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Epigallocatechin-3-Gallate Ameliorates Alcohol-Induced Liver Injury in Rats

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Abstract: Endotoxemia is a common event in alcoholic liver disease. Elevated intestinal permeability is the major factor involved in the mechanism of alcoholic endotoxemia and the pathogenesis of alcoholic liver disease. This study examined the effect of epigallocatechin-3-gallate (EGCG) on alcohol-induced gut leakiness, and explored the related mechanisms involved in its protection against alcohol-induced liver injury in rats. Four groups of female Sprague-Dawley rats were studied. Alcohol and alcohol/EGCG groups rats received fish oil along with alcohol daily via gastrogavage for 6 weeks, and dextrose and dextrose/EGCG groups rats were given fish oil along with isocaloric dextrose instead of alcohol. The dextrose/EGCG and alcohol/EGCG groups received additional treatment of EGCG (100mg.kg^{-1} body weight) daily intragastrically by gavage. Intestinal permeability was assessed by urinary excretion of lactulose and mannitol (L/M ratio). Liver injury was evaluated histologically and by serum alanine aminotransferase (ALT). Plasma endotoxin and serum tumor necrosis factor-α (TNF-α) levels were assayed; liver malondialdehyde (MDA) contents determined. CD14 and inflammatory factors, such as TNF-α, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mRNAs in the liver were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Rats given fish oil plus alcohol had gut leakiness (L/M ratio was increased), which was associated with both endotoxemia and liver injury. The above responses were accompanied by increased CD14, TNF-α, COX-2 and iNOS mRNA expressions in the liver. EGCG supplementation partly blocked the gut leakiness, reduced endotoxemia and lipid peroxidation, and blunted the elevated expressions of CD14, TNF-α, COX-2 and iNOS, all of which were associated with improved liver injury. These results show that EGCG can

block alcohol-induced gut leakiness, reduce endotoxemia, and inhibit inflammatory factors expressions in the liver, thereby ameliorates alcohol-induced liver injury.

Keywords: epigallocatechin-3-gallate; alcohol-induced liver injury; intestinal permeability; endotoxemia; CD14; cyclooxygenase-2; inducible nitric oxide synthase.

1. Introduction

Alcoholic liver disease (ALD) is a major health and economic problem in the western world [1]. In recent years, the morbidity of ALD in China has risen quickly. Among drinkers, the morbidity has reached 6.1% [2]. Gut-derived endotoxemia is a common event in ALD. Elevated intestinal permeability is the major factor involved in the mechanism of alcoholic endotoxemia and the pathogenesis of alcoholic liver disease [3]. Endotoxin may activate Kupffer cells through binding CD14 /toll-like receptor-4, followed by release of a variety of inflammatory mediators, e.g. tumor necrosis factor-α (TNF-α), lipid metabolites, as well as reactive oxygen intermediates, all of which subsequently cause liver damage [4]. Thus, blocking endotoxemia and inhibiting Kupffer cell activation may be an important strategy in the prevention of alcohol-induced liver injury.

Catechins are naturally occurring polyphenolic compounds which are found in abundance in green tea. Epigallocatechin-3-gallate (EGCG) is the major constituent of the catechins, and has been shown to possess numerous biological functions, including antioxidant, anti-inflammatory, anticancer effects [5-7]. It was reported that green tea or its extract protects against alcohol-induced liver injury in rats [8, 9]; however, the mechanisms are not fully defined. In this study, we employed ethanol plus fish oil gavage female rat model of alcohol-induced liver disease, and investigated the effects of EGCG on intestinal permeability and endotoxemia, and inflammatory factors, such as TNF-α, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressions in the liver. The results suggest that EGCG partly blocks elevated intestinal permeability and endotoxemia, and suppresses inflammatory factors expressions in the liver, thereby ameliorating alcohol-induced liver injury.

2. Experimental Section

2.1. Chemicals and Reagents

Epigallocatechin-3-gallate was purchased from Sichuan Leshan Yujia Tea Science & Technology Development Co., Ltd. Malondialdehyde (MDA) assay kit was obtained from Nanjing Jiancheng Bioengineering Co., Ltd; enzyme-linked immunosorbent assay kit (ELISA) for rat TNF-α purchased from Shanghai Senxiong Biotech industry Co., Ltd; endotoxin quantitation kit from Shanghai Yihua Medical Science & Technology Co., Ltd. TRIzol reagent was purchased from Invitrogen (USA); DL2000 DNA ladder marker from TaKaRa Biotech Co., Ltd; M-MLV reverse transcriptase and its buffer, deoxyribonucleotide (dNTP, 10 mM) and oligo$(dT)_{15}$ primer were from Promega Corp.(Madison, USA); Taq DNA polymerase and its buffer, rRNasin Ribonuclease inhibitor from

Biostar (Canada). Polymerase chain reaction (PCR) primers for TNF-α, CD14, iNOS, COX-2 and GAPDH, were synthesized by Sai-Bai-Sheng Biocompany (Shanghai, China).

2.2 Animal Model and Treatments

Female Sprague-Dawley rats, weighing 180-200g, were obtained from the Experimental Animal Center of Wuhan University. After acclimation for 6 to 7 days, animals were given 0.5-0.6ml (about 2.5ml/kg) fish oil along with ethanol or isocaloric dextrose intragastrically by gavage. The initial dose of ethanol was $6g.kg^{-1}.day^{-1}$ (solutions maximally containing 56% alcohol), and the dose was progressively increased during week 1 to a maintenance dose of 8 g/kg/day that was continued for 5 more weeks. All rats had regular standard rat chow available throughout the 6-week period. Rats were weighted three times per week. Four treatment groups were studied: 1) dextrose (D, n=5); 2) dextrose/EGCG (D/E, n=5); 3) alcohol (A, n=8); 4) alcohol/EGCG (A/E, n=8). Rats in dextrose/EGCG and ethanol/EGCG groups received an oral dose of EGCG (100mg/kg body weight) via gastrogavage concurrently with dextrose or ethanol. At the end of 6 weeks, animals were anaesthetized with urethane (20%, $1.0g.kg^{-1}$) and sacrificed by bleeding from femoral arteries and veins. Two blood samples were collected from each rat, one of which (1ml) was placed in an endotoxin-free and heparin-coated tube. Immediately after exsanguination, the livers were harvested. Small portions of the livers were kept frozen at –70°C for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, whereas another portion was separated and immersed in 10% buffered formalin solution for histological examination. All animals were given humane care in compliance with the institutional guidelines.

2.3 Measurement of blood alcohol

Blood was taken from the tail vein 1h after gavage, 2 weeks after initiation of alcohol. Blood alcohol levels (BAL) were measured using the alcohol dehydrogenase kit from Sigma Chemical Co.

2.4 Intestinal Permeability Assay

Intestinal permeability in rats was assessed after 8h fast except that the animals received intragastric administration of 2ml of a solution containing lactulose (L) 66mg/kg and mannitol (M) 50mg/kg [10]. Rats were housed individually in metabolic cages. Urine was collected for 12h, with the volume recorded and 0.2ml mercury salicylosulfide added. To promote urine output, each rat also received 5ml Ringer-lactate solution subcutaneously, just prior to sugar administration. Then 5ml urine specimen was stored at –20°C until measured. Urinary sugar levels were measured using a high-pressure liquid chromatograph (LC-9A, Shimadzu, Japan) with ion-exchange column (Transgenomic Co., USA). Calibration was performed on a daily basis with authentic standards at multiple concentrations. An increase in the urinary L/M ratio was used as an index of increased intestinal permeability [11].

2.5 Pathological Evaluation

Liver specimens, with approximate size $1.0\times0.5\times0.3cm^3$, were processed for light microscopy. This processing consisted of fixing the specimens in 10% formaldehyde for 12-24h, embedding them in paraffin, slicing sections of 5μm in thickness and staining the sections with hematoxylin and eosin. Histological assessment of steatosis, necrosis and inflammation, was performed by a pathologist who

was blinded to the identification of the treatment groups. It was done as follows: steatosis (the percentage of liver cells containing fat), 1+, <25% of cells containing fat; 2+, 26% to 50%; 3+, 51% to 75%; and 4+, >75%. Necrosis was evaluated as the number of necrotic foci/mm^2; inflammation was scored as the number of inflammatory cells/mm^2 [12].

2.6 Serum Alanine Aminotransferase and TNF-α Assay

Blood samples were allowed to clot, and the sera were isolated by centrifugation at 1000rpm for 10min and kept at –20°C before the following determination. Enzymatic activity of alanine aminotransferase (ALT) was measured using a commercial kit by a RA1000 Automatic Biochemical Analyzer (Japan).

Serum TNF-α levels were measured using the sandwich ABC-ELISA method according to the manufacturer's suggested protocol. Samples were compared with the standard curve.

2.7 Plasma Endotoxin Assay

Blood samples (1ml) collected in heparin-coated tubes were centrifuged at 500rpm for 5min, and the plasma obtained was stored at –20°C in endotoxin-free ependorf tubes. The assay was done within 48h after collection. An aliquot of 0.1ml plama was taken and added with pyrogen-free 0.9% saline solution 0.2ml and Tris-HCl buffer 0.2ml, mixed, then incubated at 100°C for 10min to remove any potential endotoxin inhibitors. After incubation, the samples were centrifuged at 3000rpm for 10min, and the supernatant was taken and used for the assay of endotoxin. An aliquot of 0.1ml supernatant was incubated with 0.05ml Limulus amebocyte lysate at 37°C for 25min. After several subsequent reactions, the samples were read spectrophotometrically at 545nm [13]. The plasma endotoxin levels were calculated against a standard curve of endotoxin (E.coli 0113:H10) concentrations of 0.5, 0.25, 0.125, 0.0625 and 0.08 $EU.ml^{-1}$.

2.8 Determination of Liver MDA Contents

Liver samples were thawed, weighed and homogenized 1:9 w:v in 0.9% saline. Then the homogenates were centrifuged at 3 000rpm for 10min at 4°C and the supernatant was taken for the assays of MDA contents and total protein.

MDA was assayed by the measurement of thiobarbituric acid-reactive substances (TBARS) levels spectrophotometrically at 532nm. Results were expressed as $nmol.mg^{-1}$ protein.

Total protein concentration was determined using the Coomassie Blue method with bovine serum albumin as standard.

Detailed procedures for the above measurements were performed according to the kits' protocol.

2.9 RT-PCR Analysis of Liver CD14, TNF-α, COX-2, iNOS mRNA Expressions

Total RNA was isolated from approximatively 50-100mg snap frozen liver tissue using the TRIzol protocol as suggested by the supplier. Two micrograms of total RNA were reverse-transcribed by adding 0.5μg oligo$(dT)_{15}$ primer, M-MLV 5×reaction buffer 5μl, dNTP (10mM, each) 1.25μl, rRNasin Ribonuclease inhibitor 25 units, M-MLV RT 200 units and DEPC-treated water to final volume of

25μl at 42°C for 60 min. The reverse transcriptase was heat-inactivated at 85°C for 5 min and cooled on ice.

The PCR reaction mixture contained 10×Taq buffer 5μl, dNTP (10mM, each) 1μl, gene specific primers (Table1. sense and antisense primers, 25 pmol.μl^{-1}, each) 1μl, Taq DNA polymerase 2.0 units and cDNA 1μl in a total volume of 50μl. Amplification was performed with 35 cycles with initial incubation at 94°C for 3 min and final extension at 72°C for 7 min, each cycle of which consisted of denaturation for 45s at 94°C, annealing for 45 s at 54°C (TNF-α, iNOS), 55°C (CD14, COX-2, GAPDH), and extension for 1min at 72°C. The quantities of cDNA producing equal amounts of GAPDH (house-keeping gene)-PCR-product were used in PCR with the primers for TNF-α, CD14, COX-2, iNOS. Following RT-PCR, 5μl samples of amplified products were resolved by electrophoresis in 2% agarose gel, stained with ethidium bromide. The level of each PCR product was semiquantitatively evaluated using a digital camera and an image analysis system (Vilber Lourmat, France), and normalized to GAPDH.

Table 1. PCR primers for CD14, TNF-α, COX-2, iNOS and GAPDH

Name	Sense	Antisense	Prod. Length (bp)
CD14	CTTGTTGCTGTTGCCTTTGA	CGTGTCCACACGCTTTAGAA	214
TNF-α	GCCAATGGCATGGATCTCAAAG	CAGAGCAATGACTCCAAAGT	357
COX-2	CCGTGGTGAATGTATGAGCATAGG	GGATGAACTCTCTCCTCAGAAGAACC	440
iNOS	TTCTTTGCTTCTGTGCTAATGCG	GTTGTTGCTGAACTTCCAATCGT	1061
GAPDH	TCCCTCAAGATTGTCAGCAA	AGATCCACAACGGATACATT	309

2.10 Statistical Analysis

Results were presented as mean ± SD unless otherwise indicated. Differences between groups were analyzed using analysis of variance (ANOVA) with post hoc LSD test. A *p* value less than 0.05 was considered to be statistically significant.

3. Results

In each of the groups studied, the rats increased their weight at a constant rate; there was no difference in weight gain among the groups. At week 2, BAL 1h after ethanol administration by gavage were similar in alcohol/EGCG group (376.6±68.1mg/100ml) and alcohol group (387.3±51.9mg/100ml).

3.1 Effect of EGCG on Alcohol-Induced Liver Injury

Animals given fish oil plus dextrose developed slight steatosis in the liver, but no inflammation or necrosis was observed (Figure 1A); whereas rats in dextrose/EGCG group showed almost normal liver histology (Figure 1B).Chronic daily fish oil plus alcohol gavage caused macrovesicular and microvesicular steatosis, spotty necrosis and mild inflammation in the liver (Table 2, Figure 1C). In alcohol/EGCG group, there was complete absence of necrosis and slight inflammation in the liver;

however, the steatosis was not significantly different but tended to decrease compared with alcohol group (Table 2, Figure 1D).

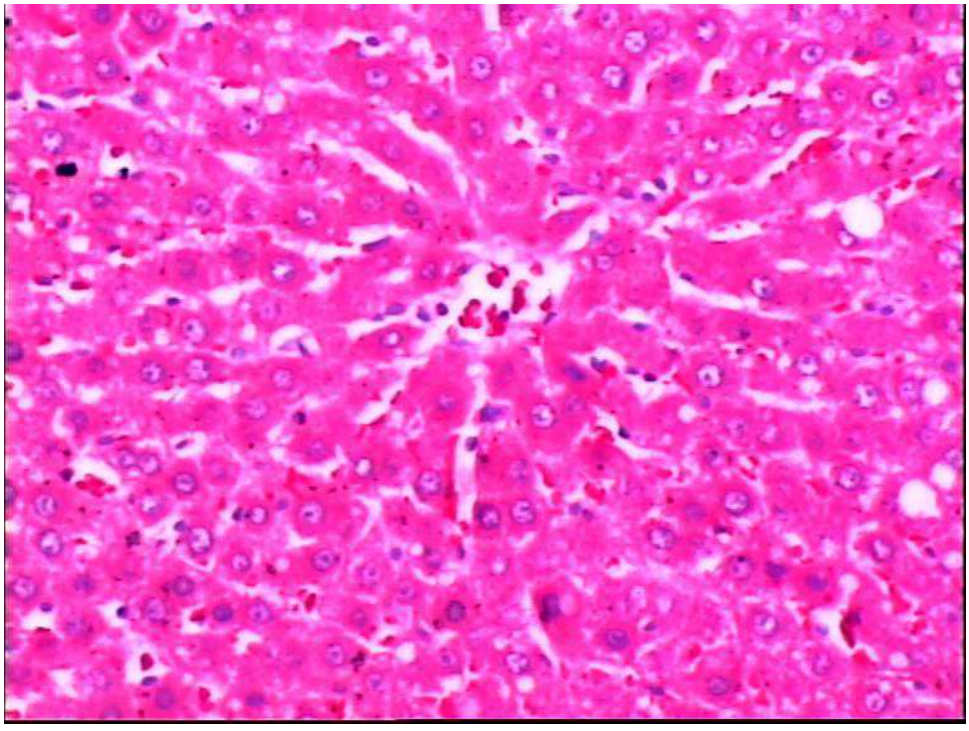
Consistent with the histological changes, serum ALT levels, an index of liver cell injury, were significantly elevated in alcohol-fed rats as compared with dextrose-fed rats ($p<0.05$). However, the increase in ALT levels was significantly reduced in alcohol/EGCG group rats compared to alcohol group rats ($p<0.05$). There was no significant difference in ALT levels between dextrose/EGCG- and dextrose-fed rats (Table 3).

Table 2. Hepatic pathological scores in the different experimental groups

Groups	Fatty liver (0-4)	Necrosis (foci/mm^2)	Inflammation (cells/mm^2)
D	0.6±0.2	0	1.6±1.5
D/E	0.2±0.1†	0	0
A	3.0±0.5†	1.0±0.5†	18.8±4.0†
A/E	2.5±0.5	0#	6.5±0.9#

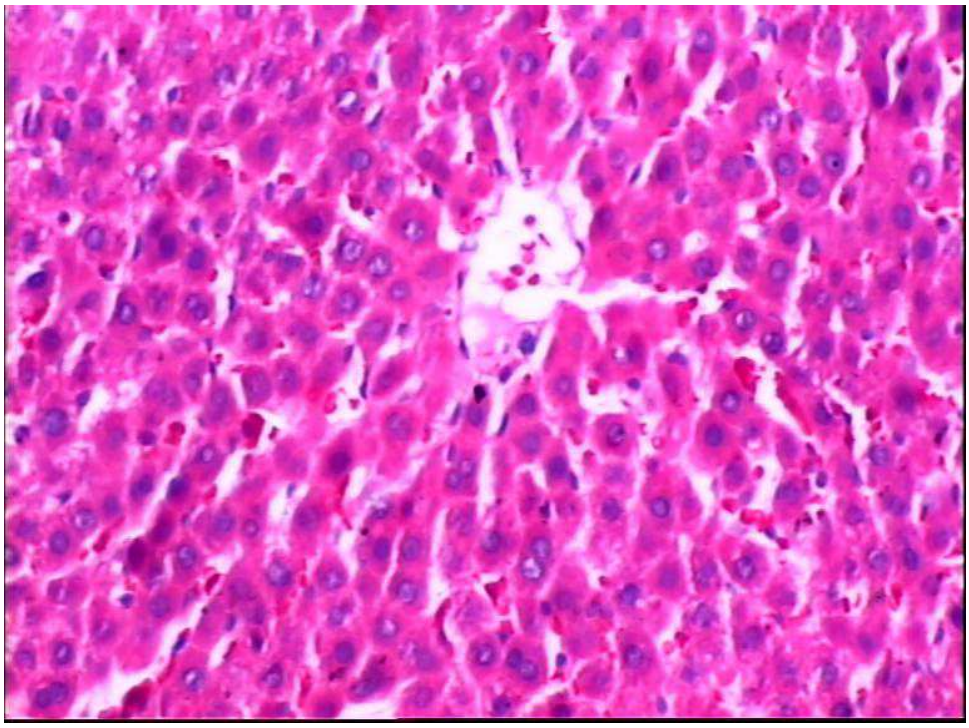
† vs D group; # vs A group, $p<0.05$

Figure 1. Representative histological sections of the liver obtained from (1A) D group, showed slight steatosis, but no inflammation or necrosis; (1B) D/E group, showed almost normal liver histology; (1C) A group, showed steatosis, spotty necrosis (short arrow) and mild infiltration of inflammatory cells (long arrow); (1D) A/E group, showed complete absence of necrosis and slight inflammation, however steatosis was still present but tended to decrease compared with A group. Original magnification, 200×.

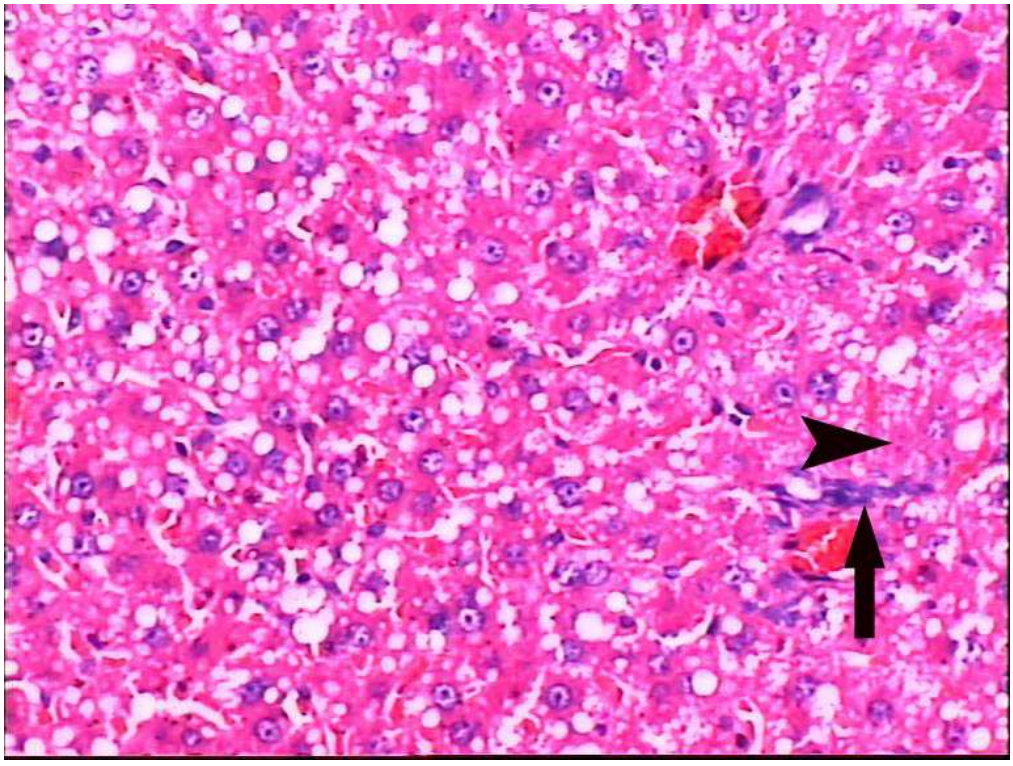


(1A) D group

(Figure 1 continued)

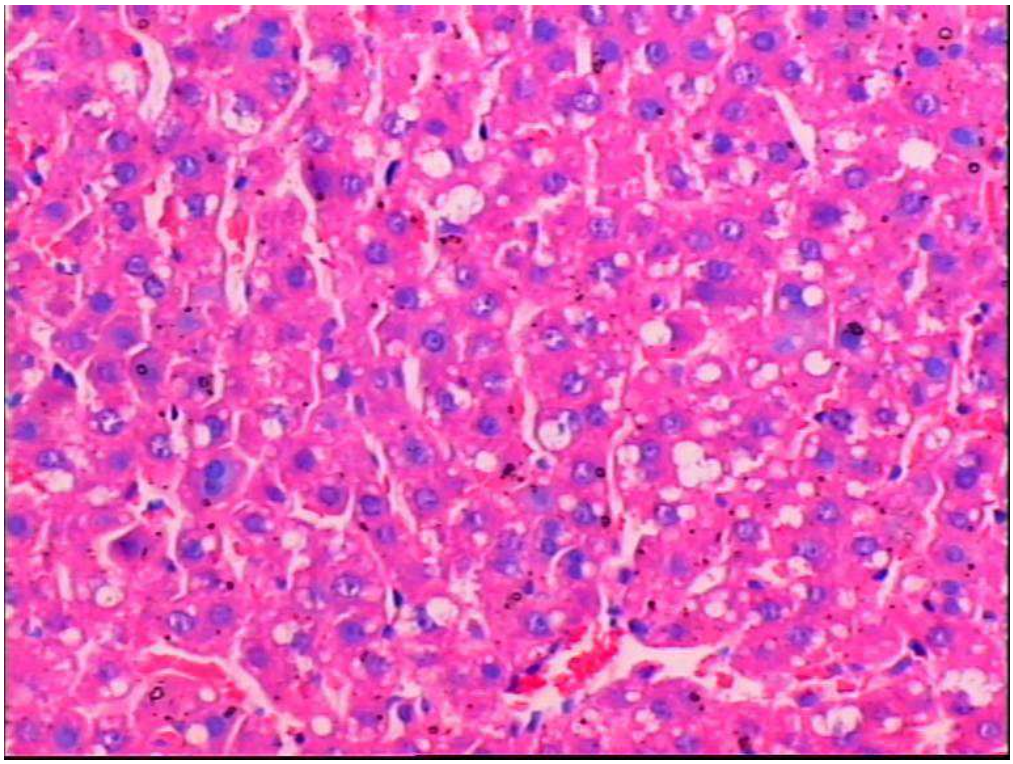


(1B) D/E group



(1C) A group

(Figure 1 continued)



(1D) A/E group

Table3. Serum ALT and TNF-α levels, plasma endotoxin levels and liver MDA contents in the different experimental groups

Groups	ALT ($U.L^{-1}$)	TNF-α ($pg.ml^{-1}$)	Endotoxin ($EU.ml^{-1}$)	MDA ($nmol.mg^{-1}$protein)
D	46.8±3.03	3.87±0.27	0.08±0.01	5.36±0.31
D/E	42.6±5.41	4.21±0.36	0.07±0.01	4.84±0.71
A	124.1±7.28†	53.65±4.46†	0.31±0.05†	9.38±0.39†
A/E	70.6±6.16#	27.70±3.83#	0.18±0.04#	7.14±0.38#

† vs D group; # vs A group, $p<0.05$

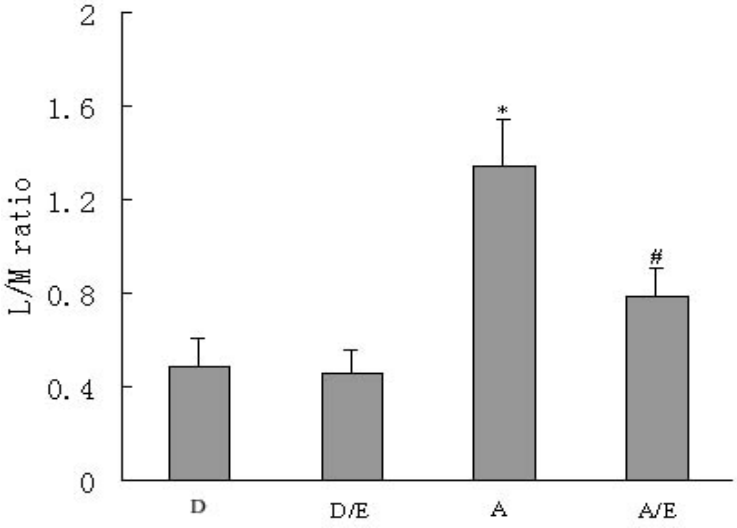
3.2 Effects of Ethanol and EGCG on Intestinal Permeability and Plasma Endotoxemia Levels

There was no significant difference in L/M ratios between dextrose (0.48±0.12) and dextrose/EGCG (0.46±0.10) groups. Chronic daily alcohol gavage caused a significant increase in intestinal permeability. The urinary L/M ratio in alcohol-fed rats at 6 weeks was 1.34±0.22, and was significantly higher than in dextrose group ($p<0.01$); concurrent treatment with EGCG blunted the increased leakiness of the gut by about 50% (0.78±0.13, $p<0.05$, Figure2).

All rats had detectable endotoxin levels in their plasma, but chronic alcohol exposure caused significant endotoxemia. The plasma endotoxin levels in dextrose/EGCG group were 0.07±0.01 $Eu.ml^{-1}$, which was comparable to dextrose group (0.08±0.01 $Eu.ml^{-1}$). The endotoxin levels in the

alcohol-fed group were 0.31±0.05 Eu.ml^{-1}, nearly 4-fold higher than dextrose group, and the increase was statistically significant (p<0.001). Plasma endotoxin levels in alcohol/EGCG-fed rats were significantly lower than in alcohol-fed rats (p<0.05, see Table3).

Figure 2. Effect of ethanol and EGCG on L/M ratios in rats. Chronic alcohol administration significantly increased the L/M ratio compared with dextrose group (* p<0.01). EGCG concurrent treatment significantly lowered the L/M ratio compared with alcohol group (# p<0.05).



3.3 Effect of ethanol and EGCG on serum TNF-α levels and liver MDA contents

In the dextrose- and dextrose/EGCG-fed groups, all rats had TNF-α serum levels of <5pg.ml^{-1}. Chronic alcohol gavage led to a significant increase in serum TNF-α. Concurrent treatment with EGCG blunted the increased levels of TNF-α by about 50% (Table3).

Liver contents of MDA, a marker of lipid peroxidation, were significantly increased after alcohol feeding compared with dextrose group. The levels of MDA were significantly lower in alcohol-fed rats with concurrent treatment with EGCG. There was no significant difference between dextrose and dextrose/EGCG groups in liver MDA contents (Table3).

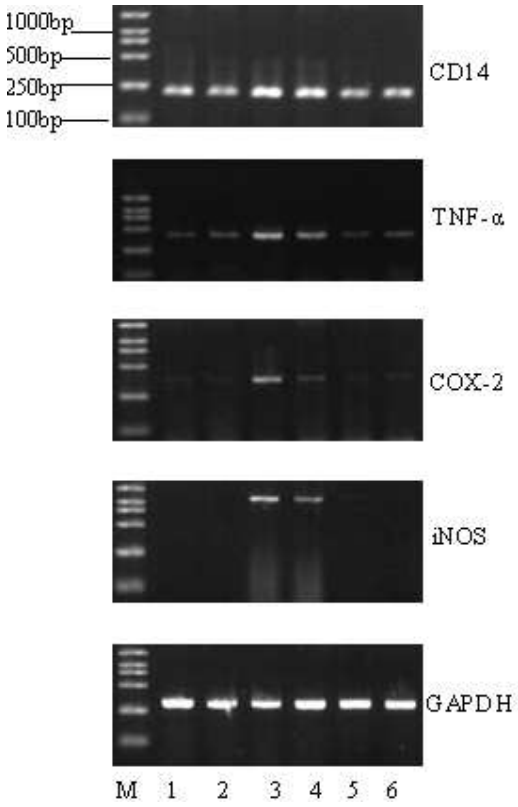
3.4 Effect of Ethanol and EGCG on CD14, TNF-α, COX-2, iNOS mRNA Expressions In the Liver

Weak or faint bands for CD14, TNF-α mRNA were detected in dextrose and dextrose/EGCG-fed groups. Significant elevation of CD14, TNF-α mRNA expression was observed in alcohol-fed rats. However, concurrent EGCG treatment significantly blunted the rises of CD14, TNF-α mRNA expression to an extent comparable to that of dextrose group (Figure3A and 3B).

COX-2 mRNA has previously been shown to be up-regulated in the rats that exhibit necro-inflammatory changes [14]. It is only detected in rats fed fish oil plus alcohol. EGCG supplementation was associated with the absence of COX-2 mRNA in rats fed ethanol (Figure3A and 3B). iNOS mRNA was barely detectable in both dextrose and dextrose/EGCG groups. However, there was a

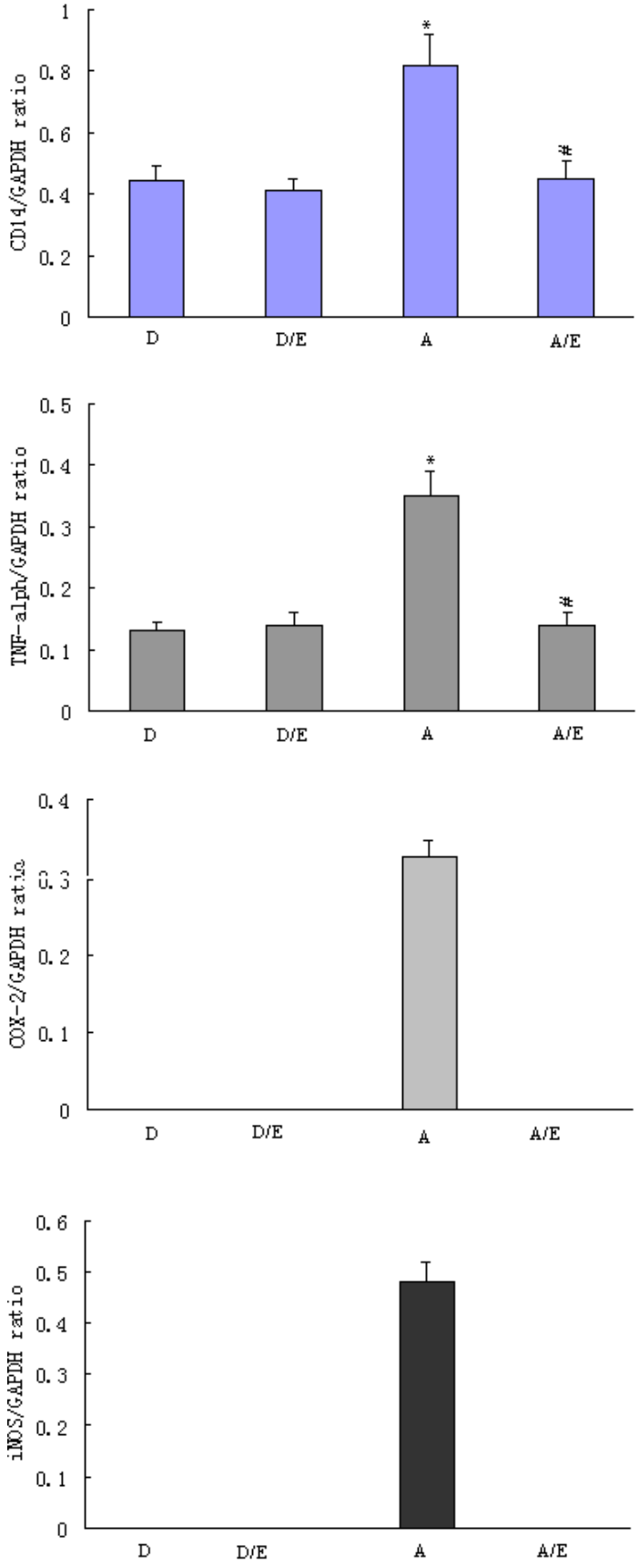
marked induction in alcohol group, which was inhibited significantly by concurrent treatment with EGCG (Figure 3A and 3B).

Figure3. RT-PCR analysis of mRNAs for CD14, TNF-α, COX-2, iNOS and GAPDH in liver samples obtained from the different groups: lane 1: D group, lane 2: D/E group, lane 3 and 4: A group, lane 5 and 6: A/E group (shown in A). The lanes represent different rats in each experimental group. Intense bands for CD14, TNF-α were observed in ethanol group, but in the other groups, only faint or weak bands were detected. COX-2 and iNOS mRNAs were detected only in fish oil plus ethanol-fed rats. None of the rats in the other groups had detectable COX-2 or iNOS mRNA. Shown in B are normalized densitometric ratios of CD14, TNF-α, COX-2 and iNOS bands to GAPDH respectively. RT-PCR analysis for each gene was performed at least once for each rat.* vs. D group, # vs. A group, $p<0.01$.



(3A)

(Figure 3 continued)



(3B)

4. Discussion

There is considerable evidence that the amount of fat in the diet is a key determinant of lesions in ALD, and that dietary lipids are a key source of the lipids accumulating in liver of ethanol-fed animals [15]. Furthermore, the saturated fatty acids are protective against ALD; in contrast, unsaturated fatty acids promote ALD [16]. In rats fed ethanol and tallow (beer fat, saturated fatty acids), no pathologic changes developed, but severe changes were seen in rats fed ethanol with coin oil (55-60% linoleic acid) [17]. Compared with rats fed coin oil with ethanol, severer liver injury, particularly necrosis and inflammation, is seen in rats fed fish oil with ethanol [18]. Alcoholic liver injury is more severe and rapidly developing in women than men [19]. The severity of necrosis, inflammation, and fibrosis was greater in ethanol-fed female than male rats [20]. In our study, we employed female rats, and used fish oil plus ethanol gavage to establish animal model of alcoholic liver injury. The rats developed endotoxemia and pathological changes in the liver after 6 weeks, e.g. pronounced macrovesicular and microvesicular steatosis, mild inflammation, and spotty necrosis. This chronic gavage of alcohol in rats is a simple experimental model that mimics key aspects of ALD in humans, including endotoxemia and liver injury, and is useful for exploring the mechanism and treatment of ALD.

EGCG is the major constituent of the catechins, and has been shown to possess antioxidant, anti-inflammatory properties [5, 6]. In the present study, EGCG concurrent treatment significantly prevented alcohol-induced liver injury as evidenced by a marked blunting of elevation in serum ALT levels and absence of necrosis in liver histology, which is consistent with Arteel GE et al.'s study with green tea extract [9]. In our study, rats in dextrose group developed slight steatosis in the liver, but almost normal liver histology was seen in dextrose/EGCG group. Alcohol/EGCG-fed rats also developed fatty liver; but compared with alcohol group, the steatosis tended to decrease although there was no statistical difference between these two groups. From the above facts, EGCG has weak antisteatotic effect. Several lines indicated that EGCG can suppress inflammatory cells infiltration [21, 22]. In our study, we also observed a significant decrease in inflammation scores in alcohol/EGCG group compared with alcohol group.

A significant body of evidence indicates that endotoxemia and endotoxin-mediated hepatocellular damage play a crucial role in the pathogenesis of ALD [3]. Plasma endotoxin levels are increased in patients with alcoholic hepatitis, and correlate with severity of liver disturbance [23]. The plasma endotoxin levels were also elevated in rats by chronic or acute administration of ethanol, which was associated with liver injury [24, 25]. Rats administered with antibiotics, polymyxin B, and neomycin, decreased plasma endotoxin levels by nearly 75%, and prevented alcohol-induced liver injury [26]. Alcoholic endotoxemia is mainly caused by increased intestinal permeability [3]. In our study, chronic alcohol gavage caused significantly increased intestinal permeability and endotoxemia in rats. However, EGCG concurrent treatment blunted the increased intestinal permeability by half and reduced endotoxemia. It was reported that ingestion of green tea reverts the intestinal mucosal and villous atrophy induced by fasting to normal, and the ingestion before fasting protects the intestinal mucosa against atrophy [27]. Mazzon E et al showed that green tea polyphenol extract attenuates colon injury induced by intracolonic instillation of dinitrobenzene sulphonic acid (DNBS) [28]. EGCG has been shown to ameliorate increased epithelial permeability induced by IFN-gamma [29]. Alcohol-induced gut leakiness is associated with myosin light chain kinase (MLCK) activation, and dissociation

of tight junction (TJ) and adherens junction protein complexes [3].The mechanism how EGCG blocks alcohol-induced gut leakiness needs further study.

Endotoxin itself is not hepatotoxic at low concentrations. However, it can evoke Kupffer cells through CD14/toll-like receptor-4 to release a variety of inflammatory mediators, including TNF-α [4]. CD14 expression in the liver is enhanced in alcoholic liver injury in rats, and correlates with the presence of pathological liver injury [30]. Alcohol-induced liver injury is reduced in CD14 knockouts mice compared with the wide-type [31]. CD14 expression is supposed to be the underlying mechanism that determines the liver's sensitivity to endotoxin toxicity [32]. In our study, chronic alcohol administration resulted in a significantly elevated mRNA expression of CD14 in the liver compared with dextrose-fed rats. In addition to CD14, serum TNF-α levels and TNF-α mRNA expression in the liver were also increased in rats fed fish oil plus alcohol. EGCG supplementation significantly blunted the increase in both CD14 and TNF-α expression in the liver, and in serum TNF-α levels. COX-2 is shown to be implicated in alcoholic liver disease and hepatocellular injury [14, 33]. COX-2-deficient mice are protected against the toxic effects of endotoxin [34]. COX-2 expression is increased in alcoholic liver injury with necroinflammatory changes and associated with endotoxemia. Kupffer cells are shown to be the primary source of COX-2 in liver [14]. In the present study, COX-2 mRNA was only detected in fish oil plus alcohol-fed rats, whose liver histology showed presence of necrosis and inflammation. EGCG concurrent treatment was associated with the absence of COX-2 mRNA expression. These above results suggest that EGCG may block Kupffer cells activation and their subsequent TNF-α and COX-2 expression.

Several lines indicated that damaging oxidant in ALD is dependent on the production of $O2^{\cdot}$- and $NO^{\cdot}$ [35, 36].Simultaneous production of $O2^{\cdot}$- and $NO^{\cdot}$ in Kupffer cells leads to formation of peroxynitrite anion ($ONOO^{\cdot}$), which may result in the stimulation of TNF-α production via activation of the oxidant-sensitive transcription factor NF-κB [37]. Hepatic nitric oxide (NO) production following endotoxemia is mediated by increased iNOS gene expression [38]. iNOS knockout mice or wild-type mice treated with N-(3-aminomethyl)benzyl-acetamindine (1400W), a highly selective iNOS inhibitor, were protected against alcohol-induced liver injury. The accumulation of 4-hydroxynonenal (lipid peroxidation) caused by alcohol was completely blocked in iNOS knockout mice [36]. In our study, liver contents of MDA, a marker of lipid peroxidation, were significantly elevated in chronic alcohol-fed rats compared with dextrose-fed rats. As expected, EGCG, the potent antioxidant, markedly blunted the increase of MDA levels. iNOS mRNA was induced in rats fed fish oil plus alcohol, but its enhanced expression was also markedly blunted after EGCG concurrent treatment.

In conclusion, this study demonstrated that EGCG partially ameliorates alcohol-induced liver injury, and that the mechanism may involve blocking alcohol-induced gut leakiness and endotoxemia, preventing Kupffer cell activation and related proinflammatory factors expressions in the liver.

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