The protective effects of myricetin against acute liver failure via inhibiting inflammation and regulating oxidative stress via Nrf2 signaling

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Abstract

This study aimed to investigate the protective effects and mechanisms of myricetin on acute liver failure in mice induced by lipopolysaccharide (LPS)/D-galactosamine (D-Gal). Our results showed myricetin (25, 50 and 100 mg/kg) pretreatment significantly improved the pathological changes of liver tissues, decreased serum ALT and AST ($p < 0.001$) induced by LPS/D-GalN. Moreover, MDA and MPO levels were reduced ($p < 0.001$), CAT and SOD activities were increased ($p < 0.001$) with myricetin (50 and 100 mg/kg) pretreatment. Likewise, inflammatory cytokines TNF-α and IL-6 mRNA in liver tissues were markedly decreased ($p < 0.001$) by myricetin. Besides, Nrf2 protein expression was drastically elevated ($p < 0.001$) by myricetin (25, 50 and 100 mg/kg). All these findings imply that myricetin may protect against acute liver failure by suppressing inflammation and regulating oxidative stress via Nrf2 signaling, and that it may be a possible strategy to avoid liver damage.

Keywords: acute liver failure; myricetin; inflammation; oxidative stress; signaling

Experimental

Chemicals and Reagents

LPS (*Escherichia coli* 055: B5), and D-GalN were purchased from sigma Chemical Co (St. Louis, MO). Myricetin (purity: 98%) was provided by Shanghai Aladdin Biochemical Technology Company Limited. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), superoxide dismutase (SOD), myeloperoxidase (MPO), and catalase (CAT) commercial reagent kits were purchased from Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). Bicinchoninic acid (BCA) protein assay reagent kit was purchased from Thermo Fisher scientific. All other chemicals were of reagent grade.

Animals and Experimental design

Male BALB/c mice (6-8 week; weight 18-22 g) were bought from Guangdong medical laboratory animal center, and were housed in controlled environment (25±1°C) with free access to food and water for one week to adapt the environment before experiment. All animal studies were approved by the Institution Animal Care and Use Committee of the Foshan University, and were performed according to the Manual of Care and Use of Laboratory Animals published by the National Institutes of Health (Permit NO. SCXK <Yue> 2018-0002).

Forty mice were randomly allocated into five groups, and each group contained eight mice: control group, LPS/D-GalN group, LPS/D-GalN + myricetin (20, 50, and 100 mg/kg) groups. Mice were intraperitoneally injected with 0.2ml 0.9% saline as control group or 500 mg/kg body weight of D-GalN and 500 μg/kg body weight of LPS as LPS/D-GalN group. For LPS/D-GalN + myricetin groups, myricetin were gave intraperitoneally once daily for three days, and 1 hour later of myricetin's last injection LPS/D-GalN was injected. The concentrations of myricetin were selected based on some published articles (Sun Y et al. 2017, Okan A et al. 2015).

Histopathological analysis

Liver tissues were collected 6 h after LPS/D-GalN injection, and were fixed in 4% paraformaldehyde. Tissues were embedded in paraffin and sectioned at 4-μM thickness. After dehydrated with graded ethanol, the sections were stained with hematoxylin and eosin staining (H&E). Finally, the histopathological changes were observed under a microscope (Echo, RVL-100, USA).

Determination of Serum ALT and AST

Blood was collected 6 h after LPS/D-GalN injection, and serum was separated by

centrifugation (4°C, 3000 rpm, 10 min). ALT and AST levels were determined according to the instructions of respective commercial kits. Briefly, serum was mixed and reacted with the matrix fluid followed by adding 2,4-dinitrophenylhydrazine to stop the above reaction. After 50 minutes in a 37°C water bathing, add sodium hydroxide solution and transfer to a 96-well plate. Finally, the plate was read using a microplate reader (Bio Rad, iMark, Japan) at a specific wave length of 510nm, and ALT or AST levels was calculated based on the OD values.

Determination of liver enzymes

Liver tissues were homogenized in physiological saline to prepare 10% liver homogenate, and then centrifugated (4°C，4000 rpm, 5 min), to collect the supernatant. The protein concentration in the liver was measured with BCA protein assay reagent kit. MDA content, MPO, SOD and GSH-Px activities were assayed using respective commercial assay kits (Jiancheng Bioengineering Institute of Nanjing) according to the instructions. First, we tested the content of MDA in high temperature (95°C) and acidic environment. MDA could undergo condensation reaction with thiobarbituric acid to form a red product with a maximum absorption peak at 532 nm. Secondly, we used colorimetric method to detect MPO activity. Because MPO could form a yellow product after a series of redox reactions, the activity of MPO could be calculated by detecting the amount of product. Finally, we tested SOD and GSH-PX activities. Under the action of the developer, hydroxylamine was oxidized to form nitrite, and it is purple-red, and SOD could inhibit the above process, so the absorbance value measured at a specific wavelength could calculate the activity of SOD. The activity of GSH-Px could be expressed by catalyzing the reaction rate of glutathione (GSH). The activity of GSH-Px was obtained by calculating the absorbance of the yellow product produced by the GSH reaction at 412nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from liver tissues using TRIzol (TransGen Biotech). Reverse transcription (RT) reaction was performed with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, San Jose, CA) in accordance with the manufacturer’s instructions. qPCR was carried out using CFX connect fluorescent quantitative PCR detection system. Primers were obtained from Sangon Biotech Co, Ltd (Shanghai, China), and were listed in Table S1.

Western blot analysis

The liver tissues were homogenized, lysed, and centrifuged (4°C，12000 rpm, 10 min). The protein concentration was determined by BCA protein assay reagent kit (Thermo Fisher Scientific). The samples containing the same number of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Merck Millipore). The membranes were blocked in 5% skim milk. After blocking, membranes were probed with specific primary antibody at 4°C overnight. Then, the membranes were incubated with secondary antibodies and washed with Tris buffered saline Tween for three times before and after. Finally, the membranes were visualized by the ECL Plus Western Blotting System (ProteinSimple, San Jose, CA, U.S. A.).

Statistical analysis

All data were expressed as mean ± standard error. Differences among different groups were calculated via one-way analysis of variance followed by Dunnett's test using GraphPad 5.0 software. A value of $p < 0.05$ was supposed to be significant.

Table S1. Primers used in this study

Gene	Primer	Sequence 5′>3′	Product size (bp)
TNF-α	Sense	ACGGCATGGATCTCAAAGAC	116
	Anti-sense	GTGGGTGAGGAGCACGTAGT	
IL-6	Sense	CCGGAGAGGAGACTTCACAG	134
	Anti-sense	CAGAATTGCCATTGCACAAC	

Figures and captions:

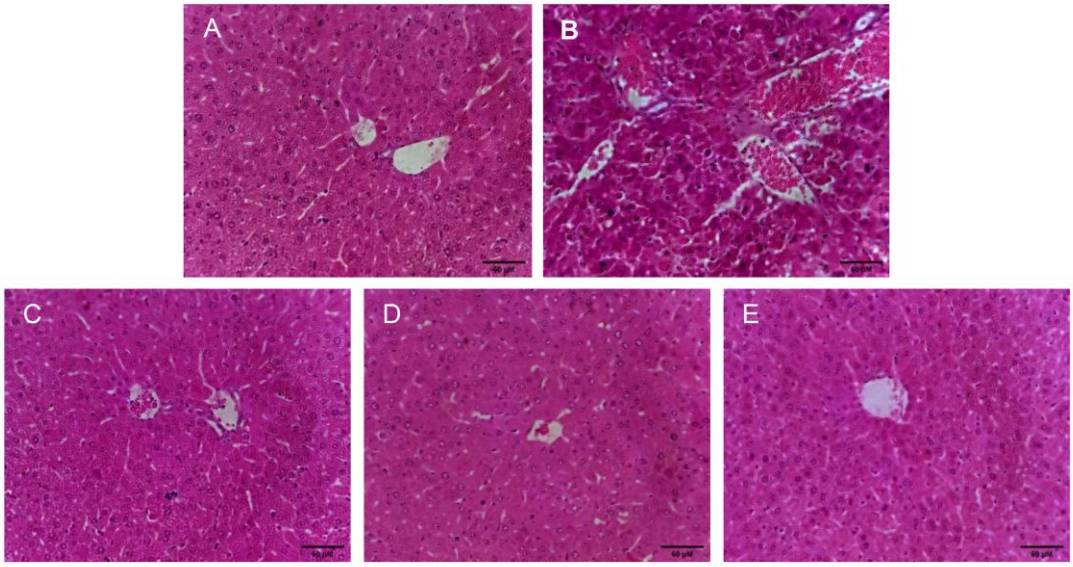

Figure S1 Effect of myricetin on liver tissues of mice with ALF induced by LPS/D-GalN. Liver tissues were collected at 6h after LPS/D-GalN injection, sectioned and stained with H&E. (A) Control; (B) LPS/D-GalN injection; (C) 25 mg/kg myricetin treatment before LPS/D-GalN injection; (D) 50 mg/kg myricetin treatment before LPS/D-GalN injection; (D) 100 mg/kg myricetin treatment before LPS/D-GalN injection. LPS/D-GalN injection obviously damaged the structure of liver tissues, leading to severe hemorrhagic necrosis and a mass of infiltration of inflammatory cells in portal area, while myricetin pretreatment decreasd these histopathological changes. Scale bar = 60 μm.

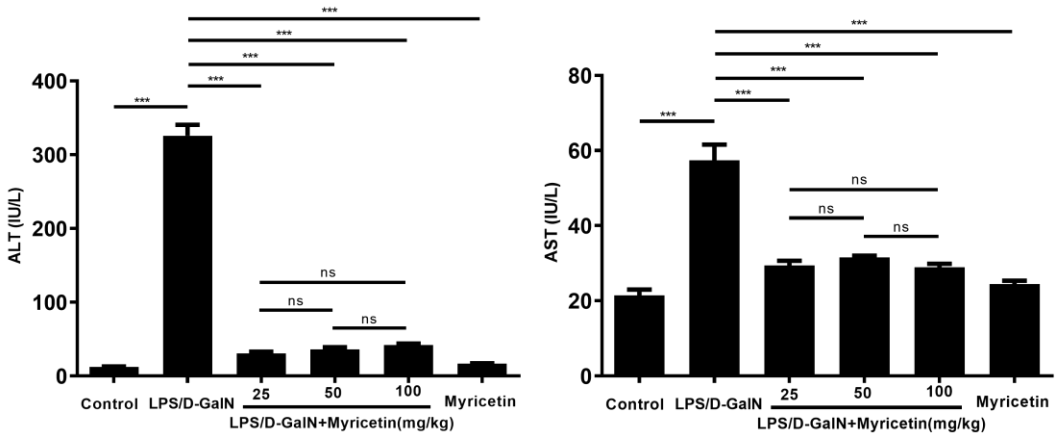

Figure S2 Effect of myricetin on serum ALT and AST in mice with ALF induced by LPS/D-GalN. Blood was collected 6 h after LPS/D-GalN injection, and serum was separated by centrifugation. ALT and AST levels were determined according to the

instructions of respective commercial kits. Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparisons test. Significant difference was defined by a p-value < 0.05 (*** p < 0.001).

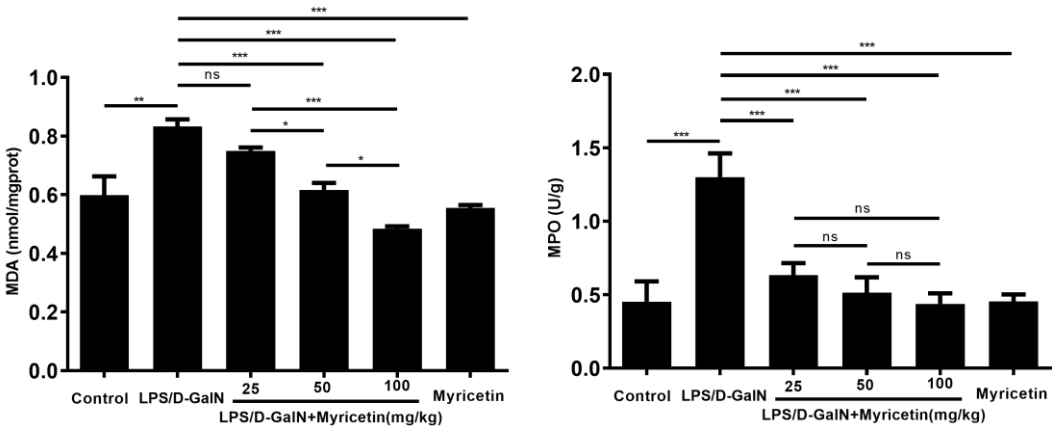

Figure S3 Effects of myricetin on MDA and MPO levels in liver tissues of mice with ALF induced by LPS/D-GalN. Liver tissues were collected at 6h after LPS/D-GalN injection, MDA and MPO levels in liver were measured using relative kits according to the instructions. Data are expressed as mean ± SEM (ns means no significance; **p < 0.01; and ***p < 0.001).

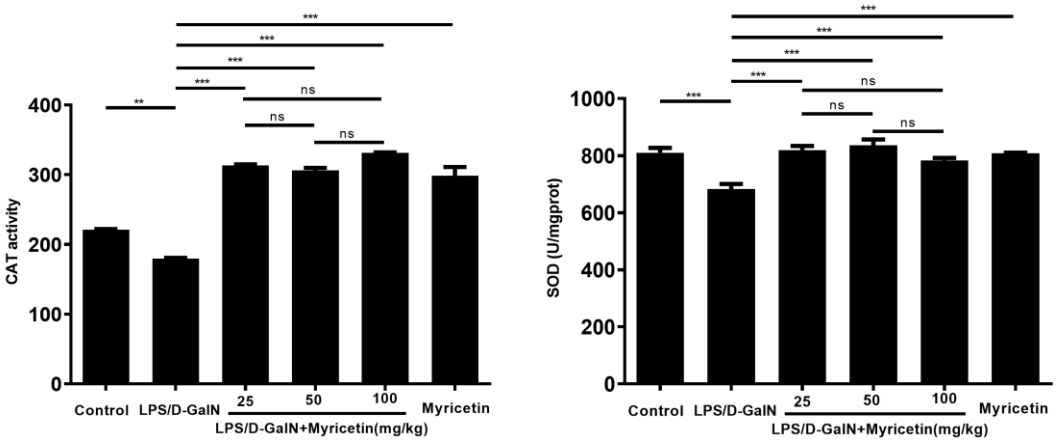

Figure S4 Effects of myricetin on CAT and SOD activities in liver tissues of mice with ALF induced by LPS/D-GalN. Liver tissues were collected at 6 h after LPS/D-GalN injection, CAT and SOD activities in liver were measured using the corresponding kits according to the instructions. Data are expressed as mean ± SEM (**p < 0.01; and ***p < 0.001).

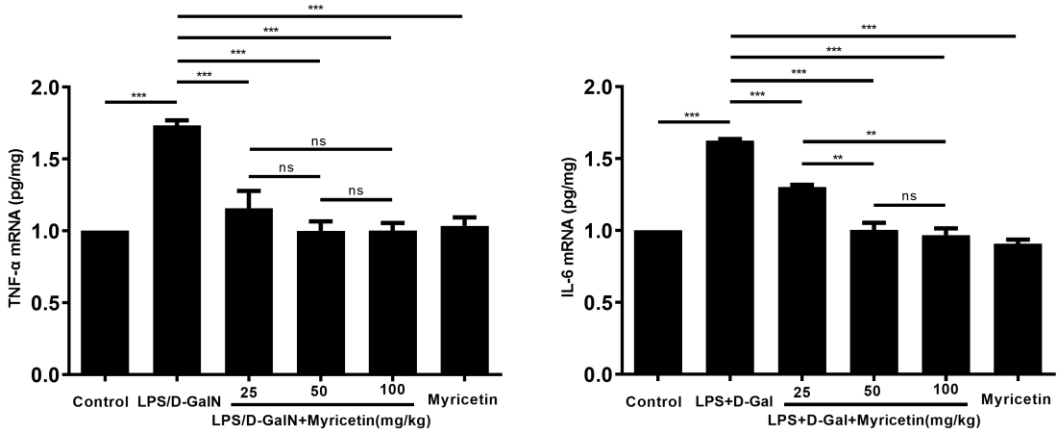

Figure S5 Effects of myricetin on the mRNA levels of TNF-α and IL-6 in liver tissues of mice with ALF induced by LPS/D-GalN. Liver tissues were collected at 6h after LPS/D-GalN injection, and total RNA was extracted using TriZol reagent. TNF-α and IL-6 mRNA levels were determined by real-time polymerase chain reaction. Data are expressed as mean ± SEM (***p < 0.001).

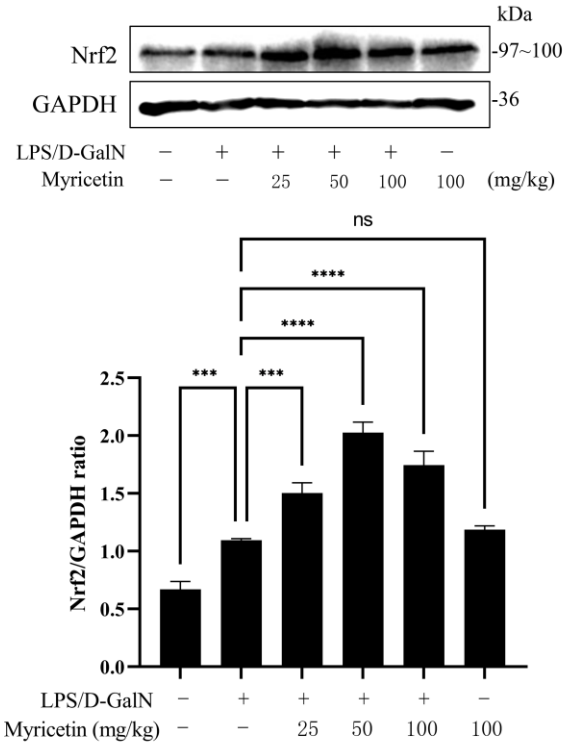

Figure S6 Effect of myricetin on Nrf2 protein expression in liver tissues of mice with ALF induced by LPS/D-GalN. Liver tissues were collected from mice 6 h after LPS/D-Gal injection. Nrf2 protein expression were analyzed by western blotting. Data are expressed as mean ± SEM (***p < 0.001; “ns” means not significant).