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ORIGINAL ARTICLE

Protective Effects of Genistein Alleviate Alcohol-Induced Liver Injury in Rats

Wanlapa Leelananthakul, Duangporn Werawatganon, Naruemon Klaikeaw[1], Maneerat Chayanupatkul, Prasong Siriviriyakul

Department of Physiology, Alternative and Complementary Medicine for GI and Liver Diseases Research Unit, [1]Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

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ABSTRACT

Background: Alcohol is a major contributor of chronic liver disease worldwide. Medical treatment for alcoholic liver disease (ALD) is limited. Due to the roles of oxidative stress in the development of ALD, genistein, a natural antioxidant, might be beneficial in alleviating alcohol-induced liver injury. **Materials and Methods:** Eighteen male Sprague–Dawley® rats were divided into three groups ($n = 6$ each). Control group received distilled water, while alcohol group received 50% alcohol (8 g/kg body weight [BW] per day), and genistein group received genistein (16 mg/kg BW per day) dissolved in 50% alcohol (8 g/kg BW per day) for 4 weeks. At the end of the study, liver tissue was obtained for histopathology and immunohistochemistry for interleukin-18 (IL-18), hepatic malondialdehyde (MDA), and glutathione (GSH) measurement. Serum samples were analyzed for alanine transaminase (ALT) and tumor necrosis factor-α (TNF-α). **Results:** Alcohol-fed rats gained significantly less weight than control and genistein ones (48.83 ± 14.59, 142.83 ± 10.06 vs. 69.17 ± 7.33 g, respectively, $P < 0.01$). Serum ALT levels were also significantly lower in genistein group than in alcohol group (32.43 ± 12.90 vs. 120.30 ± 75.30; $P < 0.05$). Hepatic MDA levels were higher in alcohol group (0.13 ± 0.02 nmol/mg protein), while the levels were comparable between genistein (0.09 ± 0.02 nmol/mg protein) and control groups (0.1 ± 0.01 nmol/mg protein). There was a trend toward a decrease in GSH levels in alcohol-fed rats as compared to control ones. On the contrary, GSH levels were significantly increased in GSH-treated rats. Markers of inflammatory responses, such as IL-18 and TNF-α, were higher in alcohol group and declined toward the control group with genistein administration. **Conclusion:** Alcohol-induced hepatic cell damages through oxidative stress and inflammatory responses. Genistein could alleviate alcohol-induced liver injury through its antioxidant and anti-inflammatory properties.

Key words: Alcoholic hepatitis, antioxidant, genistein, inflammation, oxidative stress

SUMMARY

- Genistein alleviated alcohol-induced liver injury through its antioxidant, anti-inflammatory, and glutathione restoration properties.

Parameter	Body weight	ALT	MDA	TNF-α	IL-18	GSH	Liver histopathology
Control	increased	normal	normal	normal	normal	normal	normal
Alcoholic hepatitis	decreased	increased	increased	increased	increased	decreased	steatosis, hepatocyte ballooning, and lobular inflammation
Genistein+ Alcohol	Slight decreased	decreased	decreased	decreased	decreased	increased	improved

Abbreviations used: ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease; ALT: Alanine transaminase; TNF-α: Tumor necrosis factor-alpha; IL-18: Interleukin-18; LPS: Lipopolysaccharide; MDA: Malondialdehyde; GSH: Glutathione; TBARS: Thiobarbituric acid-reactive substances; ELISA: Enzyme-linked immunosorbent assay; DMSO: Dimethyl sulfoxide; DAB: Diaminobenzidine.

Correspondence:

Prof. Duangporn Werawatganon,
Department of Physiology, Faculty of Medicine,
Chulalongkorn University, Bangkok 10330, Thailand.
E-mail: dr.duangporn@gmail.com

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INTRODUCTION

Alcoholic liver diseases (ALDs) cause significant health problems worldwide. Globally, in 2010, alcohol-attributable liver cirrhosis was responsible for 493,300 deaths and 14,544,000 disability-adjusted life years.[1] The spectrum of ALD ranges from simple steatosis, alcoholic steatohepatitis, progressive fibrosis to cirrhosis and hepatocellular carcinoma.[2] The current medical therapies for ALD are limited and with disappointing efficacy. Prednisolone, a standard treatment for severe alcoholic hepatitis, has been shown in a recent randomized controlled trial to have no benefits in long-term survivals and increased risk of infectious complications.[3] Therefore, the quest for safe and effective treatment for alcoholic hepatitis has been an area of active research.

Liver plays a pivotal role in alcohol metabolism. Once absorbed, alcohol is metabolized to acetaldehyde by alcohol dehydrogenase enzyme and subsequently to acetic acid by aldehyde dehydrogenase. Moreover, alcohol can be metabolized through microsomal ethanol-oxidizing system using cytochrome P450 2E1. This process creates reactive oxygen species (ROS).[4,5] Both acetaldehyde and ROS create lipid peroxidation leading to the production of end products such as malondialdehyde (MDA).[5-7] In addition, acetaldehyde can deplete the natural antioxidants such as mitochondrial glutathione (GSH) and S-adenosylmethionine, further intensifying the oxidative stress.[5] These chain reactions lead to hepatocyte injury, inflammatory responses, hepatic stellate cell activation, and extracellular matrix production.

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Other than the direct effects from its metabolites, alcohol can increase intestinal permeability and liver exposure to lipopolysaccharide (LPS).[8] LPS then binds with Toll-like receptor-4 (TLR-4) on Kupffer cells leading to inflammatory cytokine releases, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6, and IL-18.[8,9] These cytokines are major players in the hepatocyte injury and apoptosis. Given the roles of oxidative stress in the development of ALD, the natural products with antioxidant properties appear to be the attractive options for the treatment of ALD. One of the leading candidates would be genistein.

Genistein is one of the major isoflavones found in soy and soy products. Genistein exerts several functions such as reducing lipid peroxidation,[10-12] enhancing fatty acid catabolism,[11] and promoting extracellular matrix degradation.[12] Despite being studied extensively in nonalcoholic fatty liver disease (NAFLD), little is known about the effects of genistein in ALD. In the present study, we aimed to evaluate the effects of genistein on alcohol-induced liver injury in rats determined by the changes in histopathology, antioxidants, lipid peroxidation, and inflammatory markers. The protocol for this study was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (Approval No. 08/2556).

MATERIALS AND METHODS

Animal preparation

Male Sprague–Dawley® rats weighing 180–220 g were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. Animals were housed in a standard room with controlled temperature of 25°C ± 1°C and a 12-h light-dark cycle. The research was conducted according to the policies and procedures proposed by the Chulalongkorn University Animal Care and Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (Approval No. 08/2556).

Experimental protocol

Eighteen rats were randomly divided into three groups ($n = 6$ in each group). All animals were fed *ad libitum* with a custom diet containing 35% of total calories from fat (corn oil), 18% from protein, and 47% from carbohydrates.[13,14] Genistein powder was purchased from Cayman Chemical Company (Michigan, USA). All animals were fed with either alcohol or distilled water twice a day through an intragastric tube for 4 weeks. Group 1 or control group received distilled water (16 mL/kg body weight [BW] per day); Group 2 or alcohol group received 50% alcohol (8 g/kg BW per day); and Group 3 or genistein group received daily genistein (16 mg/kg BW per day) dissolved in 50% alcohol (8 g/kg BW per day) for the entire 4 weeks of the experiment. All rats were weighed on the 1st day of the study before drug administration and the end of the study before euthanasia to calculate the changes in BW.

At the end of 4 weeks, all rats were euthanized with sodium thiopental overdose after a 12-h fast. Total hepatectomy was performed. Liver was immediately washed with 25°C saline and then cut into several pieces. Three small pieces of the liver were frozen in liquid nitrogen and stored at −80°C until MDA and GSH analyses. The remaining liver specimens were fixed in 40 g/L formaldehyde for IL-18 expression analysis. Blood samples were obtained by cardiac puncture and kept at room temperature for 2 h. Specimens were then centrifuged at 2000 × *g* for 20 min, and serum samples were collected for alanine transaminase (ALT) and TNF-α analysis.

Hepatic malondialdehyde determination

MDA level was measured using a commercial assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The test involves the measurement of thiobarbituric acid reactive substances production rate under high temperature and acidic conditions. The methodology is as follows: 1 g of liver tissue was homogenized in radioimmunoprecipitation assay buffer containing protease inhibitor and sonicated on ice for 15 s. Supernatants were obtained after centrifugation at 1600 × *g* for 10 min at 4°C. The absorbance of supernatant fraction was read at a wavelength of 532 nm. MDA levels were calculated from a standard curve and expressed as nmol/mg protein.

Hepatic glutathione determination

GSH level was quantified using a commercial assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Liver tissues were washed with phosphate-buffered saline (PBS) solution. Tissues were then homogenized with cold (morpholino) ethanesulfonic acid buffer before being centrifuged at 10,000 × *g* for 15 min at 4°C. Supernatants were collected and deproteinated. The absorbance of supernatant fraction was read at a wavelength of 405 nm, and GSH values were calculated from a standard curve and expressed as nmol/mg protein.

Immunohistochemistry for hepatic IL-18 expression

After being fixed in formaldehyde, liver samples were embedded in paraffin and sliced at a thickness of 3 μm. Tissue sections were then deparaffinized with xylene and ethanol for 10 min. The antigen retrieval was achieved by treating the slides with citrate buffer pH 6.0 and heating in a microwave for 13 min. Slides were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity for 5 min and with 3% normal horse serum to block nonspecific binding for 20 min. Tissues were then washed with PBS solution. Subsequently, sections were incubated with mouse monoclonal antibody against IL-18 (GeneTex, Inc., Irvine, CA, USA) at a dilution of 1:200 for 1 h at a room temperature and washed again with PBS solution. Slides were then incubated with secondary antibody for IL-18 (Dako Denmark AS, Glostrup, Denmark) for 30 min at a room temperature. When the color development with diaminobenzidine was detected, sections were counterstained with hematoxylin. Under light microscopy, IL-18-positive cells were defined as Kupffer cells with dark brown-stained nuclei. Images of each sample were taken at high magnifications (×40). Five hundred Kupffer cells were manually counted for each rat, and the proportions of IL-18-positive cells were calculated.

Serum tumor necrosis factor alpha determination

Serum levels of TNF-α were determined by a solid-phase enzyme immunoassay technique using commercially available kits (R and D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. TNF-α levels were expressed as pg/mL.

Pathological examination

After fixation in 10% formalin, liver tissues were processed by routine histology procedures and embedded in paraffin. The 5-μm thick tissue sections were stained with hematoxylin and eosin (H and E) and then put on glass slides for light microscopy. An experienced pathologist blinded to the experiment evaluated all samples. The entire sections were examined for histologic grading of steatosis (0–3), hepatocyte ballooning (0–3), and lobular inflammation (0–3) according to the criteria described by Brunt *et al.*[15]

Statistical analysis

Continuous data were presented as mean ± standard deviation. One-way ANOVA and *post hoc* Tukey's honestly significant difference tests were used for comparisons among groups. $P < 0.05$ was considered statistically significant. All statistical analyses were

performed using SPSS Statistics for Windows version 17 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Effect of genistein on body weight changes

As depicted in Figure 1a, rats in the alcohol group gained significantly less weight than those in genistein and control groups with the BW changes of 48.83 ± 14.59, 69.17 ± 7.33, and 142.83 ± 10.06 g in alcohol, genistein, and control groups, respectively ($P < 0.01$). Comparing with alcohol group, rats in the genistein group gained more weight at 4 weeks (48.83 ± 14.59 g in alcohol group and 69.17 ± 7.33 g in genistein group, $P < 0.05$).

Serum alanine aminotransferase level

The level of serum ALT in alcohol group was higher than control group (120.30 ± 75.30 vs. 59.6 ± 9.78 U/L). However, serum ALT level significantly decreased in rats treated with genistein when compared with alcohol group (32.43 ± 12.90 vs. 120.30 ± 75.30 U/L, $P < 0.05$) [Figure 1b].

Effect of genistein on liver oxidative stress marker and hepatic glutathione levels

Hepatic MDA levels in alcohol group were significantly higher than in control group (0.13 ± 0.02 vs. 0.1 ± 0.01 nmol/mg protein, respectively; $P < 0.05$). With genistein coadministration, hepatic MDA levels

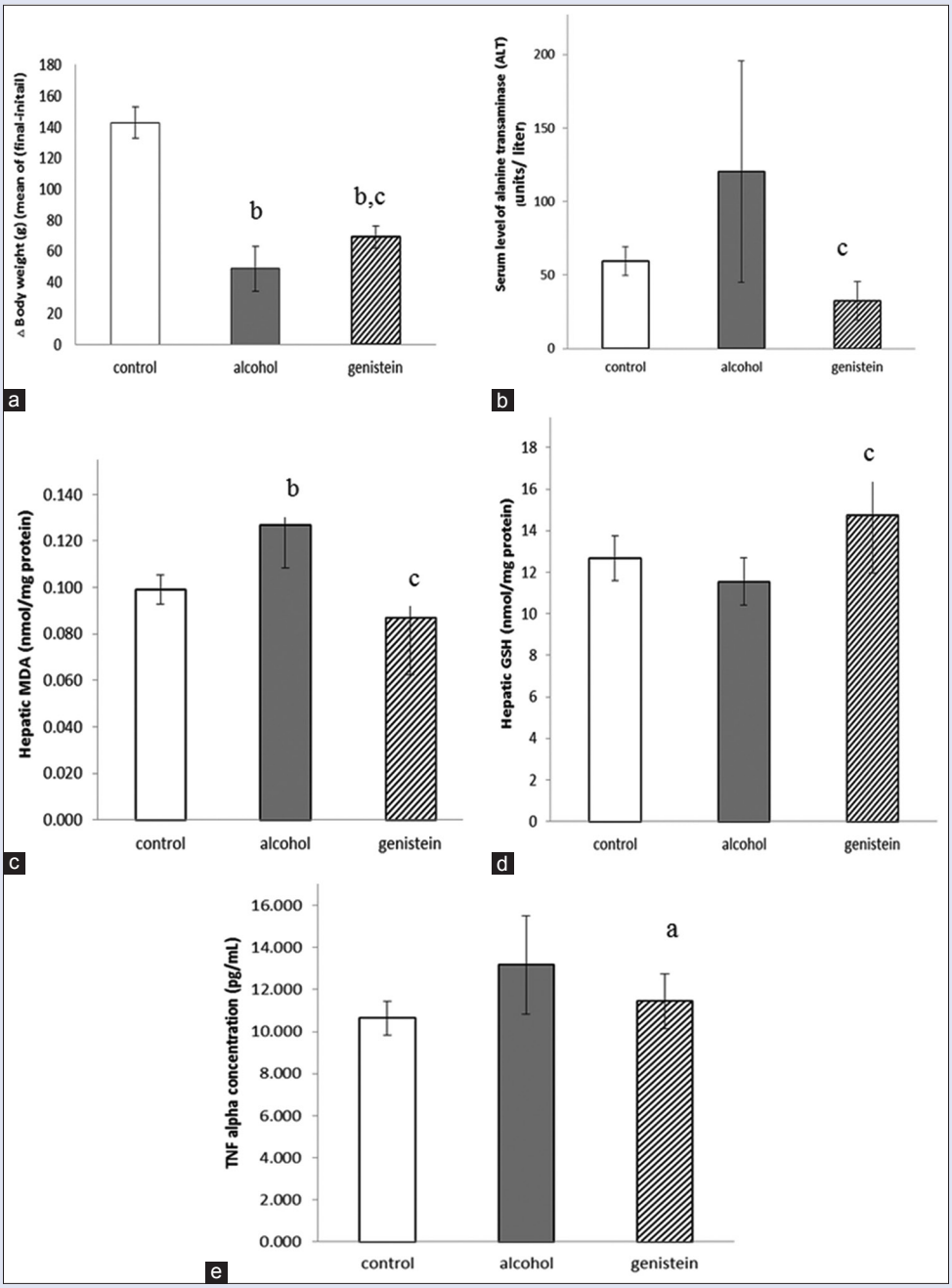

Figure 1: Effects of genistein on body weight change; (a) serum alanine transaminase; (b) hepatic malondialdehyde; (c) hepatic glutathione; (d) and serum tumor necrosis factor - α; (e) in rats with alcohol-induced liver injury. [a]$P < 0.05$, [b]$P < 0.01$ versus control group; [c]$P < 0.05$ versus alcohol group. Data are expressed as mean ± standard deviation

in genistein group decreased significantly and closely resembled those in control group (0.09 ± 0.02 vs. 0.13 ± 0.02 vs. 0.1 ± 0.01 nmol/mg protein in genistein, alcohol, and control groups, respectively; $P < 0.01$) [Figure 1c].

Hepatic GSH levels in the control group were higher than in alcohol group, albeit not statistically significant (12.66 ± 1.08 vs. 11.55 ± 1.15 nmol/mg protein, respectively). However, hepatic GSH levels were significantly higher in genistein group than in alcohol and control ones (14.77 ± 2.80 vs. 11.55 ± 1.15 vs. 12.66 ± 1.08 nmol/mg protein, respectively; $P < 0.05$) [Figure 1d].

Effect of genistein on inflammatory responses

Serum TNF-α levels in alcohol group were significantly higher than in control group (13.17 ± 2.34 vs. 10.64 ± 0.8 pg/mL, respectively; $P < 0.05$), while a fall in serum TNF-α levels was noted in rats receiving both genistein and alcohol as opposed to those receiving alcohol alone (11.43 ± 1.31 vs. 13.17 ± 2.34 pg/mL; $P < 0.05$, respectively) [Figure 1e]. As presented in Figure 2, the percentage of IL-18 positive cells in alcohol group was significantly higher than in control group (57%. 4 ± 2.54% vs. 16.87% ± 5.01%, respectively; $P < 0.01$). The receipt of genistein led to a decline in IL-18 positive cells in genistein group comparing with alcohol group (19.52% ± 10.9% vs. 57.4% ± 2.54%, respectively; $P < 0.01$).

Effect of genistein on liver pathology

As shown in Figure 3 and Table 1, liver histopathology from alcohol group exhibited liver injury features, including steatosis, lobular inflammation, and moderate ballooning degeneration of hepatocytes. On the other hand, liver histopathology in genistein group improved compared with alcohol group.

DISCUSSION

Malnutrition is common among patients with chronic alcohol consumption due to ingestion of calories devoid of protein, vitamins, and minerals and possibly the direct effect of alcohol on nutrient absorption.[16] In the present study, we found that rats in alcohol group gained significantly less weight than those in control and genistein group. These findings were in line with the study by Zhuo *et al.*, which showed that alcohol-fed rats had less weight gain than control rats and this could partially be restored by receiving taurine, epigallocatechin gallate, and genistein.[17] Interestingly, weight changes were more prominent in control group than genistein one. We hypothesized that less weight gain in genistein group happened as a result of genistein effects on the reduction of total body fat and leptin production.[18,19]

Oxidative stress and lipid peroxidation happened as a result of ROS production from alcohol metabolism through microsomal ethanol-oxidizing system and from acetaldehyde effect.[20] In this study, we used hepatic MDA level as a marker for oxidative stress and found that hepatic MDA levels were significantly higher in alcohol group than in control group, and this detrimental effect of alcohol was attenuated by genistein administration as evidenced by the comparable MDA levels between genistein and alcohol groups. Our results were similar to a previous study by Teare *et al.*,[21] which showed a significant increase in hepatic MDA levels in alcohol-fed rats compared with control rats, suggesting that oxidative stress is a mechanism of alcohol-induced liver damage. Studies in rat models of alcoholic and NAFLD demonstrated that genistein could alleviate oxidative stress and lipid peroxidation as shown by the decline in MDA levels in genistein-treated rats.[22,23]

In addition to increased ROS production, alcohol could also cause depletion in hepatic GSH, further increasing liver susceptibility to oxidative stress.[22-25] In the current study, we found a trend toward a decline in GSH levels in alcohol group. Studies have shown that acute

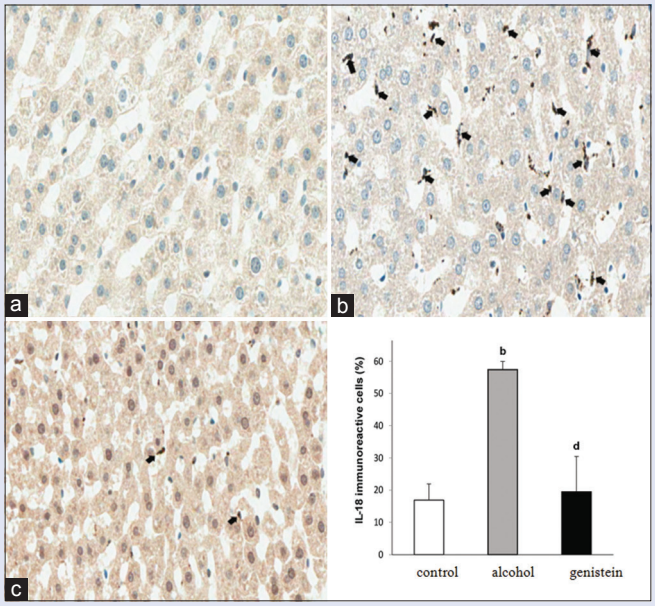

Figure 2: Effect of genistein on immunohistochemical staining of interleukin-18 in rat liver: (a) control group; (b) alcohol group; (c) genistein group. Nuclear counterstaining was performed; Positive-stained cells contain dark-brown nuclei (arrows). Images were obtained at ×40. $^{b}P < 0.01$ versus control group; $^{d}P < 0.01$ versus alcohol group

Table 1: Summary of steatosis, lobular inflammation, and ballooning degeneration score in all groups

Group	*n*	Steatosis				Lobular inflammation				Ballooning degeneration			
		0	1	2	3	0	1	2	3	0	1	2	3
Control	6	6	-	-	-	6	-	-	-	6	-	-	-
Alcohol	6	-	4	2	-	-	4	2	-	-	3	2	1
Genistein + alcohol	6	5	1	-	-	5	1	-	-	3	3	-	-

alcohol exposure could reduce GSH levels through the binding of its metabolites with GSH and the inhibition of GSH synthesis.[26] On the contrary, chronic alcohol exposure could lead to increased GSH efflux into hepatic sinusoids, GSH synthesis, and GSH levels.[27,28] The relatively unchanged GSH levels in alcohol-fed rats in our study could happen as a result of the balance between the direct effects of alcohol metabolites on GSH and the consequences of chronic alcohol exposure on GSH synthesis and efflux.

Unequivocally, GSH levels significantly increased in genistein-treated group as compared with alcohol and control groups. There are several proposed mechanisms of how genistein promotes GSH synthesis. The rate of GSH synthesis is mainly controlled by the activity of γ-glutamylcysteine ligase, which is regulated by redox-sensitive transcription factor, nuclear factor erythroid-2-related factor 2 (Nrf2).[29] An *in vitro* study showed that genistein administration could induce the expression of *Nrf2* gene and increase superoxide dismutase, catalase, and GSH levels.[30] Other antioxidant effects of genistein include the induction of GSH peroxidase 1[31] and (nicotinamide adenine dinucleotide-phosphate quinone oxidoreductase 1,[32] the enzymes responsible for the prevention of ROS formation.

Alcohol can also cause liver injury through its effects on gut dysbiosis and LPS exposure to the liver.[8] LPS then binds to TLR-4 on Kupffer cells, which in turn release inflammatory cytokines such as TNF-α and IL-18.[33] IL-18 plays a pivotal role in LPS-activated *Propionibacterium*

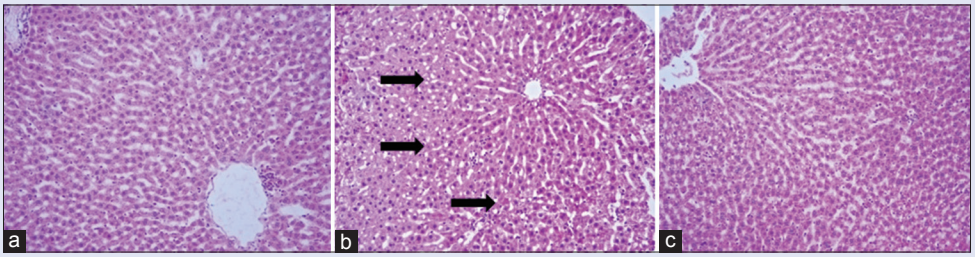

Figure 3: Hematoxylin-eosin stained liver sections (×400) in all groups. (a) control group showed normal liver histopathology; (b) alcohol group showed hepatic steatosis, lobular inflammation, and ballooning (arrow); (c) genistein group showed improvement of hepatic injury

acnes-induced liver injury through the augmentation of TNF-α release and the induction of interferon- γ and Fas ligand.[9,34,35] In this study, we found the rise in serum TNF-α levels and IL-18 positive cells in alcohol-fed rats, supporting the role of LPS in alcohol-induced liver injury. These effects could be attenuated by genistein use. Our results were similar to a study by Zhao *et al.*, which demonstrated that genistein treatment could reduce TNF-α levels and partially protect against hepatocyte apoptosis from alcohol.[22] *In vitro* studies also suggested that genistein could reduce LPS-induced inflammation through the inhibition of TNF-α, IL-6, and nuclear factor kappa B production.[36] Despite not being studied directly in ALD, genistein has been shown to reduce IL-18 levels in other conditions such as metabolic syndrome.[37]

CONCLUSION

Genistein exerted the antioxidant and anti-inflammatory properties in rat models of alcohol-induced liver injury and could prove beneficial in the prevention or treatment of ALD. Human studies are warranted to confirm its usefulness in clinical practice.

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Conflicts of interest

There are no conflicts of interest.

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