protein concentration of tissue samples was spectrophotometrically estimated using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Statistical analysis

All data are expressed as the mean±S.D. One-way ANOVA followed by Fisher's test was used to determine the statistical differences among groups using SAS software vers. 8.0 (SAS Institute, Cary NC, USA). Statistical significance was assigned at the $P<.05$ level.

Table 2
Initial and final body weights and the relative liver weights of rats in each group

Group	Initial body weight (g)	Final body weight (g)	Relative liver weight (g/g×100%)
CC	298.9±18.1^{ab}	451.4±20.3^{a}	2.2±0.2^{a}
GCG	286.1±23.0^{a}	446.0±20.9^{a}	2.2±0.2^{a}
CE	297.1±17.0^{b}	428.2±21.8^{ab}	2.8±0.2^{b}
GE	284.8±15.8^{ab}	393.6±46.9^{c}	2.6±0.2^{c}
GEG	281.0±17.0^{ab}	414.5±38.1^{bc}	2.7±0.3^{bc}

Values are expressed as the mean±S.D. (n=10). Means in the same column with different superscript letters significantly differ ($P<.05$).
Relative liver weight: (liver weight/body weight)×100%.

3. Results

The average energy intake in the five groups was as follows: CC group (74.8±8.6 kcal/day), GCG group (75.4±9.0 kcal/day), CE group (75.5±8.5 kcal/day), GE group (69.5±9.6 kcal/day), and GEG group (72.2±9.4 kcal/day). The average ethanol intake levels in the groups consuming ethanol were as follows: CE group (3.8±0.4 kcal/day), GE group (3.5±0.5 kcal/day) and EGE group (3.6±0.5 kcal/day). There were no differences in the energy or ethanol intake levels among these groups.

The initial and final body weights are shown in Table 2. The initial body weight in the CE group was significantly higher than that of the GCG group ($P<.05$). However, the final body weight in the GE group was significantly lower than those of the CC and GCG groups ($P<.05$). The relative liver weights in the CE, GE and GEG groups were significantly higher than those in the CC and GCG groups ($P<.05$), and the relative liver weight in the GE group was significantly lower than that in the CE group ($P<.05$).

The liver function of the experimental animals was monitored through AST and ALT activities. The results of the initial and final AST and ALT activities are shown in Table 3. There were no differences in the initial AST activities of the various groups. The initial ALT activity in the GCG group was significantly higher than that in the CC group ($P<.05$), and the ALT activities in the GCG, CE, GE, and GEG groups did not differ. The final AST and ALT activities in the CE, GE, and GEG groups significantly increased compared to those in the CC and GCG groups ($P<.05$). The final AST and ALT activities in the groups which were given an ethanol diet and pretreatment with glutamine (GE group) were significantly lower than those in the CE group ($P<.05$). Moreover, the final AST activity in the GEG group was also lower than that in the CE group ($P<.05$). Fig. 1 shows the representative photomicrographs of livers in the five groups. The results of semiquantitative histological assessment are shown in Table 4. The scores of fatty change and inflammation in the CE, GE, and GEG groups were higher than those in the CC and GCG group ($P<.05$). However, microvesicular fat accumulation in the GEG group was significantly lower compared to the CE group ($P<.05$). The scores of inflammation in the GE group were significantly lower than that in the CE group ($P<.05$). The scores

Table 3
Initial and the final AST and ALT activities of rats in each group[1]

Group[2]	Initial AST activity (U/L)	Initial ALT activity (U/L)	Final AST activity (U/L)	Final ALT activity (U/L)
CC	77.3±9.2^{a}	25.8±7.5^{a}	87.2±12.3^{a}	28.2±4.0^{a}
GCG	77.8±8.6^{a}	21.2±3.8^{b}	82.6±7.9^{a}	24.2±4.7^{a}
CE	77.6±13.4^{a}	22.0±2.2^{ab}	152.6±27.6^{b}	58.4±11.1^{c}
GE	78.7±10.7^{a}	22.6±4.2^{ab}	110.9±20.6^{c}	44.6±10.8^{b}
GEG	76.5±10.0^{a}	22.3±4.5^{ab}	118.1±30.1^{c}	53.9±18.3^{bc}

[1] Values are expressed as the mean±S.D. (n = 10). Means in the same column with different superscript letters significantly differ ($P<.05$).
[2] Details are as same as those described in Table 2.

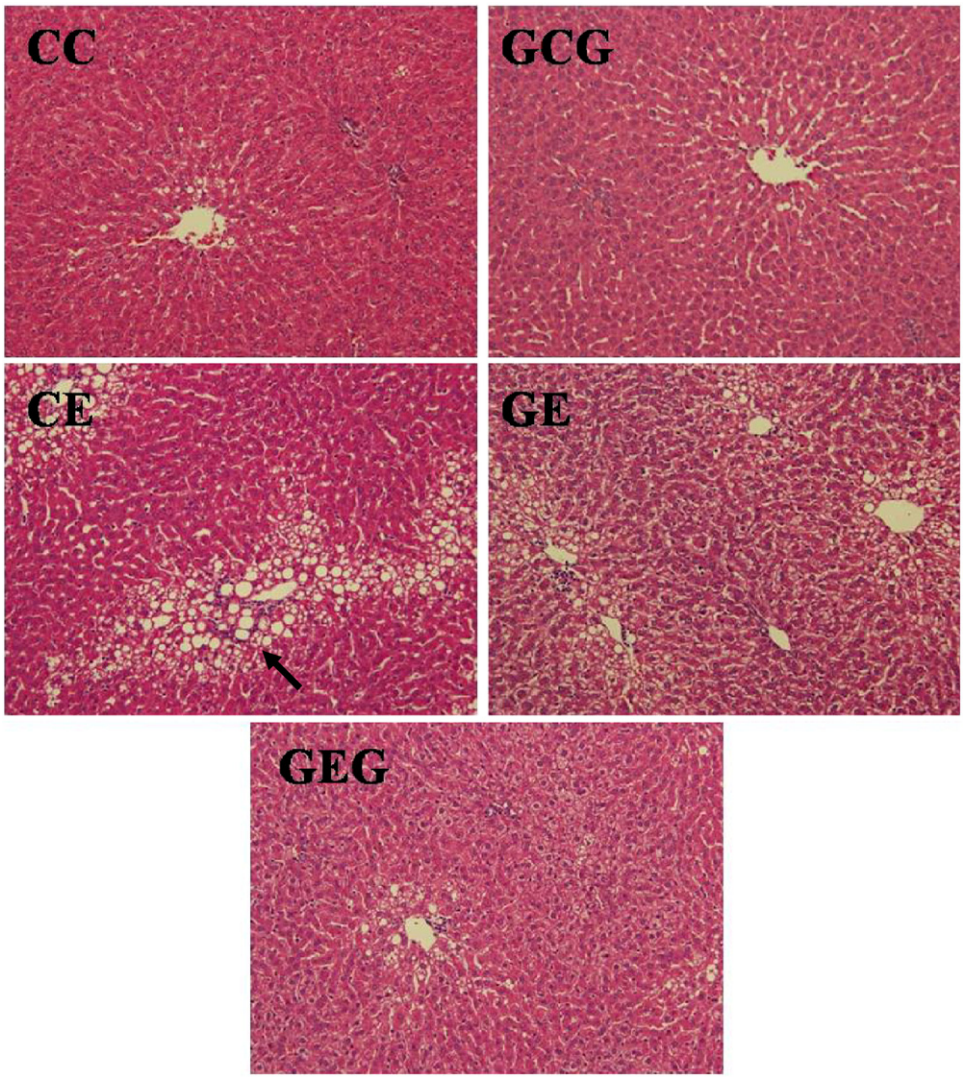

Fig. 1. Representative photomicrographs of livers. CC, control diet; GCG, control diet with glutamine; CE, control diet in the first week, and an ethanol diet during the second to eighth weeks; GE, control diet with glutamine in the first week, and an ethanol diet during the second to eighth weeks; GEG, control diet with glutamine in the first week, and an ethanol diet with glutamine during the second to eighth weeks. The CC group showed no histopathological change (hematoxylin and eosin; original magnification ×200). The CE group showed microvesicular and macrovacuolar fat accumulation (arrow).

of fibrosis were no significant differences in the five groups (data not shown). The results of hepatic lipids are shown in Table 5. Hepatic TG levels in the CE, GE, and GEG groups were significantly higher than those in the CC and GCG groups ($P<.05$); however, there were no differences among values in the CE, GE and GEG groups. Hepatic TC levels in the CE and GE groups were significantly higher than that in the CC group ($P<.05$). In addition, hepatic TC concentration in the GEG group did not differ compared to those of the CC and GCG groups.

Plasma TNF-α, IL-1β and IL-6 concentrations are shown in Table 6. TNF-α and IL-1β levels significantly increased in the CE group ($P<.05$). However, TNF-α and IL-1β levels were significantly lower in the GEG

Table 4
Hepatic histopathology scores of rats in each group[1]

Group[2]	Injury of scores			
	Fatty change (vacuole formation)		Necrosis	Inflammation
	Micro	Macro		
CC	0.3±0.2^{ab}	0.0±0.0^{a}	0.0±0.0^{a}	0.3±0.2^{a}
GCG	0.2±0.2^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.2±0.2^{a}
CE	1.1±0.4^{c}	0.7±0.5^{b}	0.1±0.2^{a}	0.8±0.3^{b}
GE	1.0±0.6^{cd}	0.6±0.6^{b}	0.0±0.0^{a}	0.6±0.3^{c}
GEG	0.7±0.3^{bd}	0.5±0.4^{b}	0.1±0.2^{a}	0.6±0.2^{bc}

[1] Values are expressed as the mean±S.D. ($n = 10$). Means in the same column with different superscript letters significantly differ ($P<.05$).
[2] Details are as same as those described in Table 2.

Table 5
Hepatic TG and TC levels of rats in each group[1]

Group[2]	TG (mg/g liver)	TC (mg/g liver)
CC	27.3±5.4^{a}	5.7±1.2^{a}
GCG	20.7±7.1^{a}	7.8±1.4^{bd}
CE	34.8±7.1^{b}	10.0±2.7^{c}
GE	36.5±8.1^{b}	9.4±2.1^{cd}
GEG	35.7±8.5^{b}	7.1±2.0^{ab}

[1] Values are expressed as the mean±S.D. ($n = 10$). Means in the same column with different superscript letters significantly differ ($P<.05$).
[2] Details are as same as those described in Table 2.

Table 6
Plasma TNF-α, IL-1β and IL-6 concentrations of rats in each group[1]

Groups[2]	TNF-α (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
CC	1.8±1.5^{ab}	9.5±2.8^{a}	79.5±25.2^{ab}
GCG	1.4±0.8^{a}	11.3±2.2^{ab}	95.1±62.3^{b}
CE	5.1±2.1^{c}	17.1±13.1^{b}	72.0±41.4^{ab}
GE	2.1±1.6^{ab}	8.4±3.7^{a}	60.8±35.3^{ab}
GEG	3.5±4.0^{bc}	11.1±6.4^{ab}	50.5±17.0^{a}

[1] Values are expressed as the mean±S.D. ($n = 10$). Means in the same column with different superscript letters significantly differ ($P<.05$).
[2] Details are as same as those described in Table 2.

group compared to the CE group ($P<.05$). The IL-6 concentration in the GCG group was significantly higher than that in the GEG group ($P<.05$). The IL-6 concentration in the GCG group did not differ compared to that of the CC group, and IL-6 concentrations in the GE and GEG groups did not differ compared to that of the CE group. Hepatic TNF-α, IL-1β, and IL-6 concentrations are shown in Table 7. Hepatic TNF-α and IL-1β levels did not differ in the various groups. Hepatic IL-6 level was significantly higher in the CE group ($P<.05$). The IL-6 level was significantly lower in the GE group compared to that in the CE group ($P<.05$), but there was no significant difference in IL-6 concentrations between the GEG and CE groups.

The antioxidant status in the liver was evaluated by the GSH/GSSG ratio, TBARS concentration, and CYP2E1 expression. The hepatic GSH/GSSG ratio and TBARS concentrations of rats in each group did not differ in the various groups (Table 8). CYP2E1 expressions in the CE, GE, and GEG groups were significantly higher than that in the CC group ($P<.05$); however, there were no differences among values in the three groups (Fig. 2).

4. Discussion

In this study, rats were fed isocaloric diets in these five groups. The average ethanol consumption in ethanol-treated groups was similar to that used in a previous report [25]. The final body weight and relative liver weight of rats fed the glutamine-containing normal liquid diet did not differ compared to the control group. This reveals that glutamine supplementation had no adverse effects. The present results showed that the final body weight significantly decreased in the ethanol-treated groups except for the CE group (Table 2). It was reported that weight gain was reduced in rats by substitution of alcohol for carbohydrates in spite of a similar energy intake [26]. However, the relative liver weight significantly increased in rats of the CE group (Table 2). Chronic alcohol consumption may cause asymptomatic hepatomegaly [27], and ethanol intake increases the relative liver weight in rats [28]. The results showed that pretreatment with glutamine can prevent the ethanol-induced relative liver weight increase in rats fed an ethanol-containing diet.

To evaluate liver damage caused by chronic ethanol feeding, plasma AST and ALT activities were determined in the present study.

Table 7
Hepatic TNF-α, IL-1β and IL-6 concentrations of rats in each group[1]

Groups[2]	TNF-α (pg/mg)	IL-1β (pg/mg)	IL-6 (pg/mg)
CC	10.9±2.5^{a}	9.0±1.2^{a}	20.6±6.9^{a}
GCG	10.2±3.6^{a}	9.6±2.3^{a}	21.2±7.0^{a}
CE	11.4±3.1^{a}	10.0±2.8^{a}	32.8±6.8^{c}
GE	9.9±3.0^{a}	8.5±2.1^{a}	24.6±5.5^{ab}
GEG	11.7±3.7^{a}	9.7±1.7^{a}	27.1±7.7^{bc}

[1] Values are expressed as the mean±S.D. ($n = 10$). Means in the same column with different superscript letters significantly differ ($P<.05$).
[2] Details are as same as those described in Table 2.

Table 8
The hepatic reduced GSH/GSSG ratio and TBARS concentrations of rats in each group[a]

Group[b]	GSH/GSSG ratio	TBARS (μM)
CC	8.9±1.6	15.0±2.0
GCG	8.9±1.3	14.6±2.1
CE	8.6±2.6	14.2±1.4
GE	9.4±2.2	14.3±2.1
GEG	9.3±1.6	14.4±2.2

[a] Values are expressed as the mean±S.D. ($n = 10$).
[b] Details are as same as those described in Table 2.

Plasma AST and ALT activities are markers of ethanol-induced liver injury [29,30]. The final plasma AST and ALT activities significantly increased in rats fed the ethanol-containing diet (Table 3). In the CE group, fat accumulation and inflammation were observed from the results of hisotopathology examinations (Table 4) and hepatic TG and TC were significantly increased (Table 5). This demonstrates that long-term ethanol administration induced liver injury in this study. In addition, the results showed that the final plasma AST and ALT activities in the GE group and final AST activity in the GEG group were significantly reduced compared to those of the CE group (Table 3). Furthermore, the injury scores of microvesicular fat accumulation were reduced in the GEG group (Table 4). Glutamine is the primary metabolic fuel of the small amino acid pool in the body, and the amide nitrogen of glutamine is the major component for the biosynthesis of nucleotides which plays a major role in cell proliferation [13,31]. In an animal model of obstructive jaundice, oral administration of glutamine reduced liver injury after bile duct ligation [32]. In addition, preceding ingestion of a 10% glutamine diet suppressed liver injury in D-galactosamine-induced acute hepatitis in rats [33]. Although the protective mechanisms of glutamine against liver damage in rats remain to be elucidated, the results are evidence that the pre-administration of glutamine showed a hepatoprotective effect against ethanol-induced liver injury.

Cytokines are produced by immune cells that act as mediators of the immune response and the response of tissues to injury. In the present study, we analyzed the systemic and hepatic cytokine levels in rats with chronic ethanol feeding. The plasma TNF-α and IL-1β levels significantly increased in the CE group compared to the control group (Table 6). These results are consistent with those of previous studies [7,34,35]. Ethanol consumption can promote bacteria overgrowth, which may cause accumulation of endotoxin. In addition,

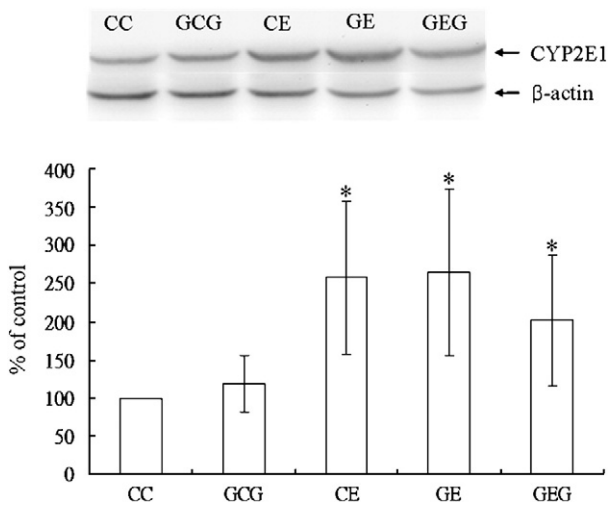

Fig. 2. Hepatic CYP2E1 protein expression of rats in each group. *Significantly differs from the CC group ($P<.05$).

ethanol-induced gut barrier disruption leads to increased intestinal permeability, and excess amount of endotoxin may transfer from intestine to the liver and general circulation and contribute to an inflammatory response [10,36]. In the present study, pretreatment of glutamine reduced plasma TNF-α and IL-1β levels in rats with chronic ethanol feeding (Table 6). The results showed that glutamine could suppress the inflammatory response. Previous studies indicated that glutamine plays an important role in the maintenance of intestinal integrity [11]. It has been reported that glutamine availability sustains recovery from decrease of transepithelial resistance and increase of permeability in Caco-2 cells [37]. Glutamine prevents acetaldehyde-induced disruption of the tight junction and adherens junction in human colonic mucosa [17]. Furthermore, glutamine prevents gut mucosal injury and improves mucosal recovery in LPS-treated rats [31]. Therefore, glutamine may provide protective effects to prevent ethanol-induced permeability increasing and endotoxin from entering the circulation and activating Kupffer cells by improving intestinal barrier function. However, plasma endotoxin level and intestinal permeability test are required in the present study to clarify the possible mechanism of glutamine's protective effect in chronic ethanol-fed rats.

The results of plasma and hepatic IL-6 levels were unanticipated in the present study. We found that plasma IL-6 levels were highest in the GCG group and lowest in the GEG group, although they did not differ in the CC and CE groups (Table 6). However, hepatic IL-6 levels significantly increased in the CE group (Table 7). Previous studies pointed that serum and hepatic IL-6 levels increased in ALD patients, suggesting that elevated IL-6 may contribute to alcoholic liver injury [8,9]. However, many studies mentioned that IL-6 may play a compensatory role in protecting against alcohol-induced liver injury [38]. IL-6 may have a protective effect by modulating the expression of the antiapoptotic proteins, bcl-2 and bcl-xL [39]. In addition, IL-6 plays a protective role by inhibiting ethanol-induced oxidative stress, lipid peroxidation, and mitochondrion permeability transition [40]. The inconsistent results of IL-6 levels between plasma and the liver suggest that confirming whether IL-6 plays a protective role or not could be a complicated. Although previous studies pointed out that glutathione supplementation attenuates the release of IL-6 in septic rats and improves survival [41,42], we could not define the effect of glutamine supplementation on IL-6 production in the present study. A further study may be necessary to elucidate the effect of glutamine on IL-6 production in ALD using IL-6-deficient mice as an experimental model [40].

The present study also investigated the oxidative status in the experimental rats. The results showed that hepatic CYP2E1 expression significantly increased when rats were fed the ethanol-containing diet (Fig. 2). The hepatic GSH/GSSG ratio and lipid peroxide products, as TBARS concentrations, showed no significant differences among the five groups (Table 8). It is believed that the increase in reactive oxygen species (ROS) generation is responsible for the high oxidative stress in the body during ethanol metabolism, and CYP2E1 plays a key role in ROS generation in the liver [1]. The results suggest that although hepatic CYP2E1 was induced by ethanol, oxidative stress had not yet increased in the liver. Glutamine via glutamate plays an important role in the intestine by producing glutathione which is considered an antioxidant substance [13]. It was also reported that glutamine-supplemented nutrition preserves hepatic glutathione in an animal model of pre-infusion with glutamine and hepatic injury [43]. However, hepatic CYP2E1 expression, the GSH/GSSG ratio, and lipid peroxide products (TBARS concentrations) in ethanol-treated rats supplemented with glutamine (the GE and GEG group) did not differ when compared to the CE group (Table 8, Fig. 2). Therefore, glutamine supplementation might not be associated with providing antioxidative protection in the liver in this study.

In conclusion, plasma TNF-α and IL-1β levels significantly increased in rats fed an ethanol-containing liquid diet for 7 weeks, suggesting that ethanol consumption induces plasma inflammatory responses and liver damage. However, pretreatment with glutamine prevented ethanol-induced liver injury by improving the ethanol-induced inflammatory response.

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References

[1] Das SK, Vasudevan DM. Alcohol-induced oxidative stress. Life Sci 2007;81:177–87.
[2] Duryee MJ, Klassen LW, Thiele GM. Immunological response in alcoholic liver disease. World J Gastroenterol 2007;13:4938–46.
[3] Schaffert CS, Duryee MJ, Hunter CD, Hamilton BCr, DeVeney AL, Huerter MM, et al. Alcohol metabolites and lipopolysaccharide: roles in the development and/or progression of alcoholic liver disease. World J Gastroenterol 2009;15:1209–18.
[4] Lumeng L, Crabb DW. Alcoholic liver disease. Curr Opin Gastroenterol 2001;17:211–20.
[5] Uesugi T, Froh M, Arteel GE, Bradford BU, Thurman RG. Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. Hepatology 2001;34:101–8.
[6] Yin M, Bradford BU, Wheeler MD, Uesugi T, Froh M, Goyert SM, et al. Reduced early alcohol-induced liver injury in CD14-deficient mice. J Immunol 2001;166:4737–42.
[7] McClain CJ, Barve S, Deaciuc I, Kugelmas M, Hill D. Cytokines in alcoholic liver disease. Semin Liver Dis 1999;19:205–19.
[8] Fujimoto M, Uemura M, Nakatani Y, Tsujita S, Hoppo K, Tamagawa T, et al. Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: relation to severity of liver disturbance. Alcohol Clin Exp Res 2000;24:48S–54S.
[9] Cameron RG, Neuman MG. Novel morphologic findings in alcoholic liver disease. Clin Biochem 1999;32:579–84.
[10] Bode C, Bode JC. Activation of the innate immune system and alcoholic liver disease: effects of ethanol per se or enhanced intestinal translocation of bacterial toxins induced by ethanol? Alcohol Clin Exp Res 2005;29:166S–71S.
[11] Reeds PJ, Burrin DG. Glutamine and the bowel. J Nutr 2001;131:2505S–8S [discussion 23S-4S].
[12] Wilmore DW, Shabert JK. Role of glutamine in immunologic responses. Nutrition 1998;14:618–26.
[13] Neu J, DeMarco V, Li N. Glutamine: clinical applications and mechanisms of action. Curr Opin Clin Nutr Metab Care 2002;5:69–75.
[14] Wischmeyer PE, Kahana M, Wolfson R, Ren H, Musch MM, Chang EB. Glutamine reduces cytokine release, organ damage, and mortality in a rat model of endotoxemia. Shock 2001;16:398–402.
[15] Tanaka T, Imano M, Yamashita T, Monna T, Nishiguchi S, Kuroki T, et al. Effect of combined alanine and glutamine administration on the inhibition of liver regeneration caused by long-term administration of alcohol. Alcohol Alcohol Suppl 1994;29:125–32.
[16] Cunningham CC, Preedy VR, Paice AG, Hesketh JE, Peters TJ, Patel VB, et al. Ethanol and protein metabolism. Alcohol Clin Exp Res 2001;25:262S–8S.
[17] Basuroy S, Sheth P, Mansbach CM, Rao RK. Acetaldehyde disrupts tight junctions and adherens junctions in human colonic mucosa: protection by EGF and L-glutamine. Am J Physiol Gastrointest Liver Physiol 2005;289:G367–75.
[18] Lieber CS, DeCarli LM. Animal models of chronic ethanol toxicity. Methods Enzymol 1994;233:585–94.
[19] Yeh SL, Lai YN, Shang HF, Lin MT, Chen WJ. Effects of glutamine supplementation on innate immune response in rats with gut-derived sepsis. Br J Nutr 2004;91:423–9.
[20] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497–509.
[21] Rudiger HA, Clavien PA. Tumor necrosis factor alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver. Gastroenterology 2002;122:202–10.
[22] Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502–22.
[23] Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 1980;106:207–12.
[24] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.

[25] Yang SC, Huang CC, Chu JS, Chen JR. Effects of beta-carotene on cell viability and antioxidant status of hepatocytes from chronically ethanol-fed rats. Br J Nutr 2004;92:209–15.
[26] Pirola RC, Lieber CS. Energy wastage in rats given drugs that induce microsomal enzymes. J Nutr 1975;105:1544–8.
[27] Morgan MY. The treatment of alcoholic hepatitis. Alcohol Alcohol 1996;31: 117–34.
[28] Aguiar AS, Boaventura GT, Abrahao RF, Freitas TL, Takiya CM, Filho PJ, et al. Ethanol in low chronic dose level attenuates major organic effects in malnourished rats. Biol Res 2009;42:31–40.
[29] Gordon H. Detection of alcoholic liver disease. World J Gastroenterol 2001;7: 297–302.
[30] Lucey MR, Mathurin P, Morgan TR. Alcoholic hepatitis. N Engl J Med 2009;360: 2758–69.
[31] Sukhotnik I, Agam M, Shamir R, Shehadeh N, Lurie M, Coran AG, et al. Oral glutamine prevents gut mucosal injury and improves mucosal recovery following lipopolysaccharide endotoxemia in a rat. J Surg Res 2007;143:379–84.
[32] Margaritis VG, Filos KS, Michalaki MA, Scopa CD, Spiliopoulou I, Nikolopoulou VN, et al. Effect of oral glutamine administration on bacterial tanslocation, endotoxemia, liver and ileal morphology, and apoptosis in rats with obstructive jaundice. World J Surg 2005;29:1329–34.
[33] Komano T, Egashira Y, Sanada H. L-Gln and L-Ser suppress the D-galactosamine-induced IL-18 expression and hepatitis. Biochem Biophys Res Commun 2008;372: 688–90.
[34] Lecleire S, Hassan A, Marion-Letellier R, Antonietti M, Savoye G, Bole-Feysot C, et al. Combined glutamine and arginine decrease proinflammatory cytokine production by biopsies from Crohn's patients in association with changes in nuclear factor-kappaB and p38 mitogen-activated protein kinase pathways. J Nutr 2008;138:2481–6.
[35] Thurman RG, Bradford BU, Iimuro Y, Frankenberg MV, Knecht KT, Connor HD, et al. Mechanisms of alcohol-induced hepatotoxicity: studies in rats. Front Biosci 1999;4:e42–6.
[36] Purohit V, Bode JC, Bode C, Brenner DA, Choudhry MA, Hamilton F, et al. Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: summary of a symposium. Alcohol 2008;42:349–61.
[37] Li N, DeMarco VG, West CM, Neu J. Glutamine supports recovery from loss of transepithelial resistance and increase of permeability induced by media change in Caco-2 cells. J Nutr Biochem 2003;14:401–8.
[38] Purohit V, Gao B, Song BJ. Molecular mechanisms of alcoholic fatty liver. Alcohol Clin Exp Res 2009;33:191–205.
[39] Hong F, Kim WH, Tian Z, Jaruga B, Ishac E, Shen X, et al. Elevated interleukin-6 during ethanol consumption acts as a potential endogenous protective cytokine against ethanol-induced apoptosis in the liver: involvement of induction of Bcl-2 and Bcl-x(L) proteins. Oncogene 2002;21:32–43.
[40] El-Assal O, Hong F, Kim WH, Radaeva S, Gao B. IL-6-deficient mice are susceptible to ethanol-induced hepatic steatosis: IL-6 protects against ethanol-induced oxidative stress and mitochondrial permeability transition in the liver. Cell Mol Immunol 2004;1:205–11.
[41] Singleton KD, Beckey VE, Wischmeyer PE. Glutamine prevents activation of NF-kappaB and stress kinase pathways, attenuates inflammatory cytokine release, and prevents acute respiratory distress syndrome (ARDS) following sepsis. Shock 2005;24:583–9.
[42] Wischmeyer P, John M. Kinney International Award for General Nutrition. Glutamine, heat shock protein, and inflammation — opportunity from the midst of difficulty. Nutrition 2004;20:583–5.
[43] Hong RW, Rounds JD, Helton WS, Robinson MK, Wilmore DW. Glutamine preserves liver glutathione after lethal hepatic injury. Ann Surg 1992;215: 114–9.