A rat model was set up by feeding ethanol and fish oil to experimental rats, which then were treated with Platycodon grandiflorum (PG) on alcohol-induced fatty liver (AFL) and investigate the possible mechanism.

Results: Treatment with PD on AFL rats significantly decreased the levels of serum ALT, AST and TBIL, coefficient of liver index directly toxic on hepatocytes, but also can indirectly damage the liver and increase the production of chemoattractant molecules from inflammatory cells (Kono et al., 2001).

Conclusion: Treatment with PD effectively protects against AFL through anti-inflammatory and anti-endotoxic process, and the confirmed mechanism is that PD treatment ameliorate alcoholic-induced liver injury mainly via TLR4-MyD88-NF-κB signal path in AFL rat.

Key words: Platycodon D, alcoholic, fatty liver, anti-inflammation, toll-like receptor 4

Introduction

High alcohol consumption results in critical problems in the body including alcohol liver diseases, which is a major health problem in the worldwide. Chronic alcohol consumption can cause steatosis, inflammation, fibrosis, cirrhosis and even liver cancer, and the most common one is alcoholic-induced fatty liver (AFL) (Williams et al., 2014). Alcohol can increase the levels of circulating endotoxin in the portal blood and the endotoxin originated from Gram-negative gut bacteria plays a crucial role in the pathogenesis of alcoholic liver injury (Bergheim et al., 2006). Accumulating clinical and animal studies have shown that acute or chronic ethanol exposure leads to increased circulating endotoxin level, which correlates well with the development of liver injury (Jokelainen et al., 2001).

Research shows (Rochaa et al., 2016) that endotoxin metabolism is related to the toll-like receptor 4 (TLR4) pathway. The cluster of differentiation 14 (CD14) is best characterized for its capability to interact with endotoxin lipopolysaccharide (LPS)-binding protein (LBP) and transfer LPS to the TLR4 accessory, molecule myeloid differential protein-2 (MD-2). The TLR4 and MD-2 form a dimer in the plasma membrane lipid. Upon LPS recognition, the complex of CD14-TLR4-MD-2 engages adaptors of myeloid differentiation primary response protein 88 (MyD88) and TNF-receptor associated factor-6 (TRAF-6), and leads to MyD88-dependent response, subsequently the CD14-TLR4-MD-2 complex was endocytosed. On the other hand, the complex recruits adaptors, tri-related adaptor molecule (TRAM) and TIR-domain-containing adaptor inducing interferon-β (TRIF), which elicits MyD88-independent response.

TLR4 signaling pathway is also an important process of inflammatory reaction. TLR4 activation initiates a pro-inflammatory response, which depends on the activation of mitogen-activated protein kinases and nuclear transcription factor kappa B (NF-κB). NF-κB is an important transcription factor and its activation plays a vital role in the production of various pro-inflammatory mediators (Liu et al., 2015) such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6). Up-regulation of TNF-α not only can be directly toxic on hepatocytes, but also can indirectly damage the liver and increase the production of chemoattractant molecules from inflammatory cells (Kono et al., 2001).

Ideal pharmacological reagents that can prevent or reverse the AFL are Chinese medicinal plants. Platycodon grandiflorum (PG) is one of the most important species and some studies have reported that the crude extract ingredients from PG, total saponins, possess intervention effect on liver damage, the main bioactive constituents of which are oleanolic acids compounds. Platycodon D
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(PD), a triterpenoid bidesmoside, is one of the saponins isolated from the roots of PG, has many effects as expectorant, anti-rheumatoid arthritis, anti-cancer, controlling obesity, anti-virus, protection of ischemia or reperfusion injury, induction apoptosis, boost immunity, and lowering blood lipid (Wu et al., 2010).

The aim of our study was designed to evaluate the ability of PD in activating TLR4 signal path of the AFL rats, and to investigate potential mechanism.

Materials and Methods
Preparation of PD

The PD (Figure 1) used in this study is the oleanolic acid, a triterpenoid bidesmoside, compose of an aglycone moiety, 3-glucose and 28-O-arabinosyl-rhamnosyl-xylosyl-apiosyl and chemically pure of 92.28%, Mr=1225.38, a polar solvent extract obtained from the roots of PG collected from Lu-mountain (Zibo, China). The structures, extracting methods and identifying methods of PD have been published previously (Wu et al., 2012).

Animals and Treatments

Female Sprague-Dawley rats, seventy days old, weighing (200±20) g, were obtained from Animal Breeding Center of Shandong University, and housed in regular cages situated in an animal room at 22 °C. The experimental rats were fed with standard rat diet and allowed to drink water at will during the acclimatization period for one week. The sixty experimental rats were randomly divided into 6 groups (n= 10 rats for each group) as normal control (NC) group, alcoholic-induced fatty liver (AFL) group, low dose PD (P DL) group, middle dose PD (PDm) group, high dose PD (PDh) group, and positive medicine (PM) group, respectively. The rats of NC group was fed on a standard laboratory fodder and given physiological saline by gavage during the entire experimental period. The other rats were given 40% ethanol 10mL and fish oil (Mission Hills, CA 91346-9606, America) 4 mL per kg body weight one day/(kg BW/day) by gavage with needle tubing, respectively, for 5 weeks, with free access to food and water ad libitum, to induce AFL. From the sixth to the ninth week, the animals of AFL group were given physiological saline, PM group were given 25 mg/kg BW/day simvastatin (Beijing shuanglu, China, H20058534), PD group were given 10, 20 and 30 mg/kg BW/day PD by gavage, respectively. Their body weight and food intake were recorded every 3 days during the sixty-three days experimental period. All experiments were indeed carried out in accordance with the guidelines of the Ethics Committee on Animal Experiments of Medical School of Shandong University (NO. 2010011).

Sample Collection

At the completion of the study, the rats were anaesthetized with intraperitoneal sodium pentobarbital and their heart blood samples were collected. The blood samples were kept at 4 °C for 12 h, centrifuged at 3 000 t/min for 15 min at 4 °C to obtain serum and stored at -80 °C before analysis. As quickly as possible, the rats were sacrificed by cervical dislocation and their livers were rapidly removed, washed in saline, dried on filter paper, and weighed. Then the fresh liver tissues were trimmed, immersed in liquid nitrogen, then transferred to the frozen pipe, stored at -80 °C before use. The other part of liver tissue per rat was homogenized in phosphate buffer saline in an ice bath, and then centrifuged at 1 500 t/min for 10 min at 4 °C, the supernatant of which was used for assay of lipid profile assessment.

Biochemistry Assay of Hepatic Function

The biochemical indicators of rats in each groups were measured using an auto-analyzer (Hitachi, Japan) and respective commercial test kits, such as serum alanine trans-aminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL) and endotoxin (Sigma, America), and Liver TG (Weifang Sanwer, China), according to the manual instructions.

Histological Observation Assay of Liver

The cubes of fresh liver tissue were fixed on object stage of the frozen microtome (Leica-cm3050s, Germany), processed
ELISA Assay of Cytokines NF-κB, TNF-α and IL-6 Protein Expression

Cytokines NF-κB, TNF-α and IL-6 were quantified by commercial ELISA kits. Briefly, 10 mg of liver tissue was homogenised in 100 μL RIPA buffer containing 1% phenylmethysulfonyl fluoride protease inhibitor (Shanghai Beyotime, China), then centrifuged at 12 000 r/min for 10 min at 4 °C, the supernatant of which were measured by ELISA kits (BioLegend, America) to study the cytokines levels. Total protein was measured by a BCA protein assay Kit (Thermo Fisher, America). The values were normalised to total protein and expressed as pg/mg of protein.

Immunohistochemical Analysis of CD14 Protein Expression

The cubes of fresh liver tissue were fixed to 8 μm sections on the free off-slides, and the sections were blew dry at room temperature, fixed in 4% paraformaldehyde for 30 min, immersed in mixed liquor (1 volume 30% H₂O₂ and 50 volumes 100% methanol) for 30 min, washed with distilled water 2 times, and then dropped in 5% BSA of closed fluid for 20 min.

Then antibodies against CD14 (Wuhan Boster, China) diluted in PBS (1:300) were dropped onto the sections and they were in incubator at the proper 37 °C for 1 h, then washed 3 times (2 min per time) with PBS (pH 7.2 ~7.6). Then the secondary antibody was added to the sections and they were at 37 °C for 20 min and then washed 3 times (2 min per times) with PBS again.

After reagents of ready-to-use strept-avidin-biotin complex (SABC, Wuhan Boster) were added onto the sections, the sections were at 25 °C for 20 min and then washed with PBS 4 times (5 min per time). Finally, target proteins were stained with reagent kit (Wuhan Boster) of 3, 3-diaminobenzidine and counterstained with hematoxylin. Resultant photomicrographs of CD14 proteins expression were taken with microscope camera (C51, Olympus, Japan) and were quantitative by software of Evaluation Image-Pro® 4.5. Images analysis on the graphs were used to determine the average gray value, representing the contents of the immune reaction of CD14 protein levels.

Immunoblot Analysis of TLR4 Protein Expression

Liver tissue sample of 100 mg was crushed in a liquid nitrogen-cooled grinding bowl, then lysed in cold RIPA buffer (Pierce Biotechnology, America), and supplemented with Halt™ Protease Inhibitor Cocktail (Pierce Biotechnology). The concentration of protein was measured with BCA protein assay kit (Thermo Fisher, America). The 40 μg of protein was loaded onto a 12% sodium dodecyl sulphate-polyacrylamide gel and transblotted onto a polyvinylidene fluoride membrane (Bio-Rad, America). Nonspecific protein was blocked with 5% nonfat dry milk in tris-buffered saline solution containing 0.1% Tween-20 for 2 h at room temperature. The membrane was immunoblotted with primary antibody of TLR4 (1:800, Cell Signaling Technology, America) at 4 °C for overnight, then incubated with a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (Zhongshan Golden bridge, China) for 2 h at room temperature, and finally detected by chemiluminescence using Enhanced NuGlo™ Chemiluminescent Substrate Kit (Alpha Diagnostic, America) followed by autoradiographic and densitometric analysis. β-actin was used as an internal control.

Real-time PCR Analysis of MD-2, MyD 88 and TRAF-6 mRNA Expression

Total RNA in liver sample obtained from each rat was isolated by TRIZOL reagents according to the procedure of the supplier (Invitrogen, America). The integrity of the extracted RNA was checked by electrophoresis on a 1% agarose gel. RNA was extracted by precipitation using 2.5 volumes of 100% alcohol and 0.1 volume of 3 mol/L sodium acetate at pH 5.2. The RNA pellet was washed with 70% alcohol, and then dissoluted in diethyl pyrocarbonate-treated water. The cDNA synthesis was done using cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad, America). LightCyler Probe Design software 2.0 was used to design primers for the target genes and the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as shown in Table 1. Applied Biosystems 7900HT Fast Real-Time PCR System (ABI, America), TOYOBO-Realtime PCR Mater Mix (SYBR Green, Toyobo, Japan), standard procedures and SDS software 2.3 were used to assess the mRNA expression of each rat. The reaction parameters were as follows: 40 cycles of 95 °C denaturation for 15 s, 60 °C annealing and extension for 60 s. After reaction the Ct values were calculated by the computer automatically, then datum calculated and counted with 2-△△Ct methods.

Table 1: The primers sequence used for real time PCR

| Genes   | Forward primer(5’-3’) | Reverse primer(5’-3’) | Amplified fragment/bp |
|---------|-----------------------|-----------------------|-----------------------|
| MD-2    | TGA AGA CAA GGC ATG GCA TGG | GTC TCC CAA GAT CAA CCG ATG | 178 |
| MyD 88  | ATA GCC ACC AGC ATG CAC | TAG GGT CCT TAC CAG GTA | 145 |
| TRAF-6  | AGC CAC AAT CCC ATG | GTC AGC GAA AGG CCG | 214 |
| GAPDH   | CCA TGG AGA AGG CTC GGG | AAG TTG TCA TGG ATG ACC | 217 |
All data were presented as mean ± SE. Differences among groups were assessed using unpaired Student’s t test and one-way ANOVA. *P value less than 0.05 was considered to be statistically significant. Calculations were performed with the SPSS 15.0 statistical software package. Significantly different at *P < 0.01 compared with AFL group, *P < 0.05 compared with AFL group, *P < 0.01 compared with NC group, *P < 0.01 compared with PM group.

Results

Physical Conditions of Rats

In the experimental process, 6 rats in the model group died. At the beginning of study, there was no difference in appearance among the groups. However, the appearance of lethargy, anorexia, sluggishness and dim fur occurred in model group at the end of the study. Body weight and liver weight were recorded of each rat in experimental process, and the liver index (liver wet weight/body weight×100) was calculated, as shown in Table 2. Alcohol consumption and drugs treatments slightly affect average body weight of the mice. For the liver, the surface of them was smooth and red-brown in NC group and relatively smooth and brown in the other four PD and PM groups, whereas enlarged hepatic volume, increased weight and noted dimmer color of liver were in AFL group.

Compared with control group, the liver weight and the liver index in AFL group were remarkably increased by 14.75% and 22.72%, respectively. The most plausible explanation is that alcohol exposure leads to severe lipid accumulation in the liver. This increase was prevented by the treatment with either simvastatin or PDh (30 mg/kg BW/d) groups, and there was no significant difference between the AFL group and other PD treatment groups (10 or 20 mg/kg BW/d).

| Groups | n  | Body weight/g | Liver weight/g | Liver index/% |
|--------|----|---------------|----------------|---------------|
| NC     | 10 | 239.61±27.61  | 8.27±1.51      | 3.74±0.11     |
| AFL    | 9  | 243.42±22.32  | 9.99±1.71      | 4.59±0.81     |
| PDl    | 8  | 238.93±31.54  | 8.66±1.23      | 4.45±0.13     |
| PDm    | 8  | 235.44±28.13  | 8.53±1.45      | 4.08±0.20     |
| PDh    | 9  | 235.91±29.45  | 8.35±2.01      | 3.82±0.21     |
| PM     | 10 | 235.65±27.98  | 8.42±1.98      | 3.81±0.19     |

PD Relieves Alcohol-induced Liver Injury

Chronic ethanol and fish oil feeding notably led to liver injury as examined by biochemistry indexes in rats, as shown in Figure 2. The plasma levels of ALT and AST in AFL group were significantly increased by 232.38% and 323.59%, respectively, compared to the control group. However, these elevations were reversed by the treatment with either PD or simvastatin, especially by PDm and PDh groups (20 and 30 mg/kg BW/d). PDl (10 mg/kg BW/d) showed a decreasing tendency in plasma ALT level in comparison to AFL group (P<0.05). Elevated ALT and AST are important indicator of liver function problems and reducing of them indicate the hepatoprotective effect of PD on alcoholic liver injury.

The TBIL is mainly used for clinical diagnosis of liver diseases, and when the liver being against inflammation can cause TBIL increase. Similarly, TG is also an important sign of liver damage. The results showed that the administration of ethanol induced a greater increase in levels of TBIL (P<0.01) in serum by 56.86% and TG in liver by 56.86% in the AFL group as compared to NC group, while treatment with PD and PM for five weeks showed a more clearly decrease of TBIL in PDm, PDh and PM groups, and of TG in all four groups (P<0.01) than AFL group. Chronic ethanol and fish oil feeding notably led to liver injury as examined by hepatic histopathological changes, as shown in Figure 3. The liver structure in NC group was normal and no obvious inflammation and hepatic steatosis were observed. In AFL group, the structure of hepatic cord was deranged and various degrees of diffuse hepatic steatosis and intra-lobular inflammation could be found obviously. Compared with AFL group, the degree of hepatic steatosis and inflammation were greatly reduced in three PD and PM intervention groups. Histological evaluation of liver specimens demonstrated that administration of PD dramatically decreased lipid accumulation.
PD Changes Endotoxin and Inflammation Response in Liver of Rats

PD changes endotoxic and inflammatory response in liver of rats, as shown in Figure 4. Ethanol and fish oil feeding caused increase by 222.91% in the level of plasma endotoxin compared with NC group. PD treatment groups and PM group significantly inhibited this ethanol-induced plasma endotoxin levels ($P<0.01$), which indicated that PD can adjust endotoxemia induced by ethanol treatment.

As expected, ethanol exposure significantly increased the hepatic levels of NF-κB p65, TNF-α and IL-6 by 426.69%, 126.64% and 95.63%, respectively. This elevation was markedly attenuated by three doses of PD and PM treatment, indicating PD treatment in AFL rats activated NF-κB to release inflammatory factors.

Figure 2: Biochemistry indexes in rats

Figure 3: Liver injury of alcoholic-reduced rats

The result of liver injury existed in the cytoplasm stained with reddish orange. 5 sections were randomly selected in each group and 3 horizons at high magnification ($\times400$) to view.

Figure 4: Endotoxin and inflammatory response in liver of rats
PD Changes TLR4-Related Enzyme Expression in Rats

The immunohistochemical resultant photomicrographs (Figure 5) of CD14 protein expression were quantitative by average gray value, representing the contents of the immune reaction of CD14 protein levels (Figure 6).

PD changes TLR4-related enzyme expression in rats, as shown in Figure 6. Ethanol exposure enhanced the expressions of TLR4, MD-2 and CD14 by 391.91%, 207.08% and 81.32%, respectively, compared to the NC group, which were decreased by PD and simvastatin treatments (P<0.01).

The mRNA levels of MyD88 and TRAF-6 in AFL groups were significantly increased by 285.71% and 219.80%, respectively, compared to the NC group. However, these elevations were reversed by the treatment with either simvastatin or PD groups, which indicate that PD can change TLR4 and related downstream enzyme gene expression in liver of rats.

NC AFL PDl
PDm PDh PM

Figure 5: Liver CD14 protein expression in rats

The result of CD14 expression products existed in the cytoplasm stained with yellow, strong positive were dark yellow or brownish yellow, yellow as positive, light yellow as weakly positive, and the background were the same color as the negative. 5 sections were randomly selected in each group and w 3 horizons at high magnification (×400) to view.

Discussion

Alcohol is a major cause of fatty liver and most of heavy drinkers to develop AFL. It is reversible with abstinence, whereas, it may progress to more severe liver disease, such as cirrhosis without abstinence, dietary intervention and reduce weight as the auxiliary treatment. An ideal and effective drug has been needed to be found to prevent and treat this disease.

Experimental model of AFL was set up successfully by ethanol gavage plus fish oil diet for five weeks in our study. Because excessive alcohol intake could maintain blood high alcohol levels and increase the levels of circulating endotoxin in the portal blood, which is the key factor for the progression of alcoholic liver diseases (Nanji et al., 1994), and fish oil rich in polyunsaturated fatty acids has played an important role in the pathogenesis of alcoholic liver disease (Polavarapu et al., 1998), the rats were fed on ethanol and fish oil to induce the AFL model. Meanwhile, female rats in the experimental model of alcohol-induced liver injury were significantly more severe liver injury than the males ones (Eagon, 2010) and simvastatin protect against liver damage by the induction related enzymes-participated signaling pathways (Habeos et al., 2008). So female rats were selected as experimental animals and simvastatin was selected as positive control to study the mechanism of liver protective function of PD against...
inflammation and endotoxin in alcohol-induced AFL rats. Our results showed that obvious hepatic injuries were observed in experimental rats and the model could mimic key aspects of alcoholic liver disease in humans. The duration of making model was significantly shortened, and the animal death rate was decreased. Although there are many methods, ethanol gavage is widely used to induce AFL in rats. This method could avoid rejection of rat to alcohol and consist with human drinking. In our study, histological observation assay and biochemical parameters such as serum ALT, AST and TBIL levels, and liver TG levels confirmed them.

PD against liver injury of AFL was assessed with histological assay and biochemical parameters, and the results showed that PD could improve the degree of hepatic injury in liver tissues, and decrease the levels of serum ALT, AST, TBIL and level of TG in liver tissue. The results suggested that the PD can inhibit the AFL effectively as well as improve the liver function.

Endotoxin is a key factor for the progression of alcoholic liver disease. It has been shown that excessive alcohol intake increases gut permeability of normally non-absorbed substances (Tang et al., 2009). Intestinal Gram-negative bacteria as well as blood endotoxin levels are increased both in alcoholic patients and feeding animal model (Zuo et al., 2001). The main chemical component in endotoxin is LPS. Alcohol and its metabolites, such as acetaldehyde, disrupt intestinal barrier function, and subsequently increase intestinal permeability, which allow the diffusion of bacterial endotoxin into the portal circulation (Bharrhan et al., 2011). Clinical and animal studies have shown that acute or chronic ethanol exposure leads to increased circulating endotoxin level, which correlates well with the development of liver injury (Jokelaizen et al., 2001). Our study found that alcohol treatment for five weeks increases the rats serum endotoxin levels and the liver damage, and PD treatment improve this situation, prompting that PD has the effects on liver injury in rats by improving the endotoxin metabolism.

TLR4 is a functional receptor expressed on the surface of various types of cells that transmit endotoxin signals. CD14 is a protein that is a component of the innate immune system, binds to LPS, and thereby subsequently presents it to dimer of TLR4 and MD-2, which can activate the intracellular signal pathway of MyD88, result in NF-κB activation (Medzhitov et al., 1997). But, two signal paths of MyD88 dependent and TRAM-TRIF are associated with TLR4. Recruitment of the TRIF adapter activates phosphorylation of interferon regulatory factor 3 (IRF3) that results in interferon regulatory Factor 3 (IFN-β) production (Yamamoto et al., 2003), just as shown that the mice deficient in IRF3 or TLR4 expression are protected from alcohol-induced liver inflammation and hepatocyte injury (Szabo et al., 2012). The LPS-TLR4 signal pathway consists of activation of transcription factors, which induces pro-inflammatory cytokines expression in the Kupffer cell in response to endotoxin (Nagy, 2003). Endotoxin plays an important role in the initiation and aggravation of alcoholic liver disease through the enhancement of pro-inflammatory cytokines (Fujimoto et al., 2000). Our results indicate that the PD was proved to increase expression of TLR4 and downstream protein of MyD88 and TRAF-6, indicating that anti-inflammatory effect of PD control is accomplished via TLR4-MyD88 signal pathway.

NF-κB is present in its inactive state in the cytoplasm as a heterotrimeric complex consisting of p50, p65 and I-κBα. The degradation of IκB and the nuclear translocation of NF-κB are considered to be crucial aspects for its activation (Ockinghaus et al., 2011). IκB is phosphorylated by IκB kinase (IKK) complex. Phosphorylation targets IκB for degradation and allows nuclear translocation of NF-κB (Liu et al., 2015). NF-κB is considered to be a key target for the treatment of inflammatory response (Zhu et al., 2015) and induction of NF-κB lead to the upregulation of pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α (Chang et al., 2015). Pro-inflammatory cytokines play a critical role in initiation and propagation of inflammatory pathogenesis in liver injury. Besides direct toxic effects on hepatocytes, TNF-α can indirectly damage the liver by increasing expression of intercellular adhesion molecule-1 (ICAM-1) in endothelial cells, as well as increasing the production of chemoattractant molecules from inflammatory cells (Zuo et al., 2003). We attempted to elucidate the molecular mechanism of the protective effects of PD by examining the expression of TLR4 and TLR4-activated NF-κB signaling pathways. Our results indicate that the PD can suppressed NF-κB activation as well as expression of TNF-α and IL-6, and the results suggest that the anti-endotoxin effect of PD is partly mediated by blocking NF-κB activation, further inhibiting the expression of inflammatory cytokines.

In conclusion, the results of this study revealed that PD exhibited anti-inflammation and anti-endotoxin effects against alcohol-induced AFL rats through the TLR4-MyD88-NF-κB pathway. These findings suggested that PD could be a potential candidate for the treatment of liver injury of AFL.

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Conflict of Interest

The authors declare no conflict of interest.

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