Myricetin protects against lipopolysaccharide−induced disseminated intravascular coagulation by anti−inflammatory and anticoagulation effect

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ABSTRACT

Objective: To explore the therapeutic effect and mechanism of myricetin on disseminated intravascular coagulation (DIC). **Methods:** The DIC model was established by injection of 60 mg/kg LPS in KM mice, and the treatment groups were injected myricetin with different concentrations (25 or 50 mg/kg) 30 min before the model was established. Both coagulation indicators and organ function were tested, including PT, APTT, fibrinogen, AST, ALT, BUN and tissue section. *In vitro*, the inflammatory model of RAW 264.7 macrophage cells were established by 10 μg/mL LPS. The treatment group was treated with 50 μmol/mL myricetin for 30 min before LPS, and the expression of TNF-α and p-NF-κB was detected, further to explore the therapeutic mechanism. **Results:** LPS-induced DIC led to a reduction of fibrinogen and a rise of PT, APTT, AST, ALT, BUN levels, but the treatment of myricetin significantly inhibited these abnormalities. Histopathology analysis also revealed that myricetin remarkably protected the liver and renal damage. *In vitro*, the expression of TNF-α and p-NF-κB induced by LPS was repressed by myricetin. **Conclusions:** This study provides new insights into the protective effects of myricetin in LPS-induced DIC by anticoagulant and anti-inflammatory via suppressing the activation of p-NF-κB which decreased TNF-α level.

1. Introduction

Disseminated intravascular coagulation (DIC) is a comprehensive pathological syndrome, characterized by activation of the systemic coagulation system, disorders of the fibrinolytic system, fibrin deposition, multiple organ thrombosis, and the severe inflammatory reaction[1,2]. DIC may secondary lead to various serious diseases such as bleeding, shock, multiple organ dysfunction syndrome (MODS) and even death[3,4]. Bacterial infection, cancer, trauma and severe liver disease are causes of DIC, which is a critical disease with high mortality and poor prognosis. When infected with Gram negative bacteria, endotoxin including LPS can activate RAW 264.7 macrophage cells to secrete inflammatory factors, such as TNF-α, causing cascade inflammatory reaction, eventually leading to the occurrence of DIC[5,6]. TNF-α is one of the most important early releasing cytokines in inflammatory reaction, and it was reported that the accumulation of TNF-α would lead to the secondary inflammatory cascades, which is regulated by NF-κB pathways[7,8]. The main clinical treatment for DIC is anticoagulant drugs, the most important of which is heparin. However, there is little evidence that heparin reverses organ dysfunction associated with DIC[9].

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Chinese medicine treatment of thrombosis, inflammation and other diseases has made great contributions. It has been found that several compounds extracted from traditional Chinese medicine have anti-inflammatory and anticoagulation effects, such as Salvianolic B (Sal B) and quercetin[10,11]. Myricetin is an important Chinese traditional medicine which exists in many natural plants such as myricaceae[12]. It has been used to treat inflammatory diseases and hypotensive with a variety of pharmacological functions such as anti-inflammatory, analgesic, anti-tumor activities and protecting liver, showing a high potential value for therapeutic application. In addition, recent researches indicated that myricetin can significantly depress the level of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) by down regulating the activation of the Akt, mTOR and NF-κB pathways, alleviating inflammation reaction to offer protective effect on various organs[13,14]. Together, we assumed that myricetin may be a potential drug for the treatment of DIC.

Although many pharmacodynamics of myricetin have been discovered, there is little research about the effect on DIC. In this experiment, we established a LPS-induced DIC mouse model to explore the pharmacological effects of myricetin on DIC, including activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen, BUN (the marker of renal injury), AST, ALT (the marker of liver injury) and so on. In this study, we established an *in vitro* inflammatory model of RAW 264.7 macrophage cells using LPS to assess the direct anti-inflammatory effect and mechanism of myricetin.

2. Materials and methods

2.1. Animals

KM mice (Female, weighing 20-25 g) were purchased from Medical Experimental Animal Center of Guangdong Province, China. All procedures utilized in this animal experiment were in accordance with institutional guidelines for animal research. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study also conformed to the Guide for Using and Caring Laboratory Animals.

2.2. Materials

Myricetin (purity >97%) was purchased from Macklin Biochemical Company (shanghai, China). LPS was from Sigma (St Louis, USA). Fatal bovine serum (FBS) and dulbeccos modified Eagles medium (DMEM) were purchased from BD Bioscience (Franklin Lakes, N.J). Heparin was purchased from Beijing Tobishi Pharmaceutical Company. All other reagents were supplied by commercial sources.

2.3. Animal models establishment and drug treatments

All procedures were conducted in accordance with the ethical guidelines of National Guide for the Care and Use of Laboratory Animals. Mice were randomly divided into five groups: two myricetin established groups, LPS group, heparin group and normal control group, with 30 mice in each group. The LPS-induced disseminated intravascular coagulation models were established by

intraperitoneally injecting (*i.p.*) 60 mg/kg purified LPS (in saline solution) in KM mice.

Before DIC induction in the LPS group, the mice were injected with saline solution. Heparin group was infused with 500 IU/kg heparin. Two myricetin established groups are as follows: High-dose group: 50 mg/kg myricetin and low-dose group: with 25 mg/kg myricetin, respectively, 30 min before LPS infusion. Simultaneously for the control group, mice were injected with saline. After that, to maintain therapeutic effects of myricetin, the same dosage was intraperitoneally injected for every 2 h.

2.4. Blood sample collection and handling

Ten mice in each group were sacrificed at 0, 2, and 8 h after LPS injection, and blood samples were collected from the abdominal aorta. The blood samples of five mice were collected in sodium citrate (1:9 v/v blood/citrate) to detect PT, APTT and fibrinogen. Serum was collected from other five mice and measured AST, ALT and BUN by the automatic analyzer (ABBOTT ARCHITECT c16000, America).

2.5. Histopathology examination of liver and renal

To determine the extent of liver and renal tissue injury in LPS-induced DIC and the protective effects of myricetin, histopathological studies were performed. After the blood sample collection, the chest cavities of mice were opened, and the livers and nephridia were excised. Livers and nephridia were fixed in 4% paraformaldehyde overnight, dehydrated with 70%, 75%, 85%, 90%, 95% and 100% alcohol, embedded in paraffin and then sliced into 5 mm thick sections. After deparaffinization with xylene and different concentration gradient of alcohol, slices were stained by hematoxylin and eosin (H&E) and phosphotungstic acid hematoxylin (PTAH). Pathological changes of the liver and renal tissue were observed using an inverted microscope.

2.6. Cell culture

The RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated FBS, and were maintained at 37 ℃ in 5% CO_2 incubators.

2.7. Cell viability assay and cytotoxicity assay

The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma–Aldrich, St. Louis, MO) assay. Briefly, RAW 264.7 macrophage cells were seeded in 96-well plates at the density of 50 000 cells/well. The control group and the three myricetin treatment groups (low, medium, high dose) were set and reagents were added accordingly: cultured medium, 0, 25, 50, 100 μmol/mL myricetin. A total of 10 mL MTT solution (5 mg/mL in PBS) was added to each well after culturing for 6 h and incubating for 4 h to induce the production of formazan crystals. After that 100 mL DMSO was added to achieve solubilization of the formazan crystal, and the optical density (OD) being determined at 490 nm using an EXL 800 micro immunoanalyzer (Bio-Tek Instruments, Burlington, VT).

2.8. Western blot analysis

RAW 264.7 macrophage cells were treated with 50 µmol/mL myricetin for 30 min and then stimulated with LPS (10 µg/mL) for 6 h. The cells were washed three times with ice-cold phosphate buffered saline (PBS), subsequently the lysis buffer [50 mmol/L Tris–Cl (pH 8.0), 150 mmol/L NaCl, 0.02% NaN_3, 1% NP40, 10 µg/mL PMSF] was added and incubated for 30 min at 4 ℃. Then the cell lysates were harvested and centrifuged at 12 000× g for 15 min to remove debris. The protein concentration was determined using BCA assay. Equal amounts of total proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Following blocking of non-specific binding sites with 5% bovine serum albumin (BSA) in TBST (TBS-Tween-20) for 1 h, the membranes were incubated overnight at 4 ℃ with appropriate dilutions of primary antibodies for detecting p-NF-κB (p-P65), TNF-α and β-actin which diluted 1/1 000 in 5% BSA with TBST. Then they were washed in TBST for three times and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally, they were exposed for curtain time to show the immunoreactive bands with a computer imaging system (Gene Co., Ltd., Hong Kong, China). Negligible loading/transfer variation was noted between samples.

2.9. Statistical analysis

All data were present as mean± standard deviation. Differences between two groups were analyzed by non-para-metric test (the Kruskal–Wallis H test). All experiments were repeated at least three times, and the data were presented as the mean± SD unless otherwise noted. A P value of 0.05 was considered to be statistically significant. Statistical analyses were conducted using SPSS.

3. Results

3.1. Protective effect of myricetin on LPS–induced DIC

To evaluate the protective effects of myricetin on DIC, we collected the blood samples in different groups at 0, 2 and 8 h after LPS treatment. In order to more intuitively visualize the differences of the indicators at 2 h and 8 h, they were presented as compared to the levels of 0 h for each group. Results showed that myricetin markedly

decreased the levels of BUN, ALT and AST ($P<0.05$). These findings indicated that myricetin was able to ameliorate liver and renal injury induced by LPS. Additionally, APTT, PT also were depressed, and fibrinogen was increased (Table 1).

3.2. Myricetin improved liver and renal function in LPS–induced disseminated intravascular coagulation

To investigate the protective effects of myricetin, the histopathological analysis of liver and renal was performed at 8 h after LPS-induced DIC KM mice injection with myricetin. In H&E stain, in the control group, normal liver histology showed that hepatic cord arrangment was organized. However, after LPS injection, liver histology showed a number of vacuolation of hepatocyte and severe thrombus. Moreover, extensive fibrin microthrombi formation appeared in hepatic lobules. Nevertheless, under treatment of myricetin, only a little cell injury situation and fibrin microthrombi formation were seen in liver compared with the LPS induced group. Myricetin treatment groups notably had a therapeutic effect on liver damage.

As for renal histopathological analysis, the result was in accordance with the liver histopathological analysis results. Normal renal H&E stain showed clear structures of glomeruli, but in the LPS group, severe thrombus were seen, and the glomerular structure was badly damaged, even degraded. PTAH stain revealed extensive thrombosis in the LPS-induced DIC rat model. On the contrary, after treatment of myricetin, the staining results revealed that glomerular structure was clear, and the thrombus were improved. Overall, myricetin treatment showed protective effects on LPS-induced disseminated intravascular coagulation.

3.3. Effect of myricetin on RAW 264.7 macrophage cells viability and cytotoxicity

In order to evaluate the effect of myricetin on cell viability and cytotoxicity, we tested the effects of myricetin on the viability of RAW 264.7 macrophage cells using MTT assay. The cell proliferation rate= (the absorbance of myricetin treatment groups/ the control group) × 100%. The results showed that the cell proliferation rate of 25, 50, and 100 µmol/L myricetin treatment groups were (88.1±3.98)%, (91.19±2.56)% and (84.48±2.34)% respectively at 6 h. It could be seen that myricetin does not have cytotoxicity to RAW 264.7 macrophage cells.

Table 1

Effect of myricetin on coagulation indicators and function indicators in plasma of LPS-induced DIC mice.

Group	BUN		ALT		AST		APTT		PT		Fibrinogen	
	2 h	8 h	2 h	8 h	2 h	8 h	2 h	8 h	2 h	8 h	2 h	8 h
Control	105.84± 6.44	102.54± 4.71	99.45± 1.74	100.90± 0.92	98.70± 1.57	101.16± 2.04	99.10± 4.36	104.28± 5.27	98.02± 3.28	102.56± 2.66	97.25± 2.70	101.75± 3.74
LPS	166.34±16.01[#]	238.46±35.94[#]	188.01±21.74[#]	201.63±22.85[#]	149.43±5.71[#]	198.58±7.35[#]	233.14±25.96[#]	453.66±15.43[#]	148.05±5.76[#]	127.92±1.95[#]	53.01±4.65[#]	34.96± 8.13[#]
Heparin	114.75±7.63[*]	142.18±13.54[*]	106.08±10.98[*]	110.81±8.65[*]	105.62±4.74[*]	133.86±5.96[*]	114.86±7.57[*]	107.44±8.56[*]	105.73±4.46[*]	103.86±3.85[*]	80.73±6.15[*]	74.96±4.22[*]
Myr 25	76.94±8.35[*]	141.73±10.53[*]	110.24±13.76[*]	118.80±6.48[*]	137.56±4.18[*]	158.12±3.75[*]	203.49±11.42	253.15±6.44[*]	101.82±5.34[*]	114.85±3.43[*]	78.60±5.42[*]	70.34±7.54[*]
Myr 50	102.94±11.35[*]	118.49±9.64[*]	130.43±8.76[*]	141.74±9.48[*]	131.25±3.70[*]	137.87±7.48[*]	159.88±8.38[*]	196.61±12.35[*]	106.84±6.44[*]	104.56±5.76[*]	85.15±3.75[*]	81.70±3.18[*]

Data were calculated as: Values detected at 2 h and 8 h after LPS treatment 100% / Baseline data. [#]$P < 0.05$ compared with the control group; [*]$P < 0.05$ compared with the LPS group. Myr 25: 25 mg/kg myricetin; Myr 50: 50 mg/kg myricetin.

3.4. Inhibition of LPS−induced TNF−α in intro by myricetin

We hypothesized that myricetin suppressed TNF-α level in RAW264.7 macrophage cells and alleviated the inflammation reaction. To verify this hypothesis, inflammatory cell models were constructed using RAW264.7 macrophage cells treated with lipopolysaccharide and the expression of TNF-α in different groups were measured by Western blot. As shown in Figure 1A and Table 2, being induced by lipopolysaccharide, inflammatory factor TNF-α in RAW264.7 macrophage cells were overexpressed, while under myricetin administration, the secretion of TNF-α was significantly decreased indicating that myricetin could inhibit lipopolysaccharide-induced inflammatory response in RAW264.7 macrophage cells.
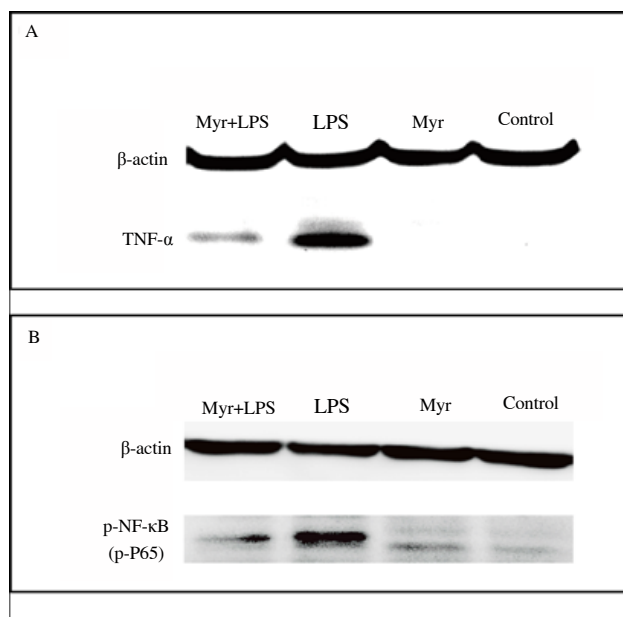
Figure 1. Western blot analysis.
(A) TNF-α expression analysis of different groups. (B) The expression of p-NF-κB (p-P65) of four different groups was analyzed. Control: control group. LPS: LPS-induced group. Myr+LPS: 50 μmol/L myricetin and 10 LPS. Myr: 50 μmol/L myricetin.

Table 2
Relative intensity analysis.

Group	TNF-α/β-actin	p-P65/β-actin
Myricetin+LPS	12.57± 0.17**	2.85±0.36**
LPS	102.34± 0.13##	13.15±0.87##
Myricetin	1.04± 0.13	1.42±0.10
Control	1	1

TNF-α level and p-NF-κB (p-P65) level were normalized to β-actin. ##$P <$ 0.01 compared with the control group; **$P < 0.01$ compared with the LPS group.

3.5. Myricetin inhibited TNF−α level by regulating p−NF− κB(p−P65) signaling

To further investigate whether the p-NF-κB(p-P65) signaling pathway was involved in myricetin inhibiting the TNF-α level,

inflammatory model cells were established with RAW264.7 macrophage cells treated with lipopolysaccharide. Compared to the lipopolysaccharide-induced group, the expression of p-NF-κB(p-P65) decreased with the treatment of myricetin indicating that p-NF-κB(p-P65) was relevant to myricetin depressing the TNF-α level (Figure 1B, Table 2).

4. Discussion

DIC is an acquired syndrome. Coagulation factors and platelets activated, thrombin increased, and extensive micro-thrombosis are common in DIC[1,15]. In addition, sepsis caused by Gram-negative bacteria, is considered a vital cause of DIC. As one of endotoxin, lipopolysaccharide, is the main component of Gram-negative bacterial cytoderm, which can directly result in tissue damage and produce many proinflammatory cytokines, such as TNF-α. These cytokines increase the expression of coagulation factors and tissue factors on vascular endothelial cells, which can directly result to the activation of coagulation factors and the release of tissue factor, leading to the activation of coagulation, the extensive formation of micro-thrombus and thus inducing the occurrence of DIC[16,17]. Therefore, researchers often establish the DIC animal model using lipopolysaccharide induction.

As we know, coagulation and anti-coagulation disturbance is the important characteristic of DIC, characterized by intravascular coagulation and the consumption of clotting factors. PT, APTT and fibrinogen are more sensitive, convenient and commonly used screening tests for coagulation systems[18,19]. Consequently, we selected APTT, PT and fibrinogen as indexes to evaluate the severity of microcirculation disorder. Additionally, with the excessive damage of fibrinolytic system and activation of coagulation, the formation and deposition of fibrin will be occurred, causing microvascular thrombosis in different organs, and prone to severe inflammatory reaction. It is believed that the mortality of DIC patients is obviously affected by severe inflammatory and organ failure, especially in the liver and kidney. Increases in plasma ALT, AST and BUN illustrate that microvascular thrombosis is involved in the impairment of renal and liver function[20,21]. Liver and renal were stained by hematoxylin and eosin (H&E) as well as phosphotungstic acid hematoxylin (PTAH), and AST, ALT and BUN were used to evaluate the severity of inflammatory and organ failure. In LPS-induced DIC mice, we found that treatment of myricetin improved the coagulation and fibrinolytic system, also controlled the damage of organ. In the coagulation system, myricetin treatment decreased APTT and PT. In the fibrinolytic system, myricetin treatment increased fibrinogen. In addition, the inflammatory reaction was inhibited. This benefit was further verified by a significant reduction in ALT, AST and BUN following myricetin treatment. These results could provide evidence of myricetin protection of the renal and liver against DIC. It is reasonable to believe that myricetin has a therapeutic effect on DIC.

In this study, we detected the toxicity of myricetin to macrophage RAW264.7 by MTT assay. There were no significant differences

between the various concentrations of myricetin treatment groups and the control group within 6 h which testified that myricetin is non-toxic. Subsequently, we constructed an inflammatory model by macrophage cells *in vitro*, followed by Western blot detection of TNF-α levels of expression. The results showed that myricetin reduced TNF-α in RAW264.7 macrophage cells (Figure 1A), suggesting that myricetin may protect the organ function and coagulation disorders by reducing the accumulation of inflammatory cytokines. Myricetin reduced TNF-α level by relating to NF-κB signaling mechanism. NF-κB is a vital pathway for the expression of inflammatory cytokines. Inhibition of NF-κB phosphorylation can inhibit the inflammatory cytokines, including the expression of TNF-α. The pathogenesis of DIC is very complex, involving pathways such as NF-κB, MAPK, ERK1/2 and so on. Specially, NF-κB signaling pathway plays an important role in inflammatory reaction. It mediates the expression of a variety of cytokines and inflammatory factors such as TNF-α[22]. An empirical application has been conducted to explore the Effects of NF-κB pathways on inflammatory model of RAW 264.7 macrophages induced by LPS. NF-κB reduces the inflammatory response by regulating TNF-α in RAW 264.7 macrophage cells, accordingly inhibiting development of DIC. Nevertheless, other pathways are still to be researched.

In conclusion, our study indicated that myricetin showed protective effect on LPS-induced DIC through in vivo and *in vitro*. This protective effect was achieved via decreasing TNF-α expression by inhibiting the activation of NF-κB signaling pathway. However, other potential mechanism of myricetin to treat DIC and the optimal dosage need more studies to prove.

Conflict of interest statement

We declare that we have no conflict of interest.

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