

Title Page

Puerarin Ameliorates Experimental Alcoholic Liver Injury by Inhibition of Endotoxin Gut-leakage, Kupffer Cell Activation and Lipopolysaccharide Receptors Expression

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a) Running Title:

Inhibition of Endotoxin signaling pathway by puerarin in ALD

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words in the Abstract, Introduction, and Discussion**

22 pages of text

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462 words in Introduction

1314 words in Discussion

d) Nonstandard abbreviations list:

ALD, alcoholic liver disease;

ALT, alanine aminotransferase;

AST, aspartate aminotransferase;

ELISA, enzyme-linked immunosorbent assay;

EtOH, ethanol;

GGT, gamma-glutamyl transpeptidase;

GAPDH, glyceraldehydes-3-phosphate dehydrogenase;

H.E., hematoxylin-eosin;

LBP, lipopolysaccharide binding protein;

LPS, lipopolysaccharide;

mCD14, membrane-bound CD14;

MD-2, myeloid differentiated protein-2;

KCs, Kupffer cells;

TLR, Toll like receptor;

TNF- α , tumor necrosis factor α ;

TEM, transmission electron micro-scope;

TG, triglyceride;

TJ, tight junctions;

ZO-1, zonula occludens-1.

e) A recommended section assignment to guide the listing in the table of contents.

Gastrointestinal, Hepatic, Pulmonary, and Renal

Abstract:

Puerarin is an isoflavone component extracted from Kudzu (*Pueraria lobata*) and has been demonstrated to alleviate alcohol-related disorder. The purpose of this study was to examine whether puerarin ameliorates chronic alcoholic liver injury through inhibition of endotoxin gut-leakage, the subsequent Kupffer cells (KCs) activation and lipopolysaccharide (LPS) receptors expression. Rats were provided with the Liber-DeCarli liquid diet for eight weeks. Puerarin (90 mg and 180 mg/kg.d) was orally administered from the beginning of the third week till the end of the experiment. Chronic alcohol intake caused increased serum ALT, AST, hepatic GGT and TG levels as well as fatty liver and neutrophil infiltration in hepatic lobules determined by biochemical and histological assay. A significant increase of liver tumor necrosis factor α (TNF- α) was detected by enzyme-linked immunosorbent assay. These pathological effects correlated with increased endotoxin level in portal vein and up-regulated protein expression of hepatic CD68, LBP, CD14, TLR2 and TLR4. Meanwhile, the intestinal microvilli were observed to be sparse, shortened and irregularity in distribution under the transmission electron microscope in conjunction with the down-regulated intestinal ZO-1 protein expression. These hepatic pathological changes were significantly inhibited in puerarin-treated animals, as well as the endotoxin level, hepatic CD68 and LPS receptors. Moreover, the pathological changes in intestinal microvillus and decreased intestinal ZO-1 were also ameliorated with puerarin treatment. These results thus demonstrate that puerarin inhibition on endotoxin gut-leakage, KCs activation and LPS receptors expression is importantly involved in the alleviation of chronic alcoholic liver injury in rats.

Introduction

Puerarin [7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-(β -D-glucopyranoside)] (supplemental Fig. 1) is an isoflavone component extracted from Kudzu (*Pueraria lobata*) which is a medicinal herb used to treat alcohol abuse in traditional Chinese medicine for more than a millennium. Puerarin has been demonstrated to alleviate alcohol-related disorder including suppression alcohol consumption and preference in rodents (Lin et al., 1996; Overstreet et al., 1996) and human beings (Penetar et al., 2012), reducing anxiety symptoms associated with alcohol withdrawal (Overstreet et al., 2003). Recently, puerarin protecting against liver injury (Zhang et al., 2006; Liu et al., 2010a; Zhao et al., 2010) were sporadically reported. But the effects of puerarin on alcoholic liver injury induced by long-term ethanol intake and the potential effective mechanisms were rarely disclosed.

Endotoxin, a toxic lipopolysaccharide (LPS) component of the gut Gram-negative bacteria, has been revealed to play an essential role in the pathological progression of alcoholic liver injury besides alcohol metabolism, oxidant stress, etc (Rao et al., 2004; Seth et al., 2011). LPS levels in the portal vein are elevated in patients with alcoholic liver cirrhosis (Szabo and Bala, 2010), which was confirmed in alcoholic liver disease (ALD) model of rodents (Tsukamoto et al., 2008). Increased plasma LPS levels in the continuous intragastric alcohol-feeding rodents are correlated with the grade of histology of liver injury (Tsukamoto et al., 2008). The mechanisms of ethanol (EtOH)-induced endotoxemia have been revealed to include diminished phagocytosis of Kupffer cells (KCs), bacterial overgrowth in the small intestine, the alteration of gastrointestinal epithelial barrier function and an increased intestinal permeability (Rao et al., 2004). Potent host response to endotoxin is involved in the ordered

interactions of endotoxin with receptors, lipopolysaccharide binding protein (LBP), membrane-bound CD14 (mCD14) or soluble CD14 and extracellular soluble myeloid differentiated protein-2 (sMD-2) or MD-2 bound to Toll like receptor 4 (MD-2-TLR4). Gut-derived endotoxin releases into the circulation, binding to LBP with high affinity. LBP increases the exposure of the normally concealed hydrophobic lipid A, catalyzing the extraction of individual endotoxin molecules by soluble CD14 or mCD14 (Lee et al., 1993; Gioannini and Weiss, 2007). The endotoxin monomer then is rapidly delivered to sMD-2 or to MD-2-TLR4, which results in activation of target cells, such as KCs, (Nagai et al., 2002; Park et al., 2009) to promote nuclear factor- κ B translocation into nuclear and transcription of proinflammatory cytokines, especially tumor necrosis factor α (TNF- α) (Su et al., 2000). TNF- α can itself further increases gut permeability, induces apoptosis and production of other cytokines, perpetuating and progressing liver injury (Bradham et al., 1998). A significant body of evidence indicates that endotoxin plays a crucial role in hepatocellular damage by activating KCs to secrete proinflammatory cytokines, especially, TNF- α . (See the illustration of mechanism of KCs activation releasing TNF- α to promote liver injury induced by gut-derived endotoxin in ALD in supplemental Fig. 2.)

In the present study, the effects of puerarin on endotoxin gut-leakage, KCs activation and endotoxin receptors expression in chronic alcoholic liver injury in rats were investigated.

Materials and Methods

Animals and Treatments

Male Sprague-Dawley (SD) rats (160 ± 10 g) were obtained from Shanghai laboratory

animal center of Chinese academy of sciences (Shanghai, China), and acclimatized for 7 days after delivery. All rats were maintained on a reverse 12h light: 12h dark cycle. Animal handling and procedures were performed according to international guidelines for the use and care of laboratory animals. The experimental protocol was approved by the local ethics committee.

Rats were divided into four groups: control (n = 10), EtOH (n = 10), EtOH plus high-dosage puerarin (180mg/kg.d, n = 10) and EtOH plus low-dosage puerarin (90mg/kg.d, n = 10) group. Lieber-DeCarli control and EtOH liquid diet were prepared according to the prescription of Lieber-DeCarli diet (Lieber and DeCarli, 1982) as described in the previous research (Fang et al., 2006). Rats in control group ingested Lieber-DeCarli control liquid diet and the others ingested Lieber-DeCarli alcohol liquid diet. EtOH provided 36% energy in the alcohol liquid diet which was substituted by dextrin-maltose in the control liquid diet. One liter liquid diet contains 1000 Kcal energy. Reagents in the Lieber-DeCarli formula were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Rats were single-cage rearing and liquid diet was intake freely without additional water or chow for eight weeks. At the beginning of the third week, rats in high and low-dosage puerarin groups were administrated with puerarin which commercially obtained from Shanghai Winherb Medical S&T Development Co.Ltd. (Shanghai, China) by gavage in 180 mg/kg.d and 90 mg/kg.d respectively, the others with equal volume of sterile water. At the end of the eighth week, rats were anaesthetized with nembutal (45mg/kg, i.p.). Liver tissue, intestinal samples and serum were collected and stores in -80°C for histological, biochemical immunohistochemical and Western blot analysis. Two milliliter blood from portal vein was

collected in pyrogen free and heparin-pretreated tube for endotoxin detection.

Histological examination

Liver and ileum tissue was formalin-fixed and embedded in paraffin. Sections (4 μ m thick) were stained with hematoxylin-eosin (H.E.) and examined under light microscope (Olympus Medical Systems Corp., Tokyo, Japan).

Frozen hepatic tissue (7 μ m thick) embedded in optimum cutting temperature compound purchased from Sakura Finetek USA, Inc. (CA., USA) was stained with oil red O reagent commercially obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) for hepatic lipid observation under light microscope (Olympus, Tokyo, Japan).

Histological liver damage was evaluated by index calculated by adding scores of macrovesicular steatosis, microvesicular steatosis, inflammation and necrosis as described in previous study (Keshavarzian et al., 2001). The severity of microvesicular steatosis was scored as 0 (no hepatocytes), 1 (less than 25% hepatocytes), 2 (26-50%), 3 (51-75%), and 4 (greater than 75% hepatocytes) containing small droplets of fat. The macrovesicular steatosis was rated 0 to 4 (according to increasing degree) similar to microvesicular steatosis. The severity of the inflammation was scored as 0 to 4 [none (0), minimal (1), mild (2), moderate (3), and severe (4)] and was based on the degree of portal and lobular inflammation and evidence of piecemeal and spotty necrosis. The degree of necrosis and necrotic hepatocytes was scored as 0 to 4 (none, minimal, mild, moderate, and severe, respectively) (Keshavarzian et al., 2001).

Intestinal injury was evaluated by following parameters: epithelial cell injury/loss, mucin (goblet cell) loss, mucosal edema, and the degree of inflammatory cells within the lamina

propria and in the epithelial layer (intraepithelial lymphocytes) (Keshavarzian et al., 2001). As described in the previous study (Keshavarzian et al., 2001), the degree of epithelial cell injury/loss was graded as follows: 0 (none), 1 (focal superficial epithelial cell injury), 2 (1-2 glands lost with or without mild superficial ulceration), 3 (3 glands lost with or without moderate areas of ulceration), or 4 (4 or more glands lost with or without severe mucosal ulceration). The degree of cellular mucin depletion was graded as 0 (none), 1 (focal), 2 (1-2 glands lost), 3 (3-4 glands), or 4 (greater than 5 glands lost). The degree of lamina propria edema was assessed by 0 (none), 1 (focal), 2 (superficial), 3 (superficial to mild), and 4 (diffuse edema). The inflammatory infiltrate was scored qualitatively as 0 (no increased inflammation), 1 (mild), 2 (moderate), 3 (severe and focal), and 4 (severe and diffuse).

Ileum samples from two random rats in every group were prepared for transmission electron micro-scope (TEM). As stated in previous study (Gul et al., 2012), ileum tissues were immersed in 2% glutaraldehyde (pH 7.4) and kept cold for 2 h. After rinsing several times in cold sodium phosphate buffer, the tissues were then postfixed in 1% osmium tetroxide solution for 2 h at 4°C. After being dehydrated in a series of graded ethanol, tissues were placed into propylene oxide and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by TEM (Philips Tecnai-12 Biotwin, Amsterdam, Netherlands).

Serum ALT and AST assay

Activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) in serum was determined with the corresponding biochemical assay kits commercially available from

Nanjing Jiancheng Bioengineering institute (Nanjing, China).

Hepatic GGT assay

Liver tissue (100 mg) was homogenized in 1 ml 0.9% NaCl and then centrifuged at 1,000g, 4°C for 15 min. The supernatant was removed into clean tubes and centrifuging at 3,000g for 10min, avoiding the upper adipose and removing the transparent for gamma-glutamyl transpeptidase (GGT) assay with commercial biochemical assay kit of Nanjing Jiancheng Bioengineering institute (Nanjing, China).

Hepatic TG assay

Liver tissue (200 mg) was homogenized in 3 ml ethanol-acetone mixture (1:1 in volume). The total hepatic triglyceride (TG) extracted in the medium at 4°C overnight and then, centrifuged at 1,000g, 4°C for 20 min, the supernatant was removed for TG assay with commercial TG analysis kits of Dongou Bioengineering Co. Ltd (Zhejiang, China).

Endotoxin assay in plasma from portal vein

Blood collected from portal vein was centrifuged at 500g, 4°C for 15 min. Plasma was removed immediately for analysis, according to the instruction of Pyrochrome® Limulus Amebocyte Lysate kit of Associates of Cape Cod, Inc. (Texas, USA).

Immunohistochemical assessment of hepatic CD68

As described in previous study (Peng et al., 2008), 4 µm thick paraffin sections were used for immunohistochemical assessment. The commercial antibodies (monoclonal anti-rat CD68, AbD Serotec, NC, USA; horseradish peroxidase -linked goat anti-mouse IgG, Santa Cruz, CA, USA) were used to detect CD68 expression. Diamino benzidine was applied as a chromogen and hematoxylin was used for floor staining. The negative control was performed on sections

from EtOH-fed rats liver where the CD68 antibody alternated with PBS buffer.

Measurement of TNF- α content by ELISA

Liver TNF- α was isolated as described in previous research (Lambert et al., 2003) and determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen Corporation, Camarillo, CA, USA) according to the manufacturer's instruction. The results were corrected by protein quantification with commercial bicinchoninic acid protein concentration assay kit of Beyotime Inst. Biotechnology (Jiangsu, China) and expressed as $\mu\text{g}/\text{mg}$ protein.

Determination of CD68 and endotoxin receptors in liver tissue and intestinal ZO-1 protein expression by Western blot

As described previously (Peng et al., 2008; Peng et al., 2009), total protein was extracted from liver and intestine tissue, analyzed with bicinchoninic acid protein concentration assay kit (Beyotime Inst. Biotechnology, Jiangsu, China). Western-blot was performed to evaluate the protein expression of hepatic CD68, LBP, CD14, TLR2, TLR4 and intestinal zonula occludens-1 (ZO-1) using the commercial antibody [mouse anti-rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) antibody, KANGCHEN Bio-Tech Inc., Shanghai, China; mouse anti-rat CD68 antibody, AbD Serotec, NC, USA; mouse anti-rat CD14 antibody, goat anti-rat LBP antibody, rabbit anti-rat TLR4 antibody and rabbit anti-rat ZO-1 antibody, Santa Cruz, CA, USA; rabbit anti-rat TLR2 antibody, Epitomics, Inc. CA, USA; goat anti-mouse IgG, goat anti rabbit-IgG peroxidase-linked antibody, Santa Cruz, CA, USA; rabbit anti goat-IgG, peroxidase linked antibody, Jackson ImmunoResearch Laboratories Inc., PA, USA). Tissue lysates were separated by electrophoresis in 10%

SDS-PAGE separating gel with Bio-Rad electrophoresis system (BioRad Laboratories, Hercules, CA, USA). The enhanced chemiluminescence kit (Pierce Biotechnology Inc., Rockford, USA) and the Furi FR-980 image analysis system (Shanghai Furi Co., Shanghai, China) were employed for revealing and quantitative analysis of the blots. Results were expressed as the ratio of protein to GAPDH.

Statistical Analysis

For parametric data, results were expressed as mean \pm S.D. The data were analyzed using a one-way analysis of variance followed by the least significant difference post hoc test. T-test is employed for the comparison of two parameters. Differences were considered statistically significant if the *P*-value <0.05 . The nonparametric data was analyzed by Kruskal-Wallis H-test for comparison of more than two groups and subsequently, Nemenyi test was employed in multiple independent groups pairwise comparisons.

Results

Liquid diets intake, body and liver weight

At the end of the eighth week, there was no significant difference in diets intake and body weight among groups ($P>0.05$, Table 1). The liver/body weight ratio of rats fed with EtOH liquid diets was significantly increased compared with that of rats in control group ($P<0.01$, Table 1) and there is no significance statistically between puerarin-treated groups and EtOH group ($P>0.05$, Table 1).

Effects of puerarin on Liver injury and steatosis induced by Lieber-DeCarli diet

Liver injury was examined by biomarkers of liver damage and histological changes in liver tissue. The serum ALT and AST activities significantly increased in EtOH group compared with that in the control (ALT, 124.02 ± 41.68 vs. 21.81 ± 7.90 U/L, $P < 0.01$; AST, 81.25 ± 30.16 vs. 26.70 ± 7.99 U/L, $P < 0.01$, Fig. 1C). The serum ALT of rats in administration with puerarin group was obviously decreased (with 180 mg/kg.d puerarin, 67.76 ± 19.60 vs. 124.02 ± 41.68 U/L, $p < 0.01$; with 90 mg/kg.d puerarin, 89.45 ± 19.21 vs. 124.02 ± 41.68 U/L, $p < 0.05$, Fig. 1C). The activity of serum AST in puerarin-treated group demonstrated similar trends (with 180 mg/kg.d puerarin, 49.70 ± 15.33 vs. 81.25 ± 30.16 U/L, $p < 0.01$; with 90 mg/kg.d puerarin, 59.28 ± 12.25 vs. 81.25 ± 30.16 U/L, $p < 0.05$, Fig. 1C). On the other hand, hepatic GGT activity increased after EtOH liquid diet intake obviously (49.75 ± 3.97 vs. 10.83 ± 2.11 U/ μ g pro., $p < 0.01$, Fig. 1C), and after puerarin administration, hepatic GGT activity decreased significantly (with 180 mg/kg.d puerarin, 15.78 ± 2.69 vs. 49.75 ± 3.97 U/ μ g pro., $p < 0.01$ and with 90 mg/kg.d puerarin, 14.01 ± 1.45 vs. 49.75 ± 3.97 U/ μ g pro., $p < 0.01$, respectively, Fig. 1C).

After chronic alcohol intake for eight weeks, the microvesicular steatosis was observed in the most regions of lobules and macrovesicular steatosis predominantly in centrilobular regions. Lipid vacuoles occupied much of the hepatocyte cytoplasm and the nucleus and other organelles were pushed to the periphery of the cell. Some hepatocytes appeared bloated, with a wispy, rarefied cytoplasm. The inflammatory cells, such as neutrophil and lymphocytes, scattered located in the pericellular region (Fig. 1A). Analysis of histological liver damage

index scores showed that the scores of alcohol intake rats (median = 9, range 6-10) increased significantly ($p=0$) compared with control (median = 0). Puerarin of high-dosage treatment ameliorated the pathological changes significantly (median = 4.5, range 3-6, $p=0.04$).

Oil red O staining was used to visualize hepatic lipid deposition. In the hepatocytes cytoplasm of chronic EtOH intake rats, there were large droplets colored with oil red O, which widespread distributed in the hepatic lobules, indicating severe steatosis in alcohol intake animals. Puerarin-treated animals with high or low-dosage, the droplets of oil red O deposit were smaller and limited, which indicated that hepatic steatosis was mitigated with puerarin administration (Fig. 1B).

The total TG extracted by liver tissue homogenization was test. As expected, long-term alcohol exposure elicited almost 5-fold increase in hepatic TG levels as compared with control animals (103.53 ± 13.59 vs. 22.39 ± 9.19 mg/g tissue, $P<0.01$, Fig. 1D). The levels of hepatic TG decreased remarkably in high-dosage puerarin-treated animals (with 180 mg/kg.d puerarin, 65.97 ± 27.54 vs. 103.53 ± 13.59 mg/g tissue, $P<0.01$; with 90 mg/kg.d puerarin, 85.53 ± 30.58 vs. 103.53 ± 13.59 mg/g tissue, $P>0.05$, Fig. 1D).

Effects of puerarin on endotoxin level and hepatic TNF- α concentration

The link between endotoxin and liver injury in ALD has been well demonstrated (Rao et al., 2004). In the present study, the endotoxin level in the portal vein was found increasing remarkably after long-term EtOH intake (0.54 ± 0.09 vs. 0.37 ± 0.05 EU/ml, $P<0.01$, Fig. 2A), and decreasing in the puerarin-treated groups (with 180 mg/kg.d puerarin, 0.40 ± 0.05 vs.

0.54±0.09 EU/L, $P<0.01$, with 90 mg/kg.d puerarin, 0.46±0.12 vs. 0.54±0.09 EU/L, $P>0.05$, Fig. 2A).

Endotoxemia activates KCs to produce pro-inflammatory factors and subsequently to promote liver injury. As the primary proinflammatory factor induced by gut-derived endotoxin, TNF- α plays an important role in mediating parenchymal cells injury in ALD. In the present study, hepatic TNF- α was observed to be increased significantly after 8-week EtOH intake comparing to that in control group (88.18±12.08 vs. 33.76±6.50 $\mu\text{g}/\text{mg}$ pro., $P<0.05$, Fig. 2B), and with puerarin administration, it decreased obviously (with 180 mg/kg.d puerarin, 61.19±13.87 vs. 88.18±12.08 $\mu\text{g}/\text{mg}$ pro., $P<0.05$; with 90 mg/kg.d puerarin, 67.13±10.35 vs. 88.18±12.08 $\mu\text{g}/\text{mg}$ pro., $P<0.05$, Fig. 2B).

Effects of puerarin on intestine injury and tight junction, ZO-1, protein expression

Endotoxins normally penetrate the gut epithelium only in trace amounts due to the intestinal barrier function (Mathurin et al., 2000; Parlesak et al., 2000; Lambert et al., 2003). The disruption of epithelial barrier function by EtOH results in the increased intestine permeability to injurious factors, such as LPS and contributes to endotoxemia in ALD (Rao et al., 2004).

In the present study, comparing to that of control, focal to superficial lamina propria edema and mild increased inflammatory but no obvious epithelial cell injury/loss or cellular mucin depletion were observed in the intestine tissue after chronic alcohol intake in H.E. staining sections (Fig. 2C). The histological injury scores of intestine showed that there was no significant difference among control (median = 0.5, range 0-1), EtOH (median = 1.5, range 1-3), 180 mg/kg.d puerarin (median = 1, range 0-3) and 90 mg/kg.d puerarin groups (median

= 1, range 0-1). These results were consistent with the results in previous researches (Persson et al., 1990; Mathurin et al., 2000; Rao et al., 2004).

However, the ultrastructural findings were more pronounced (Fig. 2D). With TEM observation, the microvilli on the epithelial cells was found to be thin and scattered, shortened and irregularity in distribution in the EtOH-diet fed rats and these ultrastructural alterations of microvilli were not obviously observed in puerarin-treated animals (Fig. 2D).

The barrier function of intestinal epithelium is provided by tight junctions (TJ), the highly specialized junctional complexes located at the apical end of epithelial cells. ZO-1 is one of the well studied TJ proteins. In the present study, ZO-1 protein expression was found to be significantly down-regulated by EtOH intake comparing to that in control (Fig. 2E). Meanwhile, with puerarin treatment, the protein expression of ZO-1 in intestine tissue was up-regulated obviously (Fig. 2E).

Effects of Puerarin on KCs activation and Protein expression of endotoxin receptors in liver

CD68/macrosialin, a transmembrane protein expressed by activated tissue macrophages (Rabinowitz and Gordon, 1991), was detected as a marker of the activated KCs. Immunohistological assay showed that few CD68-positive staining was observed in the hepatic sinusoidal of control rats (Fig. 3A), and the CD68 positive staining in hepatic tissue were strong and diffuse, especially accompanied with steatosis in the livers of EtOH-diet raised rats (Fig. 3A). While, the CD68 positive staining were thin and decreased in the liver sections of puerarin administrated rats (Fig. 3A) comparing to EtOH group. (See the negative

control of CD68 immunostaining in supplemental Fig. 3.) The protein expression tested by western-blotting assay revealed CD68 expression in liver was up-regulated significantly after chronic alcohol-intake and with puerarin treatment, protein of CD68 was down-regulated remarkably comparing to that of EtOH group (Fig. 3B)

LBP, the essential protein for LPS-transferring in the circulation, is predominantly produced by liver (Su et al., 1994), which binds to LPS of with high affinity to form the LPS-LBP complex and transfer of LPS to the surface receptors on target cells (Kupffer cells), such as mCD14. The pattern recognition receptors, TLRs, recognize the signals of LPS delivered by LBP and CD14 and activate the downstream cascades. As been disclosed in protein expression assay, in EtOH-diet group, the protein expression of endotoxin receptors in liver tissue, such as LBP, CD14, TLR4 and TLR2, were increased remarkably compared to that in control group. While, administrated with puerarin, the protein expression of endotoxin receptors was down-regulated significantly (Fig. 4).

Discussion

ALD encompasses a spectrum of injury, ranging from simple steatosis, hepatitis and cirrhosis, even the fatty drops disappear on abstinence, steatosis still increase the susceptibility of hepatocyte to further injury (Teli et al., 1995; Day and James, 1998). The continued ingestion of alcohol results in the subsequent steatohepatitis characterized with neutrophil infiltration, hepatocyte degeneration, ballooning and oncotic necrosis (Bautista, 2002; French, 2002) that rarely recover to normal hepatic histology even with ethanol withdrawal (French, 2002). Alcoholic seatohepatitis trigger the pathological progress to

fibrosis and then cirrhosis, so as to this stage appears to represent a rate-limiting step in the progression of ALD (Galambos, 1972; Diehl, 2002). Therefore, blocking or reversing the early-phase histopathologic changes (steatosis and steatohepatitis) is the key strategy of ALD treatment.

In the present study, the effects of puerarin on alcoholic liver injury and steatosis were confirmed in Lieber-DeCarli model, a mimic model of human being's early-stage of ALD. The hepatic histological alteration and biomarkers of liver damage in serum or liver tissue induced by EtOH diet were ameliorated with puerarin treatment. On the other hand, lipid deposition as well as increased hepatic TG concentration by EtOH intake was also inhibited remarkably in puerarin-treated animals.

Endotoxin-induced liver injury plays an important role in ALD. Plasma LPS levels in patients of ALD are higher compared with those in normal subjects (Parlesak et al., 2000) and nonalcoholic liver disease (Fukui et al., 1991). It has been observed the alcoholic liver injury in rats is associated with increased levels of plasma endotoxin (Mathurin et al., 2000; Tsukamoto et al., 2008). Furthermore, administration of antibiotics (Adachi et al., 1995) and probiotic bacteria (Wang et al., 2012) to rodents reduces the growth of gram-negative bacteria in the intestinal lumen and prevents ethanol-induced endotoxemia and liver injury. It has been suggested that, in ALD, the endotoxin levels at specific locations such as portal blood might be more important than its level in systemic plasma (Lambert et al., 2003). In the present study, endotoxemia in the portal vein and obvious heightened TNF- α in liver tissue observed in the EtOH intake rats reproduced the important role of gut-derived endotoxin in pathogenesis of ALD. Meanwhile, the inhibition on endotoxemia and hepatic TNF- α release

by puerarin supports the hypothesis that besides inhibition alcohol intake (Lin et al., 1996; Overstreet et al., 1996), reducing oxidation and modulation activity of cytochrome P450 (CYP) (Zhao et al., 2010), the potential mechanisms of puerarin amelioration alcoholic liver injury probable related to the pathway of gut-derived endotoxin activating KCs.

One mechanism by which alcohol induces endotoxemia is the alteration of gastrointestinal epithelial barrier function which subsequently increases intestinal permeability to endotoxins (Rao et al., 2004). The barrier function of intestinal epithelium is provided by the epithelial cells and the paracellular apical junction complex, including tight junctions and adherence junctions (Farhadi et al., 2003). Tight junctions are composed of several transmembrane proteins such as occludin and claudins and intracellular molecules such as ZO-1. In the present study, the gross morphological changes of ileum, ultrastructure of epithelial cells and ZO-1 protein expression in intestine tissue were observed. As been demonstrated in the previous researches (Persson et al., 1990; Mathurin et al., 2000; Rao et al., 2004), no obvious morphological changes were observed under light microscope after long-term EtOH exposure as well as puerarin orally administration. While the ultrastructure findings of pathological changes on microvilli of epithelial cells accompanied with decreased protein expression of intestinal TJ, ZO-1, in the EtOH diet-fed rats indicated the intestine barrier function injury. With puerarin administration, the pathological changes of microvilli were mitigated and ZO-1 protein expression was up-regulated, which probably associated with the mechanisms of puerarin inhibition gut-leakage induced by alcohol. Moreover, oxidative stress was suggested to critically mediate alcohol-induced intestinal barrier dysfunction (Kaur et al., 1998; Keshavarzian et al., 2009). Inflammatory response and cytokines, especially TNF- α , were also

found to increase the tight junction permeability (Suenart et al., 2002; Ma et al., 2004). The inhibition of oxidative stress (Hwang et al., 2011; Liu et al., 2012) and TNF- α production by puerarin (Liu et al., 2010b; Huang et al., 2012) demonstrated in different diseases provided indirect explanations on its protection on gut epithelial barrier function after alcohol intake. However, the precise mechanism puerarin inhibiting gut-leakage induced by alcohol needed more investigation.

KCs are the resident macrophages and one of the major sources of TNF- α in liver induced by LPS. They ensure maximal liver function by removing bacteria and phagocytosing foreign materials (Ajakaiye et al., 2011). KCs are quiescent in the absence of stimulatory agents. In ALD, KCs could be activated by gut-derived endotoxins and release several inflammatory cytokines, most significantly TNF- α , contributing to subsequent liver damage. CD68 is a marker of activated KCs (Rabinowitz and Gordon, 1991) that was found increasing in the liver tissue of EtOH diet-fed rats in the present study, indicating more KCs activated induced by alcohol, which was confirmed by the remarkable heightened hepatic TNF- α content in the EtOH diet group. Meanwhile, in animals that were treated with puerarin, the expression of CD68 protein was down-regulated obviously indicating inhibition of KC activation by puerarin.

To activate KCs, endotoxin signals delivery depends on the series of endotoxin receptors. LBP is produced mostly by hepatocytes (Su et al., 1994) and secreted into the bloodstream, where it binds with high affinity to the lipid A portion of LPS and catalyses the transfer of individual LPS molecules to cell surface receptors, such as mCD14, forming a monmeric LPS-CD14 complex (Lee et al., 1993; Gioannini and Weiss, 2007). With the stimulation of

proinflammatory cytokines, LBP is constitutively synthesized in hepatocytes (Elsing et al., 2011), which is consistent with the result obtained in the present study, in the rats intaking alcohol diet for a long-term, the protein expression of hepatic LBP was up-regulated markedly and down-regulated by puerarin administration.

Molecular CD14 anchors on the membrane of peripheral or liver resident microphage (Kupffer cell) through glycosylphosphatidylinositol (GPI). LPS-CD14 complex activated cells through TLR4 (Poltorak et al., 1998; Hoshino et al., 1999). It has been clearly established that TLR4 is the specific receptor of LPS from gram-negative bacteria (Uesugi et al., 2001; Su, 2002). Signaling through TLR4 requires MD-2, a secreted protein that is closely associated with the extracellular domain of TLR4 (Shimazu et al., 1999). In the present research, the protein expression of CD14 and TLR4 in liver tissue were found to be up-regulated by chronic alcohol diet intake and inhibited significantly by puerarin administration.

Other bacterial components, in addition to LPS, are likely translocated to the portal blood in alcoholics, since disruption of intestinal barrier by ethanol increases permeability for macromolecular substances in general (Rao, 2009). TLR2 appears to primarily response to gram-positive bacteria-derived lipoteichoic acid, peptidoglycan and mycobacterial lipoarabinomannan (Akira et al., 2001). In addition to TLR4, increased expression of TLR2 was observed in wild-type mice using the Lieber-DeCarli chronic alcohol feeding model, and feeding with alcohol resulted in sensitization to liver inflammation and damage because administration of TLR2 ligands increased expression of TNF- α (Gustot et al., 2006). On the other hand, MD-2 also enable TLR2 to response to the endotoxin protein-free LPS and

enhance TLR2-mediated responses to both gram-negative bacteria and their LPS (Dziarski et al., 2001). Similarly, the cytokins induced by LPS, such as IL-1 β or TNF- α , up-regulate TLR2 mRNA expression in rat hepatocyte in *vivo* and in *vitro*, either (Liu et al., 2000). In the present research, we found that the protein expression of TLR2 was remarkably up-regulated in the liver tissue of alcohol diet raised rats and also inhibited by puerarin.

In the present research, puerarin was supported to be a potential reagent to protect against chronic alcoholic liver injury and steatosis. The inhibition of puerarin on alcohol induced endotoxin gut-leakage, KCs activation and the protein expressions of endotoxin receptors probably associated with the potential mechanisms of its pharmacological effects on alcoholic liver injury.

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Authorship Contributions

Participated in research design: Hu and Peng.

Conducted the experiments: Peng, Cui, Huang, Chen, Xu.

Performed data analysis: Zhao, Xu and Feng

Wrote or contributed to the writing of the manuscript: Peng, Xu and Hu

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Footnotes

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Figure legends

Fig.1. Effects of puerarin on liver injury and hepatic lipid deposition induced by chronic alcohol intake

(A) H.E. staining for liver tissue. Severe macrovesicular steatosis was observed along with neurophil and lymphocytes infiltration in the EtOH-fed group. Puerarin treatment (180 mg/kg.d and 90 mg/kg.d) alleviated these pathological changes. (B) Oil red O staining for liver tissue. Large droplets colored with oil red O wide spread in the hepatic lobules of EtOH-fed rats. In puerarin-treated groups (180 and 90 mg/kg.d), the droplets of oil red O deposit were smaller and limited. (C) Serum ALT, AST and hepatic GGT activity determined by biochemical assay kits. (D) Hepatic TG content determined by biochemical assay kit. * $P < 0.05$, ** $P < 0.01$ compared with control. # $P < 0.05$, ## $P < 0.01$, compared with EtOH. Values are means \pm S.D., n=10 animals/group.

Fig. 2 Effects of puerarin on Endotoxin level in the portal vein, hepatic TNF- α concentration, intestine injury and ZO-1 protein expression.

(A) Endotoxin level in the portal vein determined by limulus ameobocyte lysate kit. (B) Hepatic TNF- α content analysed by ELISA. Values are means \pm S.D., n=10 animals/group. (C) Pathological changes of intestine were observed in H.E. staining sections. No obvious intestinal injury in control and EtOH-fed rats was observed. (D) Intestinal ultrastructure was observed by TEM. Microvilli was sparse, shortened and irregularity in distribution in EtOH-fed rats. In puerarin-administrated rats, the pathological changes of microvilli of intestine were not obviously observed. (E) Western-blot analysis for intestinal ZO-1 protein expression. These data were

representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with control; # $P < 0.05$, ## $P < 0.01$ compared with EtOH.

Fig. 3. Effect of puerarin on CD68 protein expression in liver.

(A) Immunohistology assay for hepatic CD68 expression. In EtOH-fed animals, the areas of CD68 positive staining were strong and diffuse in the hepatic sinusoidal, especially accompanied with steatosis, comparing to control. While, with puerarin treatment, CD68 positive staining was thin.

(B) Western-blot analysis for hepatic CD68 protein expression. * $P < 0.05$, ** $P < 0.01$ compared with control; # $P < 0.05$, ## $P < 0.01$ compared with EtOH. These data were representative of three independent experiments.

Fig. 4. Effects of puerarin on protein expression of endotoxin receptors in liver.

Western-blot analysis for LBP, CD14, TLR2 and TLR4 protein expression in liver tissue. These data were representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with control; # $P < 0.05$, ## $P < 0.01$ compared with ethanol.

Table 1. Effect of ethanol and puerarin treatment on dietary intake, body and liver weights

Data are means \pm S.D. Rats were fed Lieber-DeCarli diets for 8 weeks. Control animals were pair-fed equal amounts of isocaloric liquid diets.

Groups (n=10)	Liquid diet intake (ml)	Body weight (g)	Liver weight (g/kg body weight)
Control	2726 \pm 75	262 \pm 8	20.06 \pm 2.99
EtOH	2707 \pm 218	257 \pm 50	28.07 \pm 5.70**
Puerarin 180 mg/kg.d	2701 \pm 214	266 \pm 31	29.30 \pm 2.73**
Puerarin 90 mg/kg.d	2715 \pm 123	264 \pm 22	30.90 \pm 3.61**

* $P < 0.05$, ** $P < 0.01$ compared with control.

Fig. 1

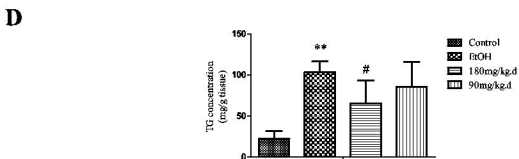
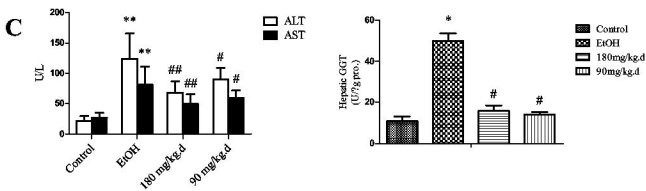
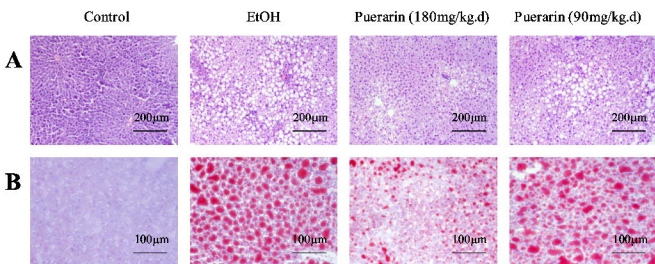


Fig. 2

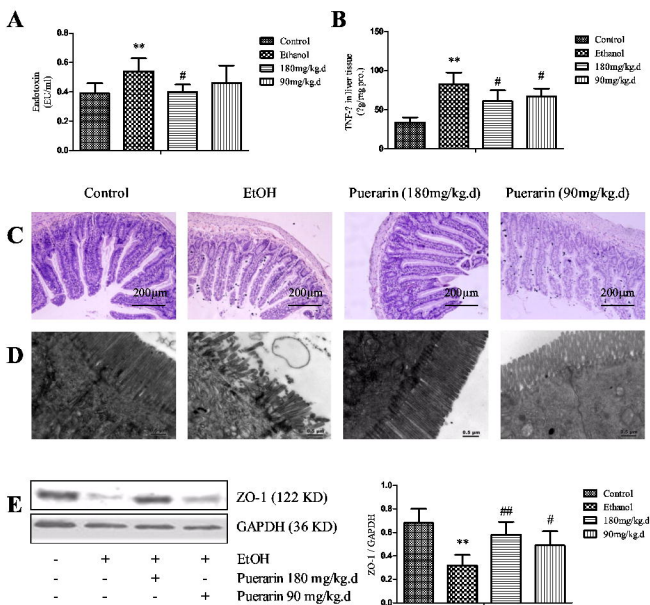
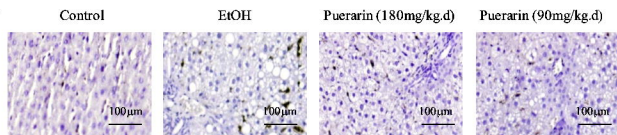


Fig. 3

A



B

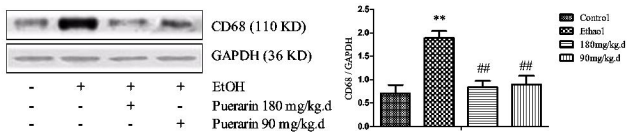


Fig. 4

