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A New Animal Model for *Polygonum multiflorum* Thunb-induced Liver Injury in Rats and Its Potential Mechanisms

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Abstract

Objective: Well accepted animal models are of the greatest importance in studying the Drug-induced Liver Injury (DILI), especially for those of which induced by the Traditional Chinese Medicine- *Polygonum multiflorum* Thunb (Chinese name: He-Shou-Wu, PMT) because of the difficulties to successfully simulate one in animals that can pattern the characteristics shown in people. This research aims to establish an early-warning animal model to investigate the potential mechanisms of DILI related to Toll-like Receptor 4 (TLR4), and to verify the effectiveness of serum microRNA-122 as a candidate biomarker for DILI. *Methods*: 2 hours after LPS injection, rats were administrated with PMT respectively for 7 consecutive days Qd. On the 2nd h, 14th h, 5th day and 8th day, serum samples were collected for ALT/ALP test and TaqMan detection of microRNA-122 expression. After weighing the organ to body ratio for each rat, liver samples were treated for histopathology observation and mTLR-4 detection by RT-PCR. *Results*: Comparing

against single administration with PMT, livers collected from animals pretreated with LPS injection were injured to various extents and performed hepatocyte degeneration. Meanwhile expression of liver mTLR4 and serum microRNA-122 increased significantly ($p<0.05$). *Conclusions*: Activated by LPS, this rat model of DILI for PMT was established successfully and its potential association with TLR4 was established. Serum microRNA-122 might be used as a candidate biomarker for the PMT- DILI.

Key words: DILI; *Polygonum multiflorum* Thunb; Animal model; TLR4; microRNA-122

Introduction

Drug-induced liver injury (DILI) is a kind of liver injury caused by the drug itself, its metabolites, special hypersensitivity constitution or tolerance reduction to certain drug.[1,2] Data from the WHO showed that in the past 50 years, DILI ranked the fifth cause of death in the world statistics. It has become the most common cause of withdrawal from the market as a result of safety concern.[3-8] While liver injury can, in general, be classified as hepatocellular, cholestasis or mixed[9-10] based on the criteria established by the Council for International Organizations of Medical Sciences (CIOMS).[11,12] The clinical diagnosis of DILI is, however, a very big challenge of exclusion. Even though the CIOMS system is also used for causality assessment of DILI by scoring parameters, as is in the Roussel Uclaf Causality Assessment Method (RUCAM) scoring system, such as time to onset of symptoms, laboratory data, additional drug regimen, known toxicity of suspected drug, non-drug causes, and response to re-challenge, other remaining discussion must also be devoted to the patterns observed in DILI with emphasis on morphological features, common drugs and differential diagnosis for each pattern.[13-15] In analysis of the reasons

that cause the current challenge in the diagnosis, prevention, and treatment of DILI, it is generally accepted that the difficulty of eliciting exposure to herbal products, over-the-counter agents and toxins, the lacking of biomarkers with liver specificity, expression stability and high sensitivity, the poor prognosis and rapid evolution of injury leading to high case fatality in along with their poorly understood mechanisms, cytochrome P450 enzymes (CYP450) metabolic activation, intracellular calcium homeostasis damage, mitochondrial damage, apoptosis, autoimmune activation, cholestasis and bile duct injury , and many others, are among the most often briefed ones.[16-19]

A new animal model for DILI by herbal and botanical drugs is in urgent need in that, taking China as an example, the proportion of Traditional Chinese Medicine (TCM) induced liver injury, exceeding one third of DILI, has grossed over anti-TB drugs to become a major cause of DILI.[20,21] Difficulties of the research rely on the complexity of TCM ingredients, unclear mechanisms of DILI, blindness of taking medicine, multiformity of pathological features and lack of specific biomarkers for diagnosis[22,23] and it is, thus, that the commonly used animal models cannot satisfy the needs of simulating the TCM-DILI. Well accepted animal models are of the greatest importance in studying the prediction, diagnosis and treatment of DILI. Currently, three animal models are extensively adopted for DILI study in vivo. The chemical-induced liver injury model, by CCl4 and APAP, leading to toxic hepatic necrosis, is for evaluating drugs with liver-protect effectivity. The immunity-induced liver injury model, BCG with LPS activation, is mainly used for studying

viral hepatitis, and the third one is the alcohol-induced liver injury model, none of which can be of any representativeness of the DILI by herbal and botanical drugs. Characterized by acute paroxysm and liver failure, the hepatotoxicity of *Polygonum multiflorum* Thunb (Chinese name: He-Shou-Wu, PMT) is commonly reported both in China and in other countries of the world in recent years.[24] PMT is one of Chinese herbs, which is traditionally valued and reported for hair-blacking, liver and kidney-tonifying and anti-aging effects as well as low toxicity. Natural compounds such as rhubarb, emodin and other anthraquinones are the main ingredients of PMT. Its origin, harvest time, processing and processing methods[16], storage and sales are likely to affect the herb quality. The unclear mechanism for its damage to liver and its complicated chemical composition undoubtedly made it difficult to do the research. Even from the perspective of the TCM protection, the first notice of amendments to oral formulations containing PMT is given by the SFDA in October 2013, and has developed to a clear prompt attention to its risk of liver injury in July, 2014.[25] As a traditional liver-protection drug, hepatotoxicity of PMT must draw our attention. Former research indicates that PMT-DILI as the idiosyncratic liver injury type may be related to immune-toxicity. [26,27] The unclear pathogenesis of hepatotoxicity, and the complex chemical composition undoubtedly make it difficult to unveil the mechanisms. One thing need to notice for the conventional animal models for PMT-DILI is that they usually cost a long period up to at least one month. What's more, the administration dosage is over 200 times more than that of the clinical usage.[28] Therefore, the aim of this work was to provide an animal model for PMT which has similar characteristics shown in humans.

It is well established that the activation of Kupffer Cells (KCs) plays a vital role in mediating idiosyncratic liver injury. The fatal thing may be the recognition and signal transduction of LPS. Available data show that the Toll-like receptors (TLRs) participate in the occurrence and development of hepatic pathological physiological process.[29] As the important receptor of LPS, TLR4 combines with LPS in the participation of LBP, CD14 and MD to activate KC. Involved in the relevant receptors-signaling molecules, TLR4 downstream signals in cells finally induce associated molecules to translocate into the nucleus and start transcription of inflammation factor and lead to liver injury.[30-32] The current study tested the simulation of a new animal model of DILI for PMT via a strategy of the pre-activation of the KCs in the liver, based on the possible mechanisms of the idiosyncratic type of DILI and in an aim of reducing the time and amount of drug needed in the conventional models. In doing so, effects of DSS and LPS, the two most commonly used activators for KCs[33-36] were compared, and LPS was chosen as the suitable inducer. Endeavors for a TLRs' role and comparisons of the serum microRNA-122[27-41] expression against the conventional biomarkers ALT and ALP for a potential biomarker were also made. The aim of this comparison was to verify the effectiveness of serum potential biomarker microRNA-122 for liver injury.

Materials and Methods

Animals

4-week male Sprague-Dawley rats (Beijing Vital River, Laboratory Animal Technology Co., Ltd.), SPF grade, weight 200±20g, were kept in GLP laboratory of National Beijing Center for Drug Safety Evaluation and Research (NBCDSER) with a 12:12 light/dark photoperiod (lights off at 18:00 h) at 20–24 °C. Each 5-rats were housed in one plastic cage and provided with filtered drinking water and food ad libitum. This study was fully conducted under protocols approved by Institutional Animal Care and Use Committee (IACUC) in NBCDSER who was authenticated by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Reagents and instruments

Acetaminophen (APAP) tablets (Beijing dawn Pharmaceutical Co., Ltd., Zhunzi H11020830, China); Trizol (P/N: 15596-018, Invitrogen Life Technologies, USA); superScript III enzyme reverse I (P/N: 18080-044, Invitrogen Life Technologies, USA); SYBR® Premix Ex Taq ™ II (Tli RNaseH Plus), ROX plus (P/N: RR82WR, TaKaRa Bio Inc., Japan); Premix Ex Taq ™ (Probe qPCR), ROX plus (P/N: RR39WR, TaKaRa Bio Inc., Japan); 5x RNA Loading Buffer (P/N: CW0611A, Beijing century goldreha co., ltd, China); RNasin (P/N: AM2682, Invitrogen Life Technologies, USA); primers and probes (Has-miR-122, U6 snRNA, Invitrogen Life Technologies, USA); LPS (L2880, Lot # 113M4068V, Sigma-Aldrich Co., USA); automatic blood analyzer (XT-2000iv, Japan Sysmex Co., Japan); vortex meter (QL-902, Lindberg Haimen Instrument Manufacturing Co., Ltd., China); centrifuge (Centrifuge 5415D, Eppendorf, Germany); spectrophotometry meter (NANODROP 2000, Thermo scientific Life Technologies, USA); fluorescence

quantitative PCR instrument (ABI7500, Applied Biosystems Life Technologies, USA). *Polygonum multiflorum* Thunb (PMT) was identified and obtained from Professor Xiao XH's research group in 302 Hospital (purchased from Beijing green Pharmaceutical Co., BN.13101701, Hubei, China). Immersing into 50% ethanol twice by cold immersion method, the two extracts of PMT were combined and concentrated to 3g ml^{-1} (raw herbs); The ethanol extracts were analyzed by LC/MS method (Agilent Technologies, 6550 iFunnelQ - TOF LC/MS). Chromatographic conditions: Agilent 300SB-C18 column (2.1mm×100mm, 1.8μm); Mobile phase was water (A) - acetonitrile (B). The linear gradient elution: 0-5min, 95%-68%A; 5-6min, 68%-45%A; 6-12min, 45% -12%A; 12 -15min, 15% - 10%A; The column temperature was 30 ℃; The volumetric flow rate was 0.3 ml/min; Detection wavelength was 280 nm; The elution time was 15 min; The Sample volume was 1 ml. The sample was prepared by 50% ethanol with a concentration of 4mg/ml for raw herb. The analyzed result is showed in figure 1.

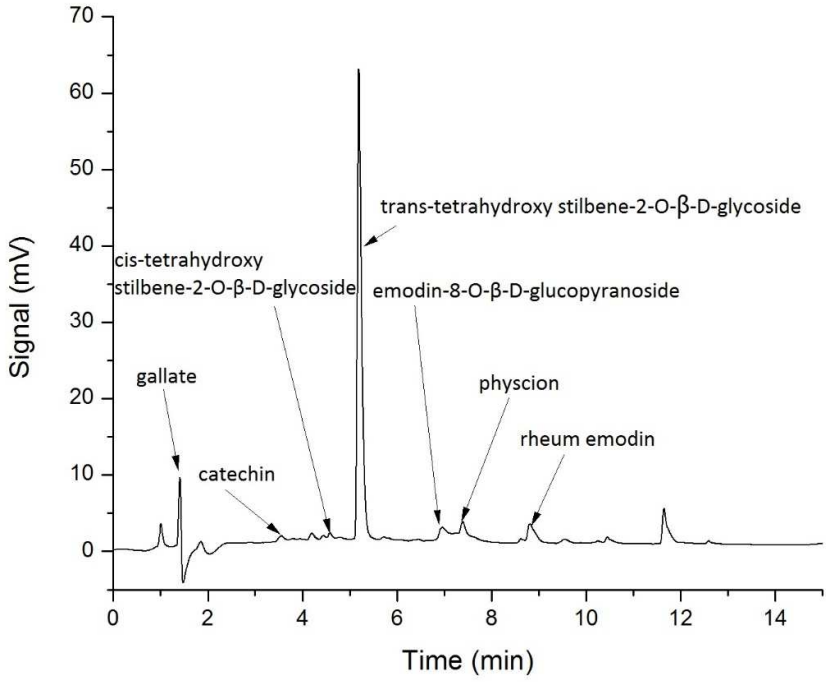

Figure 1. The UPLC fingerprint of ethanol extracts of *Polygonum multiflorum* Thunb.

Establishment of Animal model for PMT**-induced liver injury**

100 rats were randomly divided into 6 groups as follows: control group (20 rats), LPS group (20 rats), APAP group (15 rats), LPS+APAP group (15 rats), PMT group (15 rats) and LPS+PMT group (15 rats). For rats in LPS, LPS+APAP and LPS+ PMT group, LPS (4mg kg^{-1}) was injected by caudal vein. 2 hours later, rats were administrated via intragastric route with APAP (325mg kg^{-1}) and PMT (6g kg-1, equivalent to 30 times the clinical dose) respectively once a day for 7 days according to each group.

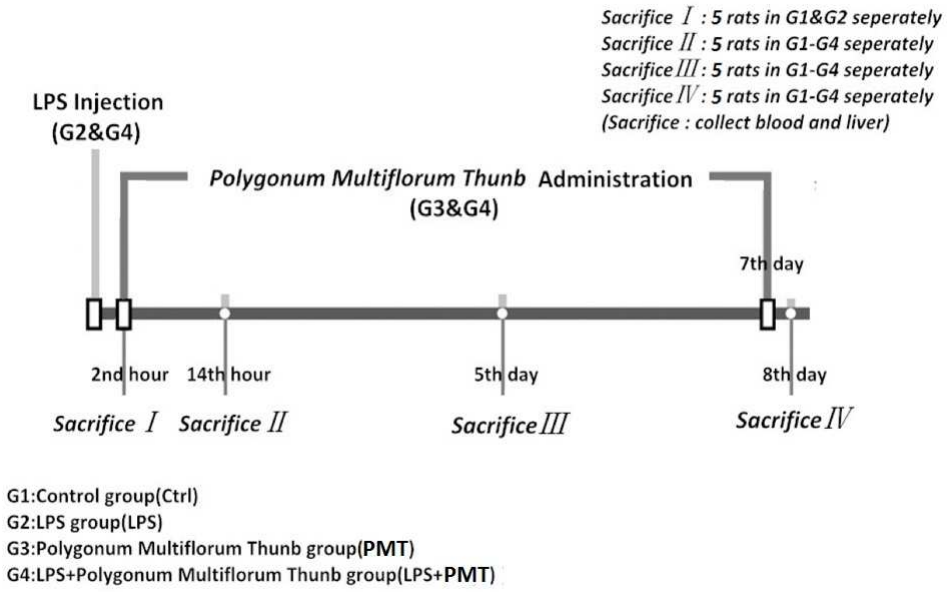

Figure 2. The entire experimental process and study design.

General clinical observations, such as body weight, were performed daily. 5 rats in the control and LPS group on the 2nd hour , 5 rats in each group on the 14th h, the 5th Day and the 8th Day were anesthetized to sacrifice with 5% Pentobarbital sodium (100mg kg^{-1} by peritoneal injection) respectively. Each blood sample was collected to clot from the abdominal aorta for further detection. After weighing the liver for each rat, livers were excised to be tested.

ALT/ALP detection in serum

The serum of rats was separated to determine the hepatic enzyme activities such as alanine aminotransferase (ALT) and alkaline phosphatase (ALP).

Histopathology Observation

4% Paraformaldehyde fixed, paraffin-embedded liver samples were cut into 4 μm thick sections. Then the sections were deparaffinized in xylene and rehydrated by ethanol and stained with hematoxylin-erosin. Histopathology observation of liver was performed under light microscopy.

Detection of liver mTLR4 expression by RT-PCR

Total RNA containing microRNA was isolated from frozen liver samples using TRIzol reagent according to the manufacturer's instructions and assessed by quantitative real-time PCR (qPCR). Then the purtity and concentration of RNA was determined using spectrophotometry meter (Thermo scientific) with an amount of 1 μL the extraction. Next, the extracted RNA was reverse-transcribed to synthesize cDNA using the SuperScript III RNAse H– Reverse transcriptase kit (Invitrogen). Thirdly, the amplification reactions were performed by product manuals using SYBR® Premix Ex Taq ™ II (Tli RNaseH Plus). SYBRgreen was used as the detection channel. The primers are listed in Table 1. The amplified samples were normalized to GAPDH. Real-time PCR data were analyzed by the relative gene expression (i.e., ΔΔCt) method.

Table 1. Primers for mTLR4

Gene	Upstream primer(5'-3')	Downstream primer(5'-3')	Length(bp)
TLR4	CTTTGCCTTCATTACAGGGACTTT	CCAGAGCGGCTACTCAGAAACT	182
GAPDH	TGGAGTCTACTGGCGTCTT	TGGAGTCTACTGGCGTCTT	138

Detection of serum microRNA-122 by TaqMan

The methods of serum Total RNA extraction was the same as mentioned in 2.2.4. Then the extracted RNA was reverse-transcribed by the TaqMan microRNA Reverse Transcription Kit and microRNA-122 stem-loop primers in accordance with the manufacturer's protocols. Premix Ex Taq™ (Probe qPCR) was used to amplify the samples by product manuals. FAM and BHQ1 were used as the detection channel. The primers are listed in Table 2. In the end, the results were calculated by the relative standard curve method and normalized to RNU6B expression.

Table 2. Primers for microRNA-122

Gene	Upstream primer(5'-3')	Downstream primer(5'-3')	TaqMan probe(5'-3')
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT	FAM-CGATACAGAGAAGATTAGCATGGCC-BHQ1
rno-miR-122-5p	TGGAGTGTGACAATGGTGTTT	GTGCAGGGTCCGAGGT	FAM-CTGGATACGACCAAACA-BHQ1
rno-miR-122-5p -loop RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAACA		

Statistical Analysis

All data are expressed as mean ± SEM. For comparison of two groups, an unpaired t test was used. For comparisons that involved multiple variables and observations, ANOVA (SPSS 19.0; GraphPrism Pad 6) was used. Having passed statistical significance by ANOVA, Turkey-Kramer test for all pair comparisons or individual comparisons were made by using the contrast method. Statistical significance is stated in the figure legends. We used relative value to compare the indicators in each group. Mean value of each indicator in control group was calculated out as the baseline value. Each value was

divided by the baseline.

Results

LPS+PMT induce weight loss of rats

In Figure 2, we observed that rats administrated with LPS+PMT had significantly greater weight loss than that in the control and PMT groups from the 2nd hour to the 8th day respectively (vs Ctrl $p<0.01$, vs PMT $p<0.01$). Rats in the LPS+APAP and LPS+PMT had similar degree of weight loss as rats in the LPS group from the 2nd hour to the 14th hour. On the 14th hour, the 5th day and the 8th day, the body weight for the rats in the LPS, LPS+PMT groups was much lower than that in the control and PMT groups. (vs Ctrl $p<0.01$, vs PMT $p<0.01$). However, rats in the LPS group had slight weight recovery from the 2nd hour to the 8th day than those in the LPS+PMT group ($p<0.05$). n=5 for each group.

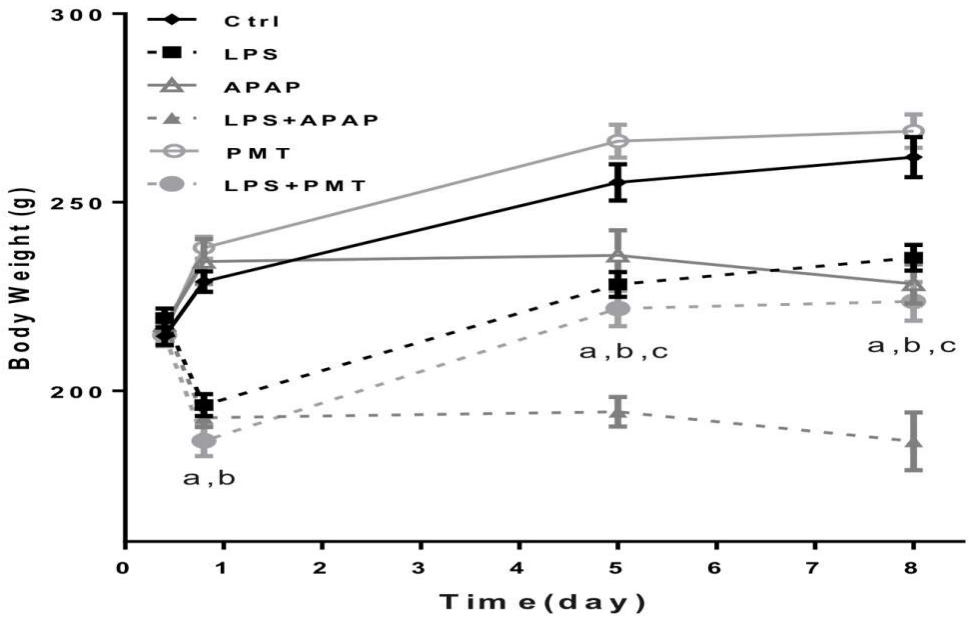

Figure 3. Evolution of the body weight in the control, LPS, APAP, LPS+APAP, PMT

and LPS+PMT groups during the experiment. The body weight for each group changed on the 2nd hour, the 14th hour, the 5th day and the 8th day. a $p<0.01$ LPS and LPS+PMT groups vs Control rats. b $p<0.01$ LPS and LPS+PMT groups vs PMT rats. c $p<0.05$ LPS vs LPS+PMT rats. Data represent the mean ± SEM, * $p<0.05$, n=5 for each group.

LPS+PMT promote slight liver swelling

Organ to body weight ratio of liver were calculated (%). Each ration was divided by the mean value for the control group on the 2nd hour, 14th hour, 5th day and 8th day respectively. Data were expressed as mean ± SEM. * $p<0.05$. n=5 for each group. After activated by LPS, rats liver enlarged to swelling. Liver to body weight ratio increased significantly on the 2nd hour. Liver enlargement in the LPS+PMT and LPS+APAP groups was more obvious than that in the PMT and APAP groups respectively (LPS+PMT vs PMT $p<0.05$, LPS+APAP vs APAP $p<0.05$). Liver enlargement in the LPS group recovered gradually. On the 8th day, liver enlargement in the LPS+PMT group was greater than that in the LPS and PMT groups (vs LPS $p<0.05$, vs PMT $p<0.05$). It suggested that the PMT-DILI model was successfully established.

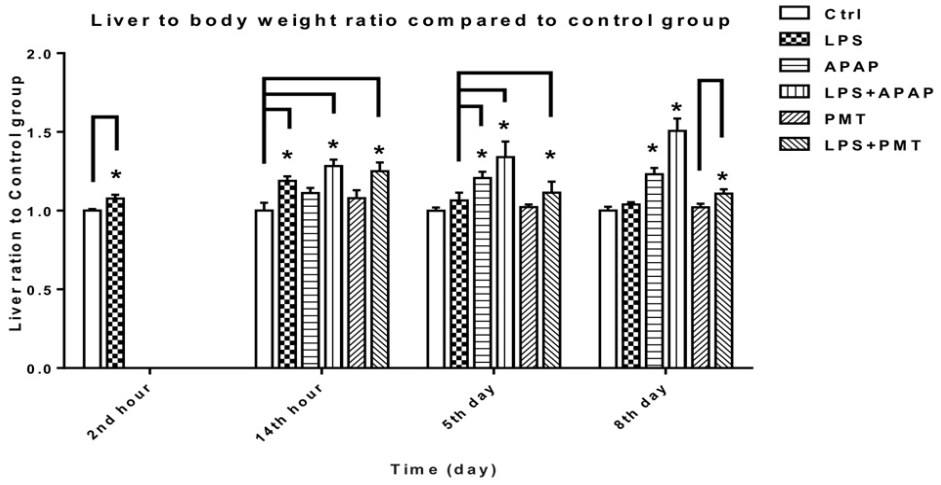

Figure 4. LPS+PMT promote slight liver swelling. On the 2nd hour, KC activated by LPS lead to liver swelling. On the 14th hour, liver swelling was shown for rats in the LPS, LPS+APAP and LPS+PMT groups. Comparing with the LPS group, the liver to body weight ratio of rats in the APAP, LPS+APAP and LPS+PMT changed significantly higher on the 5th day (vs APAP $p<0.05$, vs LPS+APAP $p<0.05$, vs LPS+PMT $p<0.05$). On the 8th day, liver to body weight ratio of rats in the LPS+PMT group was significantly higher than those in the LPS and PMT groups respectively (LPS+PMT vs LPS $p<0.05$). Data were expressed as mean ± SEM. * $p<0.05$.n=6 for each group.

ALT/ALP of LPS+PMT **change in the normal range**

Administrated with LPS+PMT, ALT/ALP changed in normal range on the 8th day. After 2 hours of Caudal vein injection of LPS, compared with the control group, serum ALT of rats in the LPS group increased significantly (LPS vs Ctrl $p<0.05$). At the following three time points, there was no obvious variation in the LPS group; serum ALT and ALP of rats in the PMT and LPS+PMT groups appeared no significant difference. The indexes varied

within normal range. Serum ALT of rats in the APAP and LPS+APAP groups increased significantly (vs Ctrl $p<0.05$). Data were expressed as mean±SD. * $p<0.05$. n=5 for each group.

Table 3. serum ALT (U L^{-1}) of rats in each group ($\bar{x}$ ±s, n=5)[a]

Time	Control	LPS	APAP	LPS+APAP	PMT	LPS+PMT
Hour 2	39.6±5.7	35.8±3.3	--	--	--	--
Hour 14	46.2±8.3	93.3±21.1*	41.8±6.3	87.8±20.7*	42.2±8.0	80.8±30.7*
Day 5	20.6±4.4	21.2±2.5	76.4±33.2	46.6±18.3	26.6±5.5	23.8±6.3
Day 8	28.4±9.1	23.8±5.4	56.6±12.6*	50.4±3.4*	30.6±6.8	28.0±5.3

[a] Note: Compared with the control group on the 2nd hour, 14th hour, 5th day and 8th day, statistically significant changes are indicated as * $p<0.05$.

Table 4. serum ALP (U L^{-1}) of rats in each group ($\bar{x}$ ±s, n=5)

Time	Control	LPS	APAP	LPS+APAP	PMT	LPS+PMT
Hour 2	175.2±15.4	229.4±49.2	--	--	--	--
Hour 14	191.2±35.0	284.8±39.5	249.0±33.1	292.4±77.1	191.4±24.3	289.4±50.1
Day 5	114.8±26.8	128.6±26.0	205.6±45.5*	182.6±45.5*	109.6±6.7	101.6±20.9
Day 8	108.0±14.7	110.2±17.3	166.4±13.1*	168.6±26.1	116.6±28.5	102.6±41.2

[a] Note: Compared with the control group on the 2nd hour, 14th hour, 5th day and 8th day, statistically significant changes are indicated as * $p<0.05$.

LPS activate the hepatotoxicity of PMT

The PMT-induced rats acute liver injury model was successfully established. On the 2nd

hour, a small amount of hepatocyte degeneration in the LPS group was observed. From the 2nd hour to the 8th day, the hepatocyte degeneration recovered gradually in the LPS group. Only a small amount of hepatocyte degeneration was observed in the PMT group; Degrees of liver injury in the LPS+PMT group deepened gradually, showing various extent of hepatocyte damage and degeneration. Chronic inflammatory lesions could be seen locally; Livers were damaged partially in the APAP group, and hepatocytes degenerated and grew necrosis.

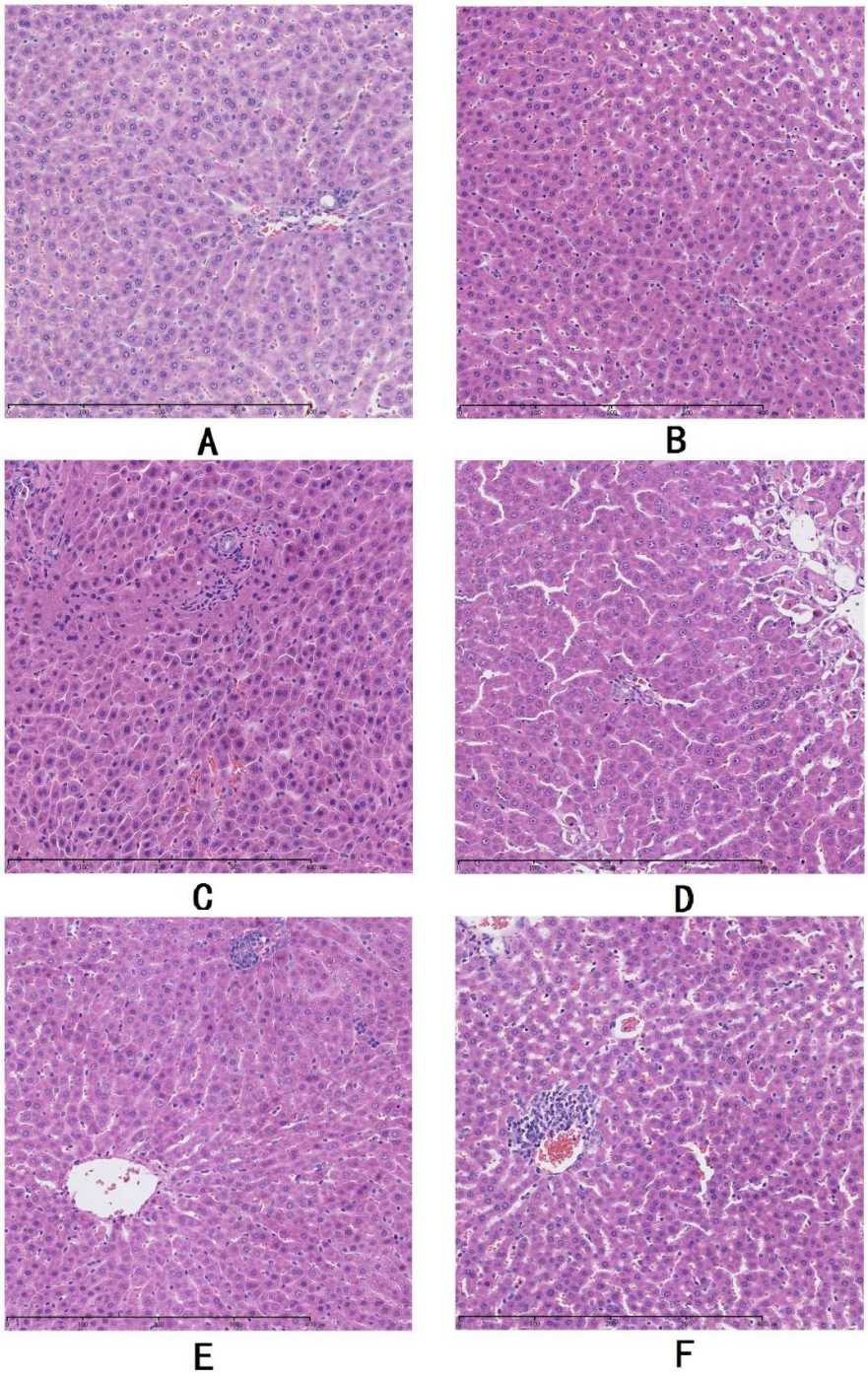

Figure 5. The histopathological examination of liver. (A). The liver tissue of rats in the control group on the 8th day. (B). A particle of liver cells degeneration recovered after LPS activation on the 8th day. (C). Acute liver injury induced by APAP on the 8th day. The injury had been shown with focal inflammation and hepatocytes degeneration. (D). The liver

tissue of rats in the LPS+ APAP group on the 8th day. The injury had been shown with the necrosis of the hepatocytes. (E). The liver tissue of rats in the PMT group on the 8th day. Administrated with PMT, few hepatocytes degenerated. (F) The liver tissue of rats in the LPS+ PMT group on the 8th day. Focal necrosis of the hepatocytes had been shown as well as a particle of hepatocyte degeneration. Figures were all stained with HE (220×).

Hepatotoxicity of LPS+PMT is associated with mTLR4 up-regulation in liver

To determine mechanism of the PMT-DILI, mTLR4 expression in liver was used for evaluating the inflammation on hepatotoxicity. Each value for all the rats was divided by the mean value of its control group on the 2^{nd} hour, the 14^{th} hour, the 5^{th} day and the 8^{th} day respectively (Figure 5). On the 2^{nd} hour, mTLR4 expression in the LPS group showed significant inhibition (Figure 5, vs Ctrl $p<0.05$). The following three time points, compared with the control group, mTLR4 expression appeared no obvious change in the PMT group. On the 5^{th} day, mTLR4 expression in the LPS+PMT group increased significantly by contrast to that of the control, LPS and PMT groups (Ctrl vs LPS+PMT $p<0.05$, LPS vs LPS+PMT $p<0.05$, PMT vs LPS+PMT $p<0.05$). While significantly increased in the APAP, LPS+APAP and LPS+ PMT groups on the 8^{th} day (Ctrl vs LPS+PMT $p<0.05$, LPS vs LPS+PMT $p<0.05$, PMT vs LPS+PMT $p<0.05$). n=5 for each group. Together, these data indicated that up-regulation of mTLR4 expression was associated with hepatotoxicity of LPS+PMT.

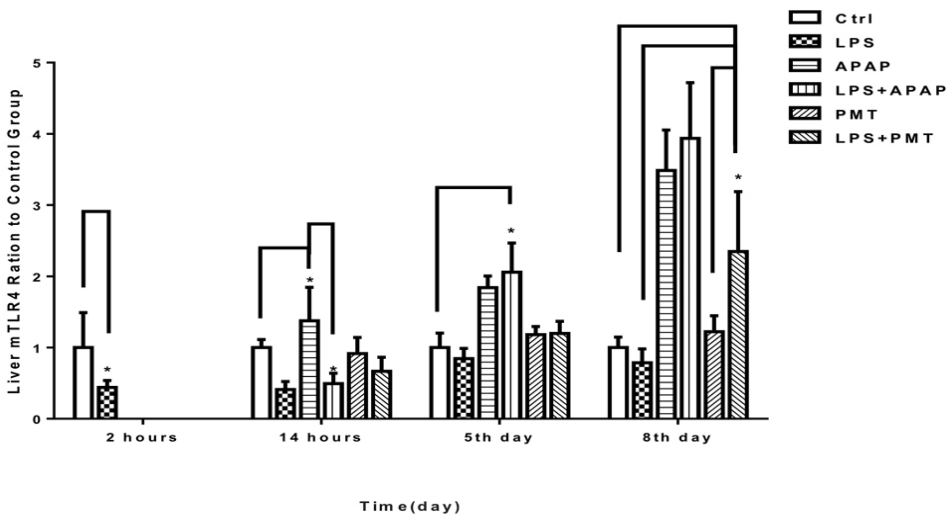

Figure 6. Hepatotoxicity of LPS+PMT on the expression of mTLR4 in liver. Expression of mTLR4 was analyzed on the 2nd hour, the 14th hour, the 5th day and the 8th day in liver tissue from each group. The mTLR4 expressions in the LPS group were obviously inhibited compared with those in the control group ($p<0.05$). Rats treated with LPS+PMT had increased the level of mTLR4 when compared to those in the LPS and PMT group respectively ($p<0.05$). Data were expressed as mean ± SEM. * $p<0.05$. n=5 for each group.

LPS+PMT upregulate microRNA-122 expression in serum

Each value for all the rats was divided by the mean value of its control group on the 2nd hour, the 14th hour, the 5th day and the 8th day respectively. After activated with LPS, microRNA-122 expression in rats serum increased significantly in the LPS group (vs Ctrl $p<0.05$) on the 2nd hour. The following three time points, compared with the control group, microRNA-122 expression in rats serum appeared no significant change in the LPS group, while significantly increased in the LPS+ PMT, APAP, LPS+APAP groups with time

passing by (vs Ctrl * p<0.05).

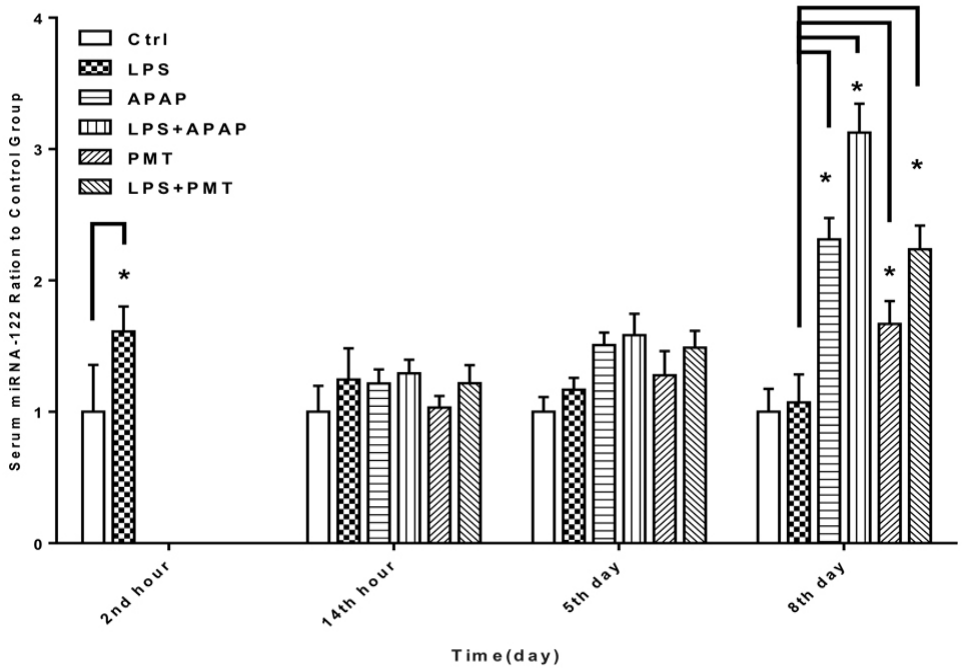

Figure 7. Ration of microRNA-122 expression in rats' serum at different time points compared with control group. Expression of microRNA-122 was analyzed on the 2nd hour, the 14th hour, the 5th day and the 8th day in serum from each group. The microRNA-122 expressions in the LPS group were obviously increased compared with those in the control group (LPS vs Ctrl p<0.05). Rats in the LPS+PMT group had increased the level of microRNA-122 when compared to those in the LPS and PMT group respectively (LPS vs LPS+PMT p<0.05, PMT vs LPS+PMT p<0.05). Data were expressed as mean ± SEM. * p<0.05. n=5 for each group.

microRNA-122 and mTLR4 are more sensitive than ALT/ALP

Each value of index, such as microRNA-122, mTLR4, ALT and ALP, was divided by the

mean of control group on the 2nd hour, 14th hour, the 5th day and the 8th day respectively. Comparing with the expression of ALT and ALP, microRNA-122 in rats serum and mTLR4 in rats liver in the LPS+PMT group elevated significantly on the 8th day. And the degree of changes was in proportion to the liver damage. The augment of indexes in the LPS+PMT group was greater than that in the PMT group. Although ALT, ALP in rats serum in the LPS+PMT group increased significantly at the beginning (vs Ctrl, $p<0.05$), the expression of the two varied within normal range of rats serum. n=5 for each group. This phenomenon indicates that microRNA-122 in serum can be used as potential biomarker for this model. Perhaps mTLR4 in liver is also a promising candidate.

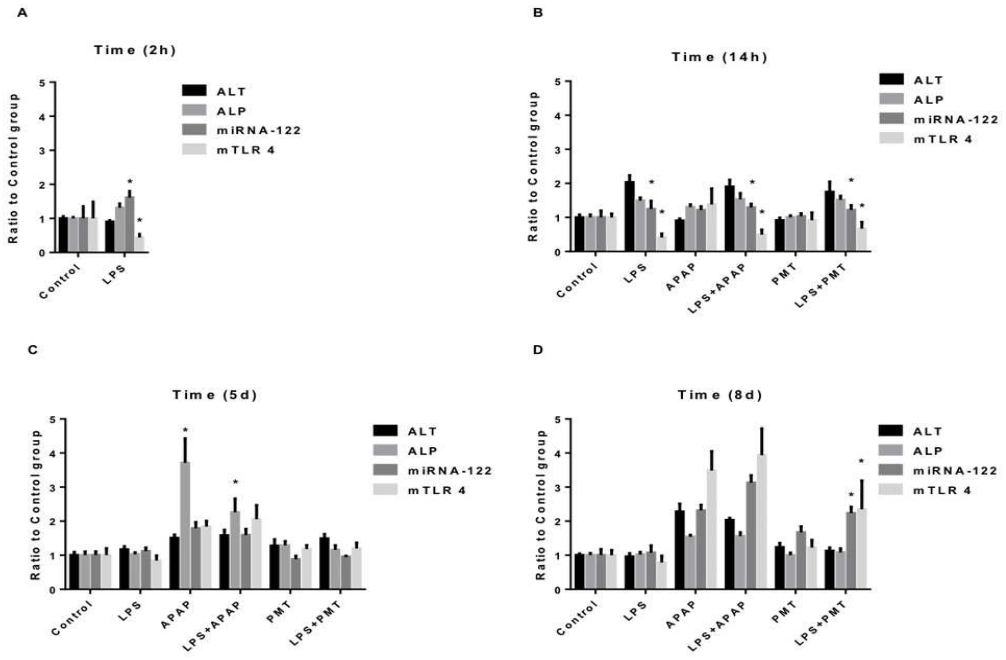

Figure 8. Comparison of ration for the expression of ALT, ALP, microRNA-122 in rats serum and mTLR4 in rats liver in each group at different time points. (A). On the 2nd hour, the expression of microRNA-122 in serum increased promptly with the activation of LPS. On the contrary, the expression of mTLR4 in liver was inhibited by LPS injection. (B).

On the 14th hour, serum ALT and ALP in the LPS, LPS+APAP and LPS+PMT groups elevated in normal range. (C). On the 5th day, each index for rats in the APAP and the LPS+APAP groups aggrandized significantly compared with the control group. (D). On the 8th day, the augment of serum microRNA-122 ration and liver mTLR4 ration in the LPS+PMT group were larger than those of ALT and ALP. By contrast, these variation in the LPS+PMT group were greater than those in the PMT group. Each index in the LPS group returned to normal. Data were expressed as mean ± SEM. * $p<0.05$. n=5 for each group.

Discussion

The pathogenesis of DILI is extremely complicated, especially for the immuno liver injury that associated with KCs activity. Liver macrophages, KCs, consist of the largest number of tissue macrophages, KCs play a vital role in regulating the immune system of the liver and even the whole body on the basis of its specific anatomic location and physiological function.[42] Its functional status depends on striking a balance between activation and tolerance of KCs. For one thing, KC, a kind of typical mononuclear phagocyte, is activated to mediate inflammatory response and lead to local immuno injury under the stimulus of enterogenous bacteria and its endotoxin. For the other thing, existed in the liver, the activity of KCs is regulated by its internal LPS signal transduction system and various cells interaction. Only the interaction between activation and tolerance reached equilibrium, could the immune response of liver remain in the normal physiological range. LPS signal transduction system of KC is activated by a mass of LPS. Then vast pro-inflammatory

factors are released to mediate inflammation and pathological injury of liver, and may eventually develop systemic inflammation.

Previous work was made to screen out an ideal model induce hepatotoxicity of PMT. DSS and LPS are commonly used to activate KCs as idiosyncratic liver injury inducers.[33-36] Our previous studies hints that LPS is a better choice to activate the immune liver injury. Clinical observation index including liver to body weight ratio, hematology test, histopathology observations were recorded to determine whether the model was successful in our previous studies. In the pre-screening model, we used 3.5% DSS for 4 consecutive days orally administration and 4mg kg^{-1} LPS once by caudal vein as inducing agents respectively. Compared with the DSS group, SD rats induced by LPS appeared loose stools on the 2nd hour. Eyelid secretion abnormalities were observed with a daily oral administration dosage of 20 times the clinical dosage for 7 consecutive days. Liver to body weight ratio of the LPS group increased significantly. Meanwhile, histopathology observation showed KCs activation, accompanied by a small number of hepatocytes degeneration. Ratio of rats with liver injury was higher than that of the DSS group. Compared with the female rats, the male rats were more sensitive to PMT-induced liver injury. It maybe results from the effect of estrogen-related anti-inflammation.[42] Thus LPS was chosen as the inducer. LPS pretreatment and PMT dosing are performed with a 2-hour interval on the basis of their the onset times of CYP450 activation.[44-46] The types of CYP450 which are used to metabolize the main ingredients of PMT are similar to those of LPS. To determine the dosage of administration, Modified Karber method was used to test

SD rat oral median lethal dose (LD50), the maximum tolerated dose (MTD) and the maximum dosage (MLD) of PMT ethanol-extract after 4mg kg^{-1} LPS injection. LD50, MTD and MLD were 30g kg^{-1}, 90g kg^{-1}, and 53.26g kg^{-1}, which were equivalent to 150, 450 and 266.3-fold of clinical dosage respectively. The 95% confidence limit for LD50 was 46.91 ~ 60.46g kg^{-1}.

Currently, the commonly used animal models are not suitable for studying the mechanism of PMT-DILI. The CCl4 model, as chemical-DILI animal model, is a classical one to accurately reflect the function, metabolism and morphology of hepatocytes. CCl4 could not only induce liver injury but also other organ injuries of experimental animals. The formation of free radicals and triggering of a chain peroxidation are the mainly accepted mechanisms. Different from the immune pathology of clinical hepatitis, liver injury that induced by chemical drugs can develop into toxic liver necrosis.[47] Another commonly used chemical model is APAP. On the condition of hypohepatia or over-dosage of APAP within a short time, the metabolism of liver is confined to deplete intracellular GSH. Then the accumulating NAPQI leads to hepatocyte degeneration and necrosis.[48,49] Secondly, alcoholic liver injury animal model is mainly used to study alcoholic fatty liver disease. The injury is mainly induced through directly affect the structure of cell membrane and function and indirectly influence its metabolites.[50,51] Thirdly, BCG with LPS activation is an immune liver injury animal model. Immune response in the liver is the important mechanism of the virus hepatitis. Direct hepatocytes damage and destruction mediate T cell immune response. T cell-mediated immune response is the direct cause for hepatocyte damage.[52]

Our studies showed that the new model with LPS activation was established successfully because of short induction period and low induction dosage. Administrated with single PMT, rats in the PMT group weighed close to those in the control group. By contrast, rats in the LPS+PMT group weighed lighter obviously than those in the control group. The different trend of weight loss of rats between the PMT and the LPS+PMT groups might be due to the pharmaceutical effect of PMT on immune system. On the contrary, LPS+PMT might have a negative effect on the animal bodies. Rats, only treated with single LPS, weighed to normal after a sharp loss. It hints that PMT have a negative effect on animal bodies with the induction of LPS. Liver to body weight ratio of rats in the LPS or PMT groups gradually returned to normal. There were no significant differences between the two. The ratio of rats in the LPS+PMT group increased on the 2^{nd} hour, and reduced to a certain level which was higher than that of the LPS or PMT groups. It suggested that PMT could induce liver injury with the participation of LPS. Ratio of rats in the APAP and LPS+APAP groups raised significantly different with that of other groups. It proved that the injury mechanism between APAP and PMT were different. For histopathology observation, acute hepatocytes degeneration could be seen after 2 hours activation by LPS. On the 8^{th} day, the morphology and structure of hepatocytes returned to normal. Liver injury in the LPS+APAP group appeared much more severe than that in the APAP group with visible hepatocytes necrosis. Minor hepatocytes degeneration could be seen in the PMT group. By contrast, hepatocytes degeneration and necrosis in the LPS+PMT group deepened gradually with the increasing times of drug administration. It proved that this model was

established successfully. LPS could activate the liver injury of PMT. Compared against the clinical usage, animal models for PMT-induced liver injury cost quite a long period up to 1 or 3 months with 200 times the clinic dosage. Its pathogenesis is different from the clinic trials. So the primary value of the research is to establish PMT-induced liver injury animal model that has similar characteristics shown in humans.

In this animal model, the pathogenesis was associated with the expression of mTLR4 in liver. After activated by LPS, mTLR4 levels of rats' liver in the LPS group returned to normal with a 7-day rise. It suggests that LPS could magnify the effect of PMT-DILI. The expression of mTLR4 in liver is proportional to the degrees of injury. TLRs, a kind of pattern recognition receptors (PRRs), can regulate the innate immunity and acquired immunity. They also play an vital role in the host resistance against microbial infection, and endogenous ligand. After the identification of exogenous and endogenous ligands, TLRs promote the inflammatory response signaling pathways and upregulate the expression of inflammatory factors to protect the tissues from injury.[53,54] Endotoxins consist of lipoprotein and lipopolysaccharide (LPS) complexes. As the main active component of endotoxins, LPS performs important pathophysiological function. Identified as critical receptor for LPS signaling, modulation of TLR4 expression is closely related to control LPS-associated inflammation. There are two primary pathways for TLR4 to induce inflammation combined by LPS activation. Namely they are the myeloid differentiation factor 88 (Myd88) dependent and MyD88 independent signaling pathways.[55] Combined

with LPS, TLR4 is transduced from the extracellular to the intracellular. It provide a bright orient for our next research.

Detection of the microRNA–122 expression in peripheral blood can help to predict the hepatotoxicity of a new drug. DILI, especially induced by TCM, is hard to predict for its acute paroxysm, rapid evolution of injury, high morbidity. Traditional biomarkers such as ALT/ALP cannot meet the requirement of early prediction of DILI. So a biomarker with high specificity, sensitivity and stability is indispensable.[56] microRNA molecules, existed in body fluid with high stability, participate in cell growth, differentiation, proliferation and apoptosis, and other important physiological processes.[57,58] The microRNA-122 only expresses in liver tissue with the highest amount expression of microRNA in hepatotoxicity.[37-41] Studies show that the microRNA - 122 is an ideal potential biomarker for liver disease.[59] In this research, we validate the effectiveness of potential serum biomarker microRNA-122 for DILI. The peak time of microRNA-122 expression in the LPS+PMT group was earlier than that of ALT/ALP. Indexes such as ALT, ALP, microRNA-122 and mTLR4 performed differently in the APAP, LPS+APAP, PMT and LPS+PMT groups. The different performance of microRNA-122 and liver to body weight ratio over time for the LPS+APAP and LPS+PMT groups indicates that the two are of different pathogenesis of liver injury.

On account of the complex ingredients of PMT extract and lack of ideal animal model, the mechanism of PMT remains unclear to prevent further injury. While the new model could

validate the relation of toxicity mechanism with TLR4 and contribute to further study. It provides a new orient for studying hepatotoxicity mechanism of chemical, herbal and botanical drugs. Liver samples of this model will be used for proteomics analysis by iTRAQ technology.[60,61] Compared with patients' liver samples, the selected proteins will be validated to unveil the mechanism of PMT-DILI by ELISA or Western Blot. With the same method, the model's blood samples will be analyzed to find out differentially expressed proteins. Then the selected proteins will be applied to identify the effectiveness of potential hepatotoxicity biomarkers for serum samples of patients with PMT-DILI.

Conclusions

This new animal model for PMT-induced Liver Injury with similar characteristics shown in humans was established successfully. Its mechanism is closely related to mTLR4 expression. More sensitive than ALT and ALP, microRNA-122 was certificated to be an ideal potential serum biomarker for this model. Meanwhile the expression trend of mTLR4 in liver is similar to that of microRNA-122 in serum. It hints that mTLR4 might also be used as a potential biomarker for this model.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Author contribution

Prof Rigao Ding, Quanjun Wang designed research; Xing Fan, Jiabo Wang, Lihua Xie, Yan-sheng Dong, Gang Han, Dan Hu, Yue Liu, Benli Yuan, Hemei Wang, Chunqi Wu and Xiaohe Xiao performed research. Xing Fan analyzed data and wrote the paper.

Reference

1 G. Abboud, N. Kaplowitz, Drug-induced liver injury, Drug Saf, 2007, 30(4), 277-294.
2 A. Reuben, DG. Koch, WM. Lee, Acute Liver Failure Study Group. Drug-induced acute liver failure: results of a U.S. Multicenter, prostective study, Hepatology, 2010, 52(6), 2065-2076.
3 A. Regev, Drug-induced liver injury and drug development: industry perspective, Semin Liver Dis, 2014 May, 34(2), 227-239.
4 NM. Chen, V. Vijay, Q. Shi, Z. Liu, H. Fang, W. Tong W, FDA-approved drug labeling for the study of drug-induced liver injury, Drug Discov Today, 2011, 16(15-16), 697-703.
5 N. Chalasani, RJ. Fontana, HL. Bonkovsky, PB. Watkins, T. Davern, J. Serrano, H. Yang, J. Rochon, Cause, clinical features, and outcomes from a prospective study of drug-induced liver injury in the United States, Gastroenterology, 2008, 135(6), 1924-1934.
6 KT. Suk, DJ. Kim, CH. Kim, SH. Park, JH. Yoon, YS. Kim, GH. Baik, JB. Kim, YO. Kweon, BI. Kim, SH. Kim, IH. Kim, JH. Kim, SW. Nam, YH. Paik, JI. Suh, JH. Sohn, BM. Ahn, SH. Um, HJ. Lee, M. Cho, MK. Jang, SK. Choi, SG. Hwang, HT. Sung, JY. Choi, KH. Han, A prospective nationwide study of drug-induced liver injury in Korea, Am J Gastroenterol, 2012, 107(9), 1380-1387.
7 ES. Bjornsson, OM. Bergmann, HK. Bjomsson, RB. Kvaran, S. Olafsson, Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland, Gastroenterology, 2013, 144(7), 1419-1425, 1425.21-3.
8 RJ. Fontana, PB. Watkins, HL. Bonkovsky, N. Chalasani, T. Davern, J. Serrano, J. Rochon; DILIN Study Group, Drug-induced Liver Injury Network(DILIN) prospective study: rationale, sesign and conduct, Drug Saf, 2009, 32, 55-68.
9 A. Regev, LB. Seeff, M. Merz, S. Ormarsdottir, GP. Aithal, J. Gallivan, PB. Watkins, Causality assessment for suspected DILI during clinical phases of drug development, Drug Saf, 2014 Nov, 37 Suppl 1, S47-56.
10 DE. Kleiner, NP. Chalasani, WM. Lee, RJ. Fontana, HL. Bonkovsky, PB. Watkins, PH. Hayashi, TJ. Davern, V. Navarro, R. Reddy, JA. Talwalkar, A. Stolz, J. Gu, H. Barnhart, JH. Hoofnagle;

Drug-Induced Liver Injury Network (DILIN), Hepatic histological findings in suspected drug-induced liver injury: systematic evaluation and clinical associations, Hepatology, 2014 Feb, 59(2), 661-670.

11 PB. Watkins, LB. Seeff, Drug-induced liver injury: summary of a single topic clinical research conference, Hepatology 2006, 43, 618–631.

12 R. Teschke, A. Schwarzenboeck, KH. Hennermann, Kava hepatotoxicity: a clinical survey and critical analysis of 26 suspected cases, Eur J Gastroenterol Hepatol, 2008, 20, 1182–1193.

13 G. Danan, C. Benichou, Causality assessment of adverse reactions to drugs. I. A novel method based on the conclusions of international consensus meetings: application to drug-induced liver injuries, J Clin Epidemiol, 1993, 46, 1323–1330.

14 C. Benichou, G. Danan, A. Flahault, Causality assessment of adverse reactions to drugs. II. An original model for validation of drug causality assessment methods: case reports with positive rechallenge. J Clin Epidemiol 1993, 46, 1331–1336.

15 RJ. Andrade, MI. Lucena MI, MC. Fernández, G. Pelaez, K. Pachkoria, E. García-Ruiz, B. García-Muñoz, R. González-Grande, A. Pizarro, JA. Durán, M. Jiménez, L. Rodrigo, M. Romero-Gomez, JM. Navarro, R. Planas, J. Costa, A. Borras, A. Soler, J. Salmerón, R. Martin-Vivaldi; Spanish Group for the Study of Drug-Induced Liver Disease, Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period, Gastroenterology2005, 129, 512–521.

16 L. Lin, B. Ni, H. Lin, M. Zhang, X. Li, X. Yin, C. Qu, J. Ni, Traditional usages, botany, phytochemistry, pharmacology and toxicology of Polygonum multiflorum Thunb: A review, J Ethnopharmacol, 2015 Jan 15, 159C, 158-183.

17 DE. Kleiner, The pathology of drug-induced liver injury, Semin Liver Dis 2009, 29, 364-372.

18 G. Lv, Z. Lou, S. Chen, H. Gu, L. Shan, Pharmacokinetics and tissue distribution of 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside from traditional Chinese medicine Polygonum multiflorum following oral administration to rats, J Ethnopharmacol, 2011 Sep 1, 137(1), 449-456.

19 YY. Wang, J. Yang, H. Liu, FQ. Lin, JS. Shi, F. Zhang, Effects of tetrahydroxystilbene glucoside on mouse liver cytochrome P450 enzyme expressions, Xenobiotica, 2014 Oct, 28, 1-7.

20 P. Zhao, C. Wang, W. Liu, G. Chen, X. Liu, X. Wang, B. Wang, L. Yu, Y. Sun Y, X. Liang, H. Yang, F. Zhang, Causes and outcomes of acute liver failure in China, PLoS One, 2013 Nov 22, 8(11), e80991.

21 Y. Zhou, L. Yang, Z. Liao, X. He, Y. Zhou, H. Guo, Epidemiology of drug-induced liver injury in China: a systematic analysis of the Chinese literature inclusing 21789 patients, Eur J Gastroenterol Hepatol, 2013, 25(7), 825-829.

22 N. Chalasani, RJ. Fontana, HL. Bonkovsky, PB. Watkins, T. Davern, J. Serrano, H. Yang, J. Rochon, Drug Induced Liver Injury Network (DILIN), Causes, clinical features, and outcomes from a prospective study of drug-induced liver injury in the United States, Gastroenterology 2008, 135, 1924.

23 FQ. Hou, Z. Zeng, GQ. Wang, Hospital admissions for drug-induced liver injury: clinical features, therapy, and outcomes, Cell Biochem Biophys 2012, 64, 77.

24 KA. Jung, HJ. Min, SS. Yoo, HJ. Kim, SN. Choi, CY. Ha, HJ. Kim, TH. Kim, WT. Jung, OJ. Lee, JS. Lee, SG. Shim, Drug-Induced Liver Injury: Twenty Five Cases of Acute Hepatitis Following Ingestion of Polygonum multiflorum Thunb, Gut Liver, 2011 Dec, 5(4), 493-499.

25 SFDA website, http://www.sda.gov.cn/WS01/CL0051/102902.html.

26 MI. Lucena, RJ. Andrade, N. Kaplowiz, M. García-Cortes, MC. Fernández, M. Romero-Gomez, M. Bruguera, H. Hallal, M. Robles-Diaz, JF. Rodriguez-González, JM. Navarro, J. Salmeron, P. Martinez-Odriozola, R. Pérez-Alvarez, Y. Borraz, R. Hidalgo; Spanish Group for the Study of Drug-Induced Liver Disease, Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex, Hepatology, 2009, 49(6), 2001-2009.

27 DE. Amacher, The primary role of hepatic metabolism in idiosyncratic drug-induced liver injury, Exper Opin Drug Metab Toxicol, 2012, 8(3), 335-347.

28 M. Chen, H. Bisgin, L. Tong, H. Hong, H. Fang, J. Borlak, W. Tong, Toward predictive models for drug-induced liver injury in humans: are we there yet? Biomark Med, 2014, 8(2), 201-213.

29 LA. O'Neill, AG. Bowie, The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling, Nat Rev Immunol, 2007, 7, 353-364.

30 YC. Lu, WC. Yeh, PS. Ohashi, LPS/TLR4 signal transduction pathway, Cytokine, 2008 May, 42(2), 145-151.

31 MC. Morris MC, EA. Gilliam, L. Li, Innate immune programing by endotoxin and its pathological consequences, Front Immunol, 2015 Jan 6, 5, 680.

32 D. Han, L. Dara, S. Win, TA. Than, L. Yuan, SQ. Abbasi, ZX. Liu, N. Kaplowitz, Regulation of drug-induced liver injury by signal transduction pathways: critical role of mitochondria, Trends Pharmacol Sci, 2013 Apr, 34(4), 243-253.

33 KC. El Kasmi, AL. Anderson, MW. Devereaux, SA. Fillon, JK. Harris, MA. Lovell, MJ. Finegold, RJ. Sokol, Toll-like receptor 4-dependent Kupffer cell activation and liver injury in a novel mouse model of parenteral nutrition and intestinal injury, Hepatology, 2012 May, 55(5), 151815-28.

34 S. Zhang, N. Yang, S. Ni, W. Li, L. Xu, P. Dong, M. Lu, Pretreatment of lipopolysaccharide (LPS) ameliorates D-GalN/LPS induced acute liver failure through TLR4 signaling pathway, Int J Clin Exp Pathol, 2014 Sep 15, 7(10), 6626-6634.

35 FF. Tukov, JF. Maddox, D E. Amacher, WF. Bobrowski, RA. Roth, PE. Ganey, Modeling inflammation-drug interactions in vitro: A rat Kupffer cell-hepatocyte coculture system, Toxicol In Vitro, 2006, 20(8), 1488-1499.

36 KC. El Kasmi, AL. Anderson, MW. Devereaux, PM. Vue, W. Zhang, KD. Setchell, SJ. Karpen, RJ. Sokol, Phytosterols promote liver injury and Kupffer cell activation in parenteral nutrition-associated liver disease, Sci Transl Med, 2013 Oct 9;5(206), 206ra137.

37 Q. Shi, H. Hong, J. Senior, W. Tong, Biomarkers for drug-induced liver injury, Expert Rev Gastroenterol Hepatol, 2010, 4(2), 225-234.

38 S. Bala, J. Petrasek, S. Mundkur, D. Catalano, I. Levin, J. Ward, H. Alao, K. Kodys, G. Szabo, Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases, Hepatology, 2012 Nov, 56(5), 1946-1957.

39 YW. Su, X. Chen, ZZ. Jiang, T. Wang, C. Wang, Y. Zhang, J. Wen, M. Xue, D. Zhu, Y. Zhang, YJ. Su, TY. Xing, CY. Zhang, LY. Zhang, A panel of serum microRNAs as specific biomarker for diagnosis of compound-and herb-induced liver injury in rats, PLoS One, 2012, 7(5), 1-11.

40 Y. Zhang, Y. Jia, R. Zheng, Y. Guo, Y. Wang, H. Guo, M. Fei, S. Sun, Plasma microRNA-122 as a Biomarker for viral-, alcohol-,and chemical-related hepatic diseases, Clinical Chemistry, 2010, 56(12), 1830-1838.

41 PJ. Starkey Lewis, J. Dear, V. Platt, KJ. Simpson, DG. Craig, DJ. Antoine, NS. French, N. Dhaun, DJ. Webb, EM. Costello, JP. Neoptolemos, J. Moggs, CE. Goldring, BK. Park, Circulating microRNAs as potential markers of human drug-induced liver injury, Hepatology, 2011, 54(5), 1767-1776.

42 J. Ananiev, M. Penkova, G. Tchernev,AA. Chokoeva, S. Philipov, C. Tana, M. Gulubova, U. Wollina, Macrophages and dendritic cells in the development of liver injury leading to liver failure, J Biol Regul Homeost Agents, 2014 Oct Dec, 28(4), 789-794.

43 L. Shi, YL. Feng, H. Lin, R. Ma, X. Cai, Role of estrogen in hepatocellular carcinoma: is inflammation the key? J Transl Med, 2014, 12, 93.

44 W. Wang, Y. He, P. Lin, Y. Li, R. Sun, W. Gu, J. Yu, R. Zhao, In vitro effects of active components of Polygonum Multiflorum Radix on enzymes involved in the lipid metabolism, J Ethnopharmacol, 2014 May 14, 153(3), 763-770.

45 J. Yu, J. Xie, XJ. Mao, MJ. Wang, N. Li, J. Wang, GT. Zhaori, RH. Zhao, Hepatoxicity of major constituents and extractions of Radix Polygoni Multiflori and Radix Polygoni Multiflori Praeparata, J Ethnopharmacol, 2011 Oct, 11, 137(3), 1291-1299.

46 W. Wang, Y. He, P. Lin, Y. Li, R. Sun, W. Gu, J. Yu, R. Zhao, In vitro effects of active components of Polygonum Multiflorum Radix on enzymes involved in the lipid metabolism, J Ethnopharmacol, 2014 May, 14, 153(3), 763-770.

47 O. Park, WI. Jeong, L. Wang, H. Wang, ZX. Lian, ME. Gershwin, B. Gao, Diverse Roles of Invariant Natural Killer T Cells in Liver Injury and Fibrosis Induced by Carbon Tetrachloride, Hepatology, 2009 May, 49(5), 1683–1694.

48 M. Coen, Metabolic phenotyping applied to pre-clinical and clinical studies of acetaminophen metabolism and hepatotoxicity, Drug Metab Rev, 2014 Dec, 23, 1-16.

49 MR. McGill, M. Lebofsky, HR. Norris, MH. Slawson, ML. Bajt, Y. Xie, CD. Williams, DG. Wilkins, DE. Rollins, H. Jaeschke, Plasma and liver acetaminophen-protein adduct levels in mice after acetaminophen treatment: dose-response, mechanisms, and clinical implications, Toxicol Appl Pharmacol, 2013, 269, 240–249.

50 G. Szabo, S. Bala, Alcoholic liver disease and the gut-liver axis, World J Gastroenterol, 2010, 16, 1321–1329.

51 E. Ceni, T. Mello, A. Galli, Pathogenesis of alcoholic liver disease: role of oxidative metabolism, World J Gastroenterol, 2014 Dec 21, 20(47), 17756-17772.

52 H. Wang, W. Wei, YX. Shen, C. Dong, LL. Zhang, NP. Wang, L. Yue, SY. Xu, Protective effect of melatonin against liver injury in mice induced by Bacillus Calmette-Guerin plus lipopolysaccharide, World J Gastroenterol, 2004 Sep 15, 10(18), 2690-2696.

53 T. Kawai, S. Akira, TLR signaling, Semin Immunol, 2007, 19, 24-32.

54 AE. Medvedev, I. Sabroe, SN. Hasday, SN. Vogel, Tolerance to microbial TLR ligands: molecular mechanisms and relevance to disease, J Endotoxin Res 2006, 12, 133-150.

55 A. Velayudham, I. Hritz, A. Dolganiuc, P. Mandrekar, E. Kurt-Jones, G. Szabo, Critical role of toll-like receptors and the common TLR adaptor, MyD88, in induction of granulomas and liver injury, J Hepatol, 2006 Dec, 45(6), 813-824.

56 JW. Eun, HJ. Bae, Q. Shen, SJ. Park, HS. Kim, WC. Shin, HD. Yang, CY. Jin, JS. You, HJ. Kang, H. Kim, YM. Ahn, WS. Park, JY. Lee, SW. Nam, Characteristic molecular and proteomic signatures of drug-induced liver injury in a rat model, J Appl Toxicol, 2015 Feb, 35(2), 152-164.

57 RJ. Hornby, P. Starkey Lewis, J. Dear, C. Goldring, BK. Park, MicroRNAs as potential circulating biomarkers of drug-induced liver injury: key current and future issues for translation to humans, Expert Rev Clin Pharmacol, 2014 May, 7(3), 349-362.

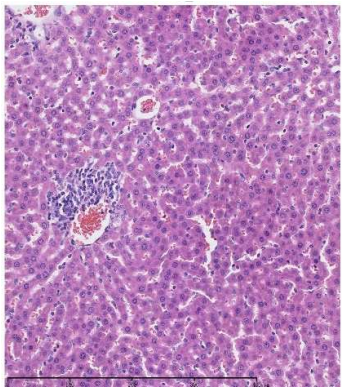
58 R. Christoph, L. Tom, Circulating microRNAs as markers of liver inflammation, fibrosis and cancer, J Hepatol, 2014 Dec, 61(6), 1434-1437.

59 RJ. Fontana, Pathogenesis of idiosyncratic drug-induced liver injury and clinical perspectives, Gastroenterology, 2014 Apr, 146(4), 914-928.

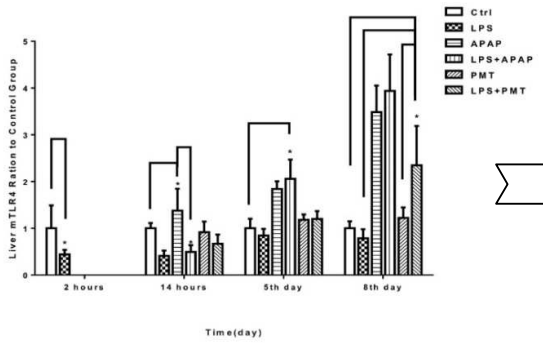
60 TA. Addona, X. Shi, H. Keshishian, DR. Mani, M. Burgess, MA. Gillette, KR. Clauser, D. Shen, GD. Lewis, LA. Farrell, MA. Fifer, MS. Sabatine, RE. Gerszten, SA.Carr, A pipeline that integrates the discovery and verification of plasma protein biomarkers reveals candidate markers for cardiovascular disease, Nat Biotechnol, 2011 Jun 19, 29(7), 635-643.

61 X. Song, J. Bandow, J. Sherman, JD. Baker, PW. Brown, MT. McDowell, MP. Molloy, iTRAQ experimental design for plasma biomarker discovery, J Proteome Res, 2008 Jul, 7(7), 2952-2958.

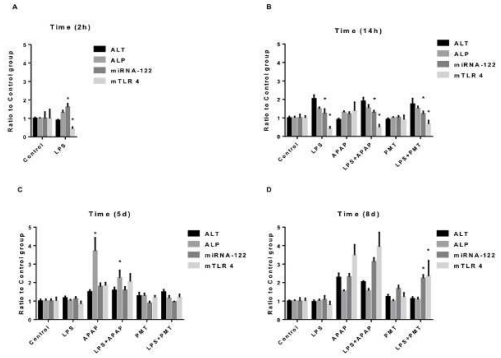
Graphical Abstracts: A new animal model for *Polygonum multiflorum Thunb (PMT)*-induced Liver Injury, more similar to clinical trials, is established successfully with the activation of LPS. Its pathogenesis is associated with the expression of mTLR4 in rats' liver. More sensitive than ALT and ALP, microRNA-122 is certificated to be an ideal potential serum biomarker for this model.



LPS injection triggered the hepatotoxicity of *PMT* in rats



The expression of mTLR4 in liver is positive proportional to the degrees of PMT-induced liver injury in this model.



Serum microRNA-122 serves as a better potential biomarker for liver injury in this model than ALP or ALT does.