



Original research article

## Apigenin inhibits ethanol-induced oxidative stress and LPS-induced inflammatory cytokine production in cultured rat hepatocytes

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## ARTICLE INFO

## Article history:

Received 24 April 2017

Received in revised form 31 July 2017

Accepted 24 November 2017

Available online 27 November 2017

## Keywords:

Apigenin

CYP2E1

Hepatocytes

Inflammatory cytokines

Oxidative stress

## ABSTRACT

Apigenin is a natural flavonoid compound that has antioxidative, anti-inflammatory, and hepatoprotective effects, but the underlying mechanisms are still unclear. In this study, the effects of apigenin on ethanol-induced oxidative stress and lipopolysaccharide (LPS)-induced inflammatory cytokine production were examined in cultured rat hepatocytes. Following pretreatment of ethanol-stimulated hepatocytes with apigenin 6–24 mM for 2 h, the levels of cytochrome P450 2E1 (CYP2E1) protein expression and supernatant alanine aminotransferase and malondialdehyde were reduced ( $P < 0.05$  or  $P < 0.01$ ), while the activities of glutathione reductase and glutathione peroxidase were increased ( $P < 0.05$  or  $P < 0.01$ ). Likewise, the pretreatment of LPS-stimulated hepatocytes with the same concentrations of apigenin could decrease the levels of nuclear factor- $\kappa$ B protein expression and supernatant tumor necrosis factor- $\alpha$  and interleukin-6 ( $P < 0.05$  or  $P < 0.01$ ), and increase the level of I $\kappa$ B- $\alpha$  protein expression ( $P < 0.05$  or  $P < 0.01$ ). In all of these results, the concentration of 24  $\mu$ M was the most effective. These findings demonstrate that apigenin may exert an inhibitory effect on ethanol-induced oxidative stress and LPS-induced inflammation in the cultured hepatocytes, and its mechanisms may be related to the reduction of CYP2E1 expression, increment of antioxidative ability, and regulation of inflammatory gene expression.

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## Introduction

The pathogenesis of alcoholic liver disease (ALD) is very complicated and has not been fully clarified, but many studies have revealed that the oxidative stress and inflammation play an important role in the process of ALD formation (Das and Vasudevan, 2007; Park et al., 2017; Wang et al., 2016). It is known that the hepatic cytochrome P450 2E1 (CYP2E1) can not only metabolize the ethanol, but also generate the reactive oxygen

species (ROS) (Cederbaum, 2010; Neuman et al., 2015), which may lead to the oxidative stress and start the lipid peroxidation (Park et al., 2017; Radosavljevic et al., 2009). The ROS may also induce inflammation via the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) inflammatory pathway (Parola and Robino, 2001), which may promote the production of some inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) (Yin et al., 1999). Therefore, the hepatic antioxidants and antioxidative enzymes, including reduced glutathione (GSH), glutathione reductase (GR), and glutathione peroxidase (GSH-PX), may markedly affect the generation and development of ALD.

Apigenin (4', 5, 7-trihydroxyflavone), a natural plant flavone, is a bioactive compound present in a variety of fruits and vegetables (Tsanova-Savova and Ribarova, 2013; Zhang et al., 2011), and has many beneficial biological effects (Zhou et al., 2017), such as antioxidative and anti-inflammatory effects (Kang et al., 2011; Xu et al., 2016), which has aroused intense interest in the development of pharmacological reagent that can prevent or reverse ALD. To our knowledge, the effects of apigenin on ethanol-induced

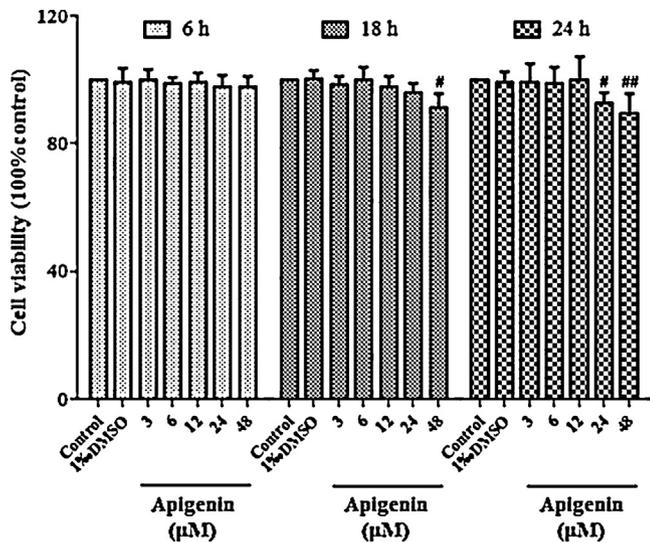
**Abbreviations:** ALD, alcoholic liver disease; BCNU, carmustine; CYP2E1, cytochrome P450 2E1; DMSO, dimethyl sulfoxide; GR, glutathione reductase; GSH, reduced glutathione; GSH-PX, glutathione peroxidase; GST, glutathione S-transferase; IL-6, interleukin-6; INH, isoniazid; LPS, lipopolysaccharide; MDA, malondialdehyde; NF- $\kappa$ B, nuclear factor- $\kappa$ B; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

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<https://doi.org/10.1016/j.jab.2017.11.006>

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**Fig. 1.** Cell viability after treatment with apigenin 3–48  $\mu\text{M}$  for 6–24 h. Each value represents the means  $\pm$  SD, with  $n=6$  per group.  $^{\#}P<0.05$ ,  $^{\#\#}P<0.01$  versus 1% DMSO group.

oxidative stress and lipopolysaccharide (LPS)-induced inflammatory cytokine production in the cultured rat hepatocytes have not been reported. The aim of our present study was to examine whether apigenin could alleviate the pathological damages and to investigate its potential mechanisms.

## Materials and methods

### Chemicals and reagents

Apigenin was kindly provided by Suzhou Baozetang Medical Technology Co., Ltd. (Suzhou, China) and solubilized in 1 %

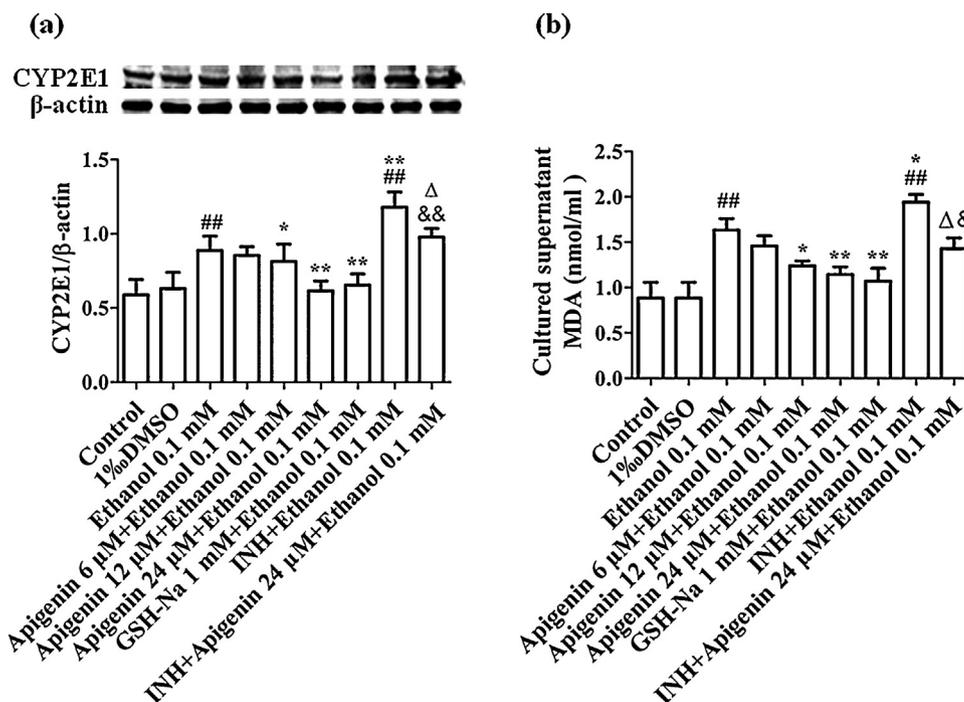
dimethyl sulfoxide (DMSO) solution, and the purity was  $>98\%$  as determined by high performance liquid chromatography. Glutathion (reduced glutathione sodium, GSH-Na) was procured from Pharminvest SPA (Italy) and dissolved in normal saline. Ethanol was a product of Chinasun Specialty Products Co., Ltd. (Changshu, China). LPS was a product of Sigma-Aldrich (St. Louis, MO, USA). Carmustine (BCNU), an inhibitor of GR, was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Isoniazid (INH) was a product of Mackiln Bio-Technology Co., Ltd. (Shanghai, China). The assay kits for GSH, GR, GSH-PX, glutathione S-transferase (GST), malondialdehyde (MDA), and alanine aminotransferase (ALT) were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The ELISA kits for rat TNF- $\alpha$  and IL-6 were purchased from Shanghai Xitang Biotechnological Co., Ltd. (Shanghai, China). Anti-CYP2E1 antibody was obtained from Proteintech Group Co., Ltd. (Wuhan, China). Anti-NF- $\kappa\text{B}$  p65, anti-I $\kappa\text{B}$ - $\alpha$ , and anti- $\beta$ -actin antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). All other reagents used in this study were of analytical grade.

### Cell culture

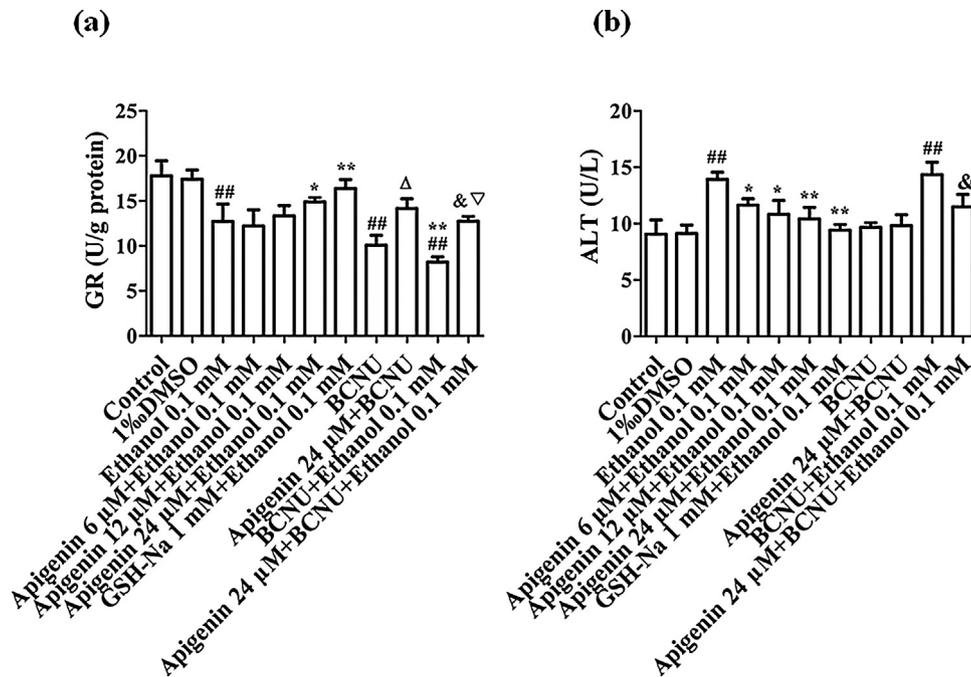
The rat BRL hepatocytes, purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Measurement of cell viability

The BRL cells ( $1 \times 10^5/\text{ml}$ ) were seeded into 96-well microtiter plates and cultured for 24 h before treatment. After the cells were treated with either 1% DMSO solution or apigenin at 3, 6, 12, 24, and 48  $\mu\text{M}$  for 6, 18, and 24 h, respectively, the cell viability was



**Fig. 2.** Hepatocellular CYP2E1 protein expression and cultured supernatant MDA level after pretreatment with different concentrations of apigenin for 2 h in rat BRL cells stimulated with ethanol or ethanol plus INH. Each value represents the means  $\pm$  SD, with  $n=3$  per group.  $^{\#\#}P<0.01$  versus control group;  $^{\#}P<0.05$ ,  $^{\#\#}P<0.01$  versus ethanol 0.1 mM group;  $^{\Delta}P<0.05$  versus INH 1 mM+ethanol 0.1 mM group;  $^{\#}P<0.05$ ,  $^{\#\#}P<0.01$  versus apigenin 24  $\mu\text{M}$ +ethanol 0.1 mM group.



**Fig. 3.** Hepatocellular GR activity and cultured supernatant ALT level after pretreatment with different concentrations of apigenin for 2 h in rat BRL cells stimulated with ethanol. Each value represents the means  $\pm$  SD, with  $n = 3$  per group.  $^{##}P < 0.01$  versus control group;  $^*P < 0.05$ ,  $^{**}P < 0.01$  versus ethanol 0.1 mM group;  $^{\Delta}P < 0.05$  versus BCNU group;  $^{\&}P < 0.05$  versus BCNU + ethanol 0.1 mM group;  $^{\nabla}P < 0.05$  versus apigenin 24  $\mu$ M + ethanol 0.1 mM group.

determined by 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to our method (Wang et al., 2015). The absorbance of control cells (untreated) was considered as 100% cell viability.

#### Measurements of CYP2E1 protein expression and supernatant MDA level in apigenin-treated cells stimulated with ethanol

The BRL cells were divided into 9 groups: control group, 1% DMSO group, ethanol group, ethanol plus apigenin 6, 12, and 24  $\mu$ M groups, ethanol plus GSH-Na 1 mM group, INH plus ethanol group, and INH plus apigenin 24  $\mu$ M plus ethanol group. First, the cells of last two groups were treated with INH 1 mM for 4 h; next, the medicine-treated cells were pretreated with apigenin or GSH-Na for 2 h; finally, ethanol (a final concentration was 0.1 mM) was added and incubated for an additional 10 h. The expression of CYP2E1 protein was detected by the Western blot method, and the level of MDA in the cultured supernatant fluid was detected by the colorimetric method following the manufacturer's instruction.

#### Measurements of supernatant ALT and intracellular GR, GSH-PX, GST, and GSH levels in apigenin-treated cells stimulated with ethanol

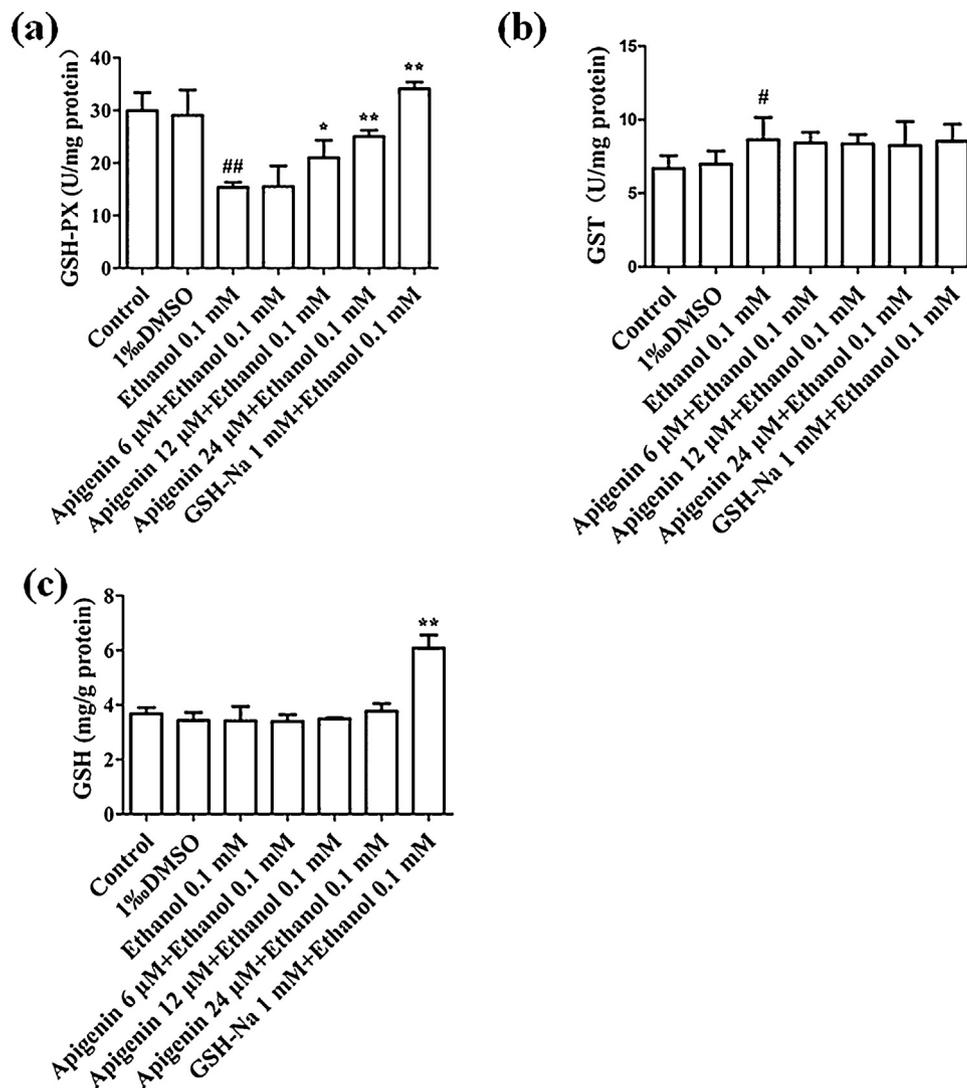
The BRL cells were divided into 11 groups: control group, 1% DMSO group, ethanol group, ethanol plus apigenin 6, 12, and 24  $\mu$ M groups, ethanol plus GSH-Na 1 mM group, BCNU group, BCNU plus apigenin 24  $\mu$ M group, BCNU plus ethanol group, and BCNU plus ethanol plus apigenin 24  $\mu$ M group. First, the cells of last four groups were treated with BCNU 0.2 mM for 2 h; next, the medicine-treated cells were pretreated with apigenin or GSH-Na for 2 h; finally, ethanol (a final concentration was 0.1 mM) was added and incubated for an additional 10 h. The supernatant ALT and intracellular GR, GSH-PX, GST, and GSH levels were detected by the colorimetric methods according to the manufacturer's instructions, respectively.

#### Measurements of NF- $\kappa$ B p65 and I $\kappa$ B- $\alpha$ protein expressions as well as supernatant TNF- $\alpha$ and IL-6 levels in apigenin-treated cells stimulated with LPS

The BRL cells were divided into 7 groups: control group, 1% DMSO group, LPS group, LPS plus apigenin 6, 12, and 24  $\mu$ M groups, and LPS plus GSH-Na 1 mM group. After the cells were pretreated with apigenin or GSH-Na for 2 h, LPS (a final concentration was 2  $\mu$ g/ml) was added and incubated for an additional 12 h. The cultured supernatants and cells were then collected, the former was used to measure the levels of TNF- $\alpha$  and IL-6 according to the ELISA methods on a VersaMax plate reader (Molecular Devices, CA, USA), and the latter was used to measure the expressions of NF- $\kappa$ B p65 and I $\kappa$ B- $\alpha$  proteins according to the Western blot methods, respectively.

#### Western blot analysis for protein expression

The Western blot assay was used to measure the expressions of CYP2E1, NF- $\kappa$ B p65, I $\kappa$ B- $\alpha$ , and  $\beta$ -actin proteins, and performed according to our previous description (Cui et al., 2014). In brief, an aliquot of 60–80  $\mu$ g protein from each sample was loaded on 10% SDS-polyacrylamide gel and separated by electrophoresis under constant current, and subsequently transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk at room temperature for 1.5 h and then incubated with the primary antibodies for CYP2E1 (1:300 dilution), NF- $\kappa$ B p65 (1:400 dilution), I $\kappa$ B- $\alpha$  (1:1000 dilution), and  $\beta$ -actin (1:1000 dilution) at 4  $^{\circ}$ C overnight, respectively. Next, the membranes were washed and incubated with the fluorescent secondary antibody at room temperature for 1 h. The protein blots were analyzed by densitometry using an Odyssey infrared imaging system and the Image J software (1.46r), and the relative ratio of the protein of interest was subjected to  $\beta$ -actin.



**Fig. 4.** Hepatocellular GSH-PX, GST, and GSH levels after pretreatment with different concentrations of apigenin for 2 h in rat BRL cells stimulated with ethanol. Each value represents the means  $\pm$  SD, with  $n = 3$  per group. # $P < 0.05$ , ## $P < 0.01$  versus control group; \* $P < 0.05$ , \*\* $P < 0.01$  versus ethanol 0.1 mM group.

#### Statistical analysis

The data are expressed as the means  $\pm$  SD of at least three independent experiments. The significance of difference between experimental groups was determined by using one-way ANOVA followed by a post hoc LSD test. The statistical analysis was conducted using SPSS 20.0 software, and  $P < 0.05$  was considered statistically significant.

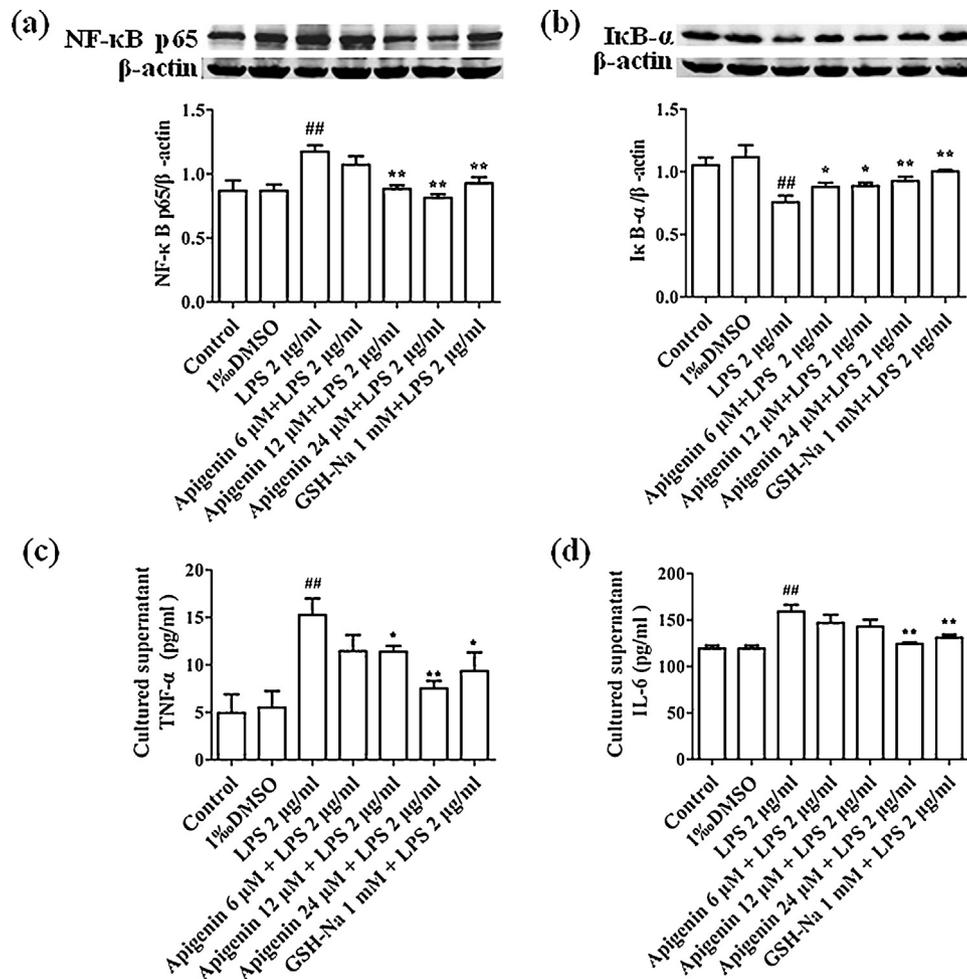
#### Results

##### Cytotoxicity of apigenin on BRL cells

The results showed that the cell survival rate did not differ obviously when BRL cells were treated with apigenin 3–24  $\mu$ M for 6–18 h, respectively (Fig. 1), indicating that the concentrations of apigenin had no cytotoxic effects for BRL cells and might be used in the experiments.

##### Apigenin decreased the levels of hepatocellular CYP2E1 protein expression and cultured supernatant MDA

The results showed that the levels of CYP2E1 protein expression and supernatant MDA between control and 1% DMSO groups were not significantly different (Fig. 2), indicating that 1% DMSO did not affect the normal status of the cultured cells. After stimulation with ethanol 0.1 mM, the levels of CYP2E1 protein expression and supernatant MDA were significantly increased as compared with the control group ( $P < 0.01$ , Fig. 2). Following pretreatment of BRL cells with apigenin 6–24  $\mu$ M for 2 h, the both indices were gradually decreased with the increasing concentrations of apigenin and close to the control levels in the 24  $\mu$ M group (Fig. 2), indicating a good dose–effect relationship. The both indices in the ethanol plus INH-treated cells were further increased as compared with the ethanol group ( $P < 0.05$  or  $P < 0.01$ ), and decreased by 24  $\mu$ M apigenin treatment ( $P < 0.05$ ). The positive control GSH-Na might also decrease the both indices ( $P < 0.01$ ).



**Fig. 5.** Hepatocellular NF-κB p65 and IκB-α protein expressions as well as cultured supernatant TNF-α and IL-6 levels after pretreatment with different concentrations of apigenin for 2 h in rat BRL cells stimulated with LPS 2 μg/ml. Each value represents the means ± SD, with  $n = 3$  per group. <sup>##</sup> $P < 0.01$  versus control group; <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$  versus LPS 2 μg/ml group.

#### Apigenin increased the activities of hepatocellular GR and GSH-PX as well as decreased the level of cultured supernatant ALT

Compared with the control group, the activities of hepatocellular GR and GSH-PX were significantly lower ( $P < 0.01$ , Figs. 3 a and 4 a), while the level of cultured supernatant ALT was significantly higher in the ethanol-stimulated group ( $P < 0.01$ , Fig. 3b). After pretreatment with apigenin, these indices could be dose-dependently reversed, especially in the 24 μM apigenin-treated group ( $P < 0.05$  or  $P < 0.01$ , Figs. 3 and 4 a). The similar results were observed in the GSH-Na-treated group. The hepatocellular GR activity in the BCNU-treated or ethanol plus BCNU-treated groups was significantly lower than that in the control group ( $P < 0.01$ , Fig. 3a), the addition of apigenin could prevent the reduction of hepatocellular GR activity ( $P < 0.05$ , Fig. 3a). However, the effects of apigenin on GST and GSH levels were not found (Fig. 4b and c).

#### Apigenin regulated the protein expressions of hepatocellular NF-κB p65 and IκB-α as well as decreased the levels of cultured supernatant TNF-α and IL-6

As shown in Fig. 5, the levels of hepatocellular NF-κB p65 protein expression and supernatant TNF-α and IL-6 were significantly higher, while the IκB-α protein expression was significantly lower in the LPS-stimulated group than in the control

group ( $P < 0.01$ ). After pretreatment with apigenin or GSH-Na, the expression of NF-κB p65 protein was decreased, while that of IκB-α protein was increased ( $P < 0.05$  or  $P < 0.01$ ). Accordingly, the levels of supernatant TNF-α and IL-6 in the apigenin-treated groups, like positive control GSH-Na, were decreased, especially in the 24 μM apigenin-treated group ( $P < 0.01$ ).

#### Discussion

A large body of evidence has demonstrated that hepatocellular CYP2E1 can be induced by ethanol, and the microsomal ethanol oxidizing enzyme is thought to be a major source of ROS production (Aroor et al., 2012; Lu and Cederbaum, 2008), which may subsequently cause the oxidative stress and lipid peroxidation, and finally lead to the liver injury (Cederbaum, 2010). The present results showed that after pretreatment with apigenin, the levels of hepatocellular CYP2E1 protein expression and supernatant MDA were reduced, especially in the 24 μM group. INH is an additional inducer of CYP2E1. We also observed that apigenin pretreatment could decrease the both indices in the ethanol plus INH-treated cells. These results suggested that apigenin might be a natural CYP2E1 inhibitor, which is different from the common antioxidants, such as reduced glutathione (Ning et al., 2011) and silymarin (Zhang et al., 2013), and is beneficial for the amelioration of oxidative stress induced by ethanol.

The ROS production can reduce the hepatic antioxidant ability, including antioxidative enzymes and antioxidants (Albano, 2006). It is known that the antioxidative system plays an important role in preventing hepatic oxidative injury (Torok, 2016). In the present study, the results showed that after pretreatment with apigenin, the activities of GR and GSH-PX were increased, especially in the 24  $\mu$ M group. The GR, an enzyme of glutathione redox cycle, may promote the biotransformation of oxidized glutathione to GSH (Kretzschmar, 1996), but the expected increment of GSH was not found in the apigenin-treated groups. The potential reason might be the increment of GSH consumption under oxidative stress. To further determine the effect of apigenin on GR, the BCNU, a specific GR inhibitor (Hojo et al., 2002), was used. The results showed that the inducible effect of apigenin on GR was reduced after preincubation with BCNU for 2 h, suggesting that apigenin might be an activator of GR. Therefore, we thought that apigenin, like antioxidants reduced glutathione (Ning et al., 2011) and silymarin (Zhang et al., 2013), might enhance the scavenging effect on CYP2E1-derived ROS via the increment of hepatocellular antioxidant ability.

The NF- $\kappa$ B is a redox-sensitive transcriptional factor that is activated by oxidative stress (Gloire and Piette, 2009) and may control the inflammatory cytokine production (Nanji et al., 1999). Under normal conditions, NF- $\kappa$ B forms a cytoplasmic complex with its inhibitory protein I $\kappa$ B- $\alpha$  (Baeuerle, 1991). On cell stimulation with inflammatory factors, the I $\kappa$ B- $\alpha$  is phosphorylated and degraded, the free NF- $\kappa$ B subsequently translocates to the nucleus where it initiates the gene transcription of inflammatory cytokines (Kanarek and Ben-Neriah, 2012), such as TNF- $\alpha$  and IL-6. To examine the effects of apigenin on the production of TNF- $\alpha$  and IL-6 in activated hepatocytes, LPS, a potent inducer of inflammatory cytokine production, was used in this study. The present results showed that apigenin could decrease the expression of NF- $\kappa$ B protein and increase the expression of I $\kappa$ B- $\alpha$  protein. The former was in accordance with previous observation (Kang et al., 2011) and attributable to the reduction of ROS. Accordingly, the levels of TNF- $\alpha$  and IL-6 in the cultured supernatants were also reduced. Based on the results, we deduced that the reductions of these inflammatory cytokines might be from the synergic regulatory effects of apigenin on NF- $\kappa$ B and I $\kappa$ B- $\alpha$  protein expressions.

In conclusion, our present results demonstrate that apigenin can exert an inhibitory effect on ethanol-induced oxidative stress and LPS-induced inflammatory response in the cultured BRL cells, and its mechanisms may be related to the reduction of CYP2E1 expression, increment of antioxidant ability, and regulation of inflammatory gene expression. These effects of apigenin may be good for the prevention and treatment of alcoholic liver injury.

### Conflicts of interest

The authors declare that they have no competing interests.

### Acknowledgments

This work was supported by the Science and Technology Funds of Jiangsu Province (No. BY2015039-09), China. Both Feng Wang and Rui-Jun Zhou contributed equally to this work.

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