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Original Research Article

Buddleoside inhibits TLR4-related pathway in a mouse model of acute liver failure, promotes autophagy, and inhibits inflammation

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Abstract

Purpose: *To study the inhibitory influence of buddleoside on TLR4-associated pathway, autophagy and inflammation in a mouse model of acute liver failure (ALF).*
Methods: *Sixty male C57BL/6 mice were assigned to 5 groups: control, model, and three dose-groups of buddleoside, with 12 mice per group. Levels of interleukin (IL)-1, IL-6, TLR4 pathway-associated proteins, and autophagy-related proteins in each group were determined; cell adhesion in each group was also analyzed.*
Results: *Levels of TLR4, MAPK and NF-κB-related pathways in model mice were significantly up-regulated, relative to control mice, but they were more down-regulated in the 3 anthocyanin groups than in model group ($p < 0.05$). There were significantly higher levels of TNF-α, IL- and IL-6 in model mice than in the control group, but they were down-regulated in high-, medium- and low-dose mice, relative to model mice. The population of adherent cells was significantly higher in ALF mice than in controls, but there were markedly lower numbers of these cells in the 3 anthocyanin-treated mice than in model mice ($p < 0.05$).*
Conclusion: *Buddleoside mitigates ALF in mice by down-regulating inflammatory factors, reducing serum levels of ALT and AST, and up-regulating autophagy-related protein expressions by activating TLR4/MAPK/NF-κB signaling pathway. Thus, buddleoside may be useful in the treatment of acute liver failure, but this has to be curtained through clinical trials.*

Keywords: *Buddleoside; Acute liver failure; TLR4-related pathway; Autophagy; Inflammatory factors*

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INTRODUCTION

Acute liver failure (ALF) is caused by acute viral infection, drugs, and autoimmune diseases. It manifests as abnormality in coagulation, jaundice, ascites and hepatic encephalopathy, and it is associated with increasing mortality rate [1]. Acute liver failure (ALF) induces massive necrosis of liver cells within a short period, leading to liver injury, hepatic encephalopathy and inflammatory response syndrome, and eventually to necrosis and failure of multiple organs [2]. Liver injury, whether acute or chronic, may lead to liver failure. The occurrence of liver injury manifests mainly as hepatocyte apoptosis and generation of a large number of

inflammatory factors which further affect liver tissues [3].

Toll-like receptor (TLR) is an important LPS receptor, and it acts *via* the recognition of injury-related molecules and antigen-related molecules [4]. The most important TLR for LPS is TLR4. It mediates MAPK and nuclear factor kappa-B (kB) which are important in the activation of inflammation and induction of ALF [5].

Eukaryotes maintain homeostasis mainly through autophagy which is crucial in degradation of unwanted components and abnormal polypeptides [6]. Studies have found that autophagy inhibits excessive inflammation produced through immune response, and also inhibit liver damage due to various factors such as alcoholic and non-alcoholic fatty liver [7].

Wild chrysanthemum belongs to *Compositae*. It *detoxifies heat*, protects the heart and reduces blood pressure [8]. Studies have found that the extract of wild chrysanthemum produced anti-inflammatory and vascular protective effects in mice, and also suppressed ear swelling, foot swelling and granuloma [9]. The main bioactive compound in wild chrysanthemum is monanthine which is a natural flavonoid. Monanthine is used in the treatment of hypertension. It lowers plasma cholesterol, and exerts a protective effect on blood vessels. However, monanthine is rarely used for treating ALF.

This research was done to unravel the influence of monanthine on autophagy and inflammation in a mouse model of ALF, and the role of TLR4-related pathway in the process.

EXPERIMENTAL

Animals

Sixty healthy male C57BL/6 mice (mean age of 7.33 ± 1.25 weeks and mean body weight of 23.86 ± 3.22 g), were used in this study. The mice were maintained in an environment with 12-h light/12-h dark cycle, and room temperature of 23 ℃. They were fed adaptively for 7 days, with adequate drinking water.

Ethical approval

This research was approved by the Animal Ethical Committee of Guizhou Education University (approval no. 2019032), and performed according to the guidelines of "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [10].

Equipment and reagents

The instruments and reagents used, and their suppliers (in brackets) were: TLR4 receptor (Shanghai Guangrui Biotechnology Co. Ltd); fetal bovine serum (Zhengzhou Jiulong Biological Products Co. Ltd); DAB Chromogenic Kit (Beijing Regen Biotechnology Co. Ltd.); PBS buffer (Shanghai Xinyu Shenwu Technology Co. Ltd); monanthine (purity ≥ 98 %; Chengdu Dest Biotechnology Co. Ltd); LPS and D-GaLN (Beijing Solebo Technology Co. Ltd); NF-κB and MAPK Pathway Kit (Shanghai Anti-Biological Technology Co. Ltd); automatic biochemical analyzer (Beijing Pulang New Technology Co. Ltd); optical microscope (Beijing Presse Instrument Co. Ltd); electronic balance (Mettler Toledo International Ltd); high-speed, low-temperature centrifuge (Shanghai Maigao Scientific Instrument Co. Ltd), and automatic slicer (Shanghai Leica Microsystem Trading Co. Ltd).

Establishment of ALF model and animal grouping

Sixty mice were assigned to 5 groups: control, model, and 3 monanthine dose groups (n = 12). Except for controls, the model and 3-dose monanthine groups were intraperitoneally injected with LPS and D-GaIN (30 μg/kg and 500 mg/kg, respectively) in order to establish a mouse model of ALF. The caudal vein of each mouse was injected with normal saline 24 h before the model was established. Mice in the 3 monanthine groups received the drug at doses of 45, 20 and 12 mg/kg, through intragastric administration 3 days once a day before the model was established. Mice in control group were given equivalent volume of 0.9 % NaCl intraperitoneally. The number of surviving mice was recorded every 2 h, and the mice were monitored continuously for 24 h. Liver tissue and blood specimens were taken 6 h after the operation. The blood samples were allowed to clot, and sera obtained after centrifugation were kept frozen at -80 ℃ in 1.5-mL Eppendorf tubes prior to analysis.

Determination of serum levels of liver enzymes

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using automatic biochemical analyzer.

Histopathological examination of liver tissues

Liver tissues of mice were fixed in 10 % formaldehyde, embedded in paraffin, sliced and

stained with hematoxylin and eosin (H & E). The slides were examined for pathological changes under a light microscope.

Determination of levels of inflammatory factors

Serum and cell supernatant concentrations TNF-α, IL-1 and IL-6 were measured using their respective ELISA kits.

Determination of expression levels of autophagy-related proteins

The expression levels of cellular proteins were measured with immunoblotting assay. Total proteins were extracted from liver tissues of mice in each group, and centrifuged. The extract protein content was estimated using BCA method. Then, equal amounts of protein were subjected to SDS-PAGE, followed by transfer to PVDF membranes which were subsequently incubated overnight at 4 °C with primary antibodies for LC3-II, ATG5), ATG7, and homologous domain protein1 (Beclin-1)-related proteins. Then, the membranes were subjected to incubation with secondary antibodies linked to horse radish peroxidase at room temperature for 1 h, followed by chemiluminescence and Grayscale analysis for calculation of relative protein expression levels.

Cell adhesion studies

The cells were inoculated in (6-well plates at a density of 1.5×10^4/well, stained, and incubated in an incubator. The cells were stimulated with monanthine solutions containing different levels of LPS for 24 h, and labeled with fluorescent labeling method.

Statistical analysis

The SPSS 20.0 software was employed for statistical analysis in this study. Measurement data are presented as mean ± SD. Two-group comparison was done with *t*-test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Serum levels of ALT and AST

There were markedly increased blood activities of transaminases in model mice, relative to controls, but the activities of these enzymes were decreased in the 3 monanthine groups, relative to those in model mice ($p < 0.05$; Table 1).

Table 1: Comparison of levels of ALT and AST amongst the different groups (mean ± SD, n = 12)

Group	ALT (U/L)	AST (U/L)
Control	95.36±8.24	81.64±8.97
Model	439.58±52.14*	523.61±49.11*
High-dose	124.24±12.44#	132.45±14.32#
Medium-dose	175.49±11.85#	169.78±15.49#
Low-dose	228.92±13.44#	241.23±16.79#

*$P < 0.05$, vs control; #$p < 0.05$, vs model

Expression of TLR4-related pathways

The expression levels of proteins related to TLR4/MAPK/NF-KB signaling pathway were markedly up-regulated in ALF model, relative to controls, but they were down-regulated in the 3 monanthine groups, when compared with ALF model group ($p < 0.05$). These results are shown in Table 2.

Table 2: Expression levels of proteins related to TLR4/MAPK/NF-KB signaling pathway (mean ± SD, n = 12)

Group	TLR4	MAPK	NF-kB
Control	0.63±0.06	0.25±0.02	0.39±0.03
Model	0.99±0.15*	1.05±0.11*	0.75±0.13*
High-dose	0.69±0.08#	0.38±0.03#	0.41±0.04#
Medium-dose	0.73±0.06#	0.62±0.05#	0.58±0.06#
Low-dose	0.85±0.05#	0.81±0.06#	0.63±0.05#

*$P < 0.05$, vs control; #$p < 0.05$, vs model

Degree of inflammatory response

Table 3 shows that there were markedly up-regulated expressions of pro-inflammatory proteins in ALF model, relative to control mice, but they were down-regulated in the 3 monanthine groups, when compared to model mice ($p < 0.05$).

Table 3: Levels of inflammatory cytokines amongst different groups (ng/L, mean ± SD, n = 12)

Group	TNF-α	IL-1	IL-6
Control	45.33±10.62	46.12±8.62	41.22±9.88
Model	359.44±19.32*	226.14±28.67*	356.72±24.62*
High-dose	134.62±21.48#	92.11±25.41#	109.56±21.66#
Medium-dose	209.68±21.77#	140.39±25.17#	160.44±19.47#
Low-dose	305.11±28.49#	193.44±21.33#	204.64±22.41#

*$P < 0.05$, vs control; #$p < 0.05$, vs ALF mice

Table 4: Comparison of expression levels of autophagy-related proteins amongst different groups (mean ± SD, n = 12)

Group	LC3-Ⅱ	ATG5	ATG7	Beclin-1
Control	0.72±0.06	1.31±0.12	0.43±0.04	0.39±0.02
Model	0.51±0.09*	0.71±0.21*	0.21±0.06*	0.09±0.01*
High-dose	2.23±0.12#	2.07±0.35#	2.18±0.18#	0.89±0.05#
Medium-dose	1.22±0.08#	1.56±0.24#	1.63±0.11#	0.52±0.03#
Low-dose	0.99±0.05#	1.22±0.25#	1.05±0.22#	0.47±0.04#

*P < 0.05, vs controls; #p < 0.05, vs ALF mice

Expression levels of autophagy-related proteins

The expressions of proteins related to LC3-Ⅱ, ATG5, ATG7 and Beclin-1 were markedly down-regulated in model mice, relative to control mice, but were markedly up-regulated in the 3 monanthine groups, relative to control group (p < 0.05). These results are presented in Table 4.

Cell adhesion

The number of adhered cells was markedly higher in ALF model than in controls, but it was lower in the 3 monanthine-treated groups than in model mice (p < 0.05; Table 5).

Table 5: Comparison of cell adhesion numbers amongst the different groups (mean ± SD, n = 12)

Group	Cell adhesion number
Control	108.62±25.79
Model	294.75±29.76*
High-dose	112.45±22.45#
Medium-dose	138.74±20.47#
Low-dose	182.79±21.75#

*P < 0.05, vs control; #p < 0.05, vs ALF mice

DISCUSSION

It is known that ALF is associated with high degree of mortality in clinical practice. The most effective treatment for ALF is liver transplantation, but due to limitations imposed by scarcity of liver donors, ALF usually results in high mortality [11]. The pathogenesis of ALF is a process affected by multiple factors, and its clinical characteristics are complex and variable, leading to poor prognosis and high fatality rate which constitute a huge challenge to clinicians [12]. Endotoxemia is a leading cause of ALF-related death [13]. Lipopolysaccharide (LPS) from dead Gram-negative bacteria forms endotoxin which stimulates Kupffer cells and causes liver injury [14]. Therefore, it is particularly important to select effective and reliable drugs for ALF treatment.

Monanthine inhibits the activity of acetylcholinesterase, and it is beneficial in cancer prevention and bone cell differentiation [15]. A study has reported that monanthine regulated hypertension through the renin angiotensin system by activating the p38MAPK pathway through angiotensin converting enzyme 2 (ACE2), thereby inhibiting the enzyme [16]. Monanthine also influences bone cell differentiation. It induced mesenchymal stem cell-to-osteoblast transformation, leading to morphogenesis of bone-specific matrix proteins [17]. Monanthine may provide high concentration of phosphate and significantly enhance alkaline phosphatase activity and mRNA expression at the early stage of osteocyte differentiation, thereby playing an anti-osteoporosis role by generating calcified nodules [18]. With respect to liver injury, monanthine reduced necrotic areas in liver failure, and reduced the occurrence of hepatic phlebitis and cell infiltration [19]. Monanthine reduces liver cholesterol level, resulting in decreased blood activities of ALT and AST. Monanthine also blocks the phosphorylation of JnK pathway and regulates oxidation reactions mediated by fatty acids, thereby alleviating liver injury caused by lipid toxicity.

Nuclear factor kappa-B (NF-κB) is crucial in inflammatory response, and it acts as a bridge in inflammatory response. Moreover, TLR4 is the most important LPS receptor, and its signaling molecules activate MAPK pathway and induce the activation of NF-KB pathway [20]. Activation of NF-κB pathway induces massive expression of adhesion molecule (ICAM-1) in vascular endothelial cells, which leads to the migration and adhesion of white blood cells along the vascular wall, resulting in vascular endothelial injury. This stimulates massive expressions of IL-1, TNF-α, and IL-6 in vascular endothelial cells and mast cells, and also induces autophagy. Thus, ERK1/2, P38 and JnK are affected through inflammatory mediators, and NF-κB is activated again, eventually forming a vicious cycle [21].

In this study, the levels of ALT and AST in high-, medium- and low-dose monanthine groups were down-regulated, relative to ALF mice, which suggests that the use of monanthine may

regulate blood levels of transaminases and reduce blood cholesterol *in vivo*. The expressions of proteins related to the TLR4/MAPK/NF-KB pathway in high-, medium- and low-dose monanthine groups were markedly reduced, relative to ALF model mice, indicating that monanthine suppressed the TLR4/MAPK/NF-KB pathway, reduced inflammatory response, and mitigated inflammatory damage *in vivo*. Inflammatory factors were lower in the 3 monanthine groups than in model mice. These results indicate that monanthine reduced the levels of inflammation, alleviated vascular injury caused by inflammatory factors, and inhibited the necrosis of liver cells.

The expression levels of LC3-Ⅱ, ATG5, ATG7 and BEClin-1 were more up-regulated in the 3 monanthine-treated mice than in control mice. Thus, monanthine activated autophagy and improved the survival of mice with ALF. The number of adherent cells was lower in the three monanthine groups than in ALF mice. These results showed that monanthine decreased adhesion of inflammation cells and the secretion of inflammatory factors.

CONCLUSION

Monanthine reduces blood transaminase activities, and up-regulates the expressions of autophagy-related proteins by activating TLR4/MAPK/NF-KB signaling pathway, thereby reducing inflammation and adhesion of inflammatory cells *in vivo.* Thus, buddleoside can potentially be developed for the management of acute liver failure.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jianfeng Cao designed the study, supervised the data collection, and analyzed the data. Jianfeng Cao interpreted the data and prepared the manuscript for publication. Jianfeng Cao, Chao Wu, Weiwei Ye, Zaiqin Yang and Zili Chang supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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