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Cimifugin Inhibits Inflammatory Responses of RAW264.7 Cells Induced by Lipopolysaccharide

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Background: RAW264.7 cells are induced by lipopolysaccharide (LPS) as a rheumatoid arthritis (RA) model. The present study investigated the effect of cimifugin on the proliferation, migration, chemotaxis, and release of inflammation-related factors and inflammation-related signaling pathways of LPS-induced RAW264.7 cells.

Material/Methods: MTS assay was used to determine the proliferation of RAW264.7 cells. Transwell assay was employed to examine the migration and chemotaxis of the cells. ELISA was performed to measure the contents of chemotactic factors and inflammatory factors in cell culture supernatants. Western blotting was carried out to detect the expression of factors related with MAPKs and NF- κ B signaling pathways.

Results: Cimifugin (0–100 mg/L) had no cytotoxicity for RAW264.7 cells. LPS stimulation induced morphological differentiation of RAW264.7 cells, but intervention by cimifugin inhibited the activation effect by LPS by about 50%. Cimifugin (100 mg/L) decreased the migration and chemotaxis of RAW264.7 cells to 1/3 of that in control cells by decreasing the release of migration- and chemotaxis-associated factors by at least 30%. Cimifugin (100 mg/L) suppressed the release of inflammatory factors from RAW264.7 cells to less than 60% of that in the LPS group. In addition, cimifugin (100 mg/L) inhibited the activities of MAPKs and NF- κ B signaling pathways.

Conclusions: The present study demonstrates that cimifugin reduces the migration and chemotaxis of RAW264.7 cells and inhibits the release of inflammatory factors and activation of related signaling pathways induced by LPS. Cimifugin may have potential pharmacological effects against RA.

MeSH Keywords: **Cytokines • Felty Syndrome • MAP Kinase Signaling System • NF-kappa B**

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Background

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized mainly by synovitis in middle-aged and elderly populations [1]. The features of RA include synovial hyperplasia and chronic erosive inflammation and autoimmune disorders, and the disease often affects the joints [2,3]. About 1% of the world's population is affected by RA [4], and the prevalence rate of the disease is about 0.32–0.36% in China [5].

Anti-inflammatory therapy has become an important direction in the basic and clinical treatments for RA [6]. The pathogenesis of RA is closely related to 2 important signaling cascades that are involved in inflammatory response: the mitogen-activated protein kinase (MAPK) signal transduction pathway [7,8] and the nuclear factor-kappa B (NF- κ B) signal transduction pathway [9]. The decrease of MAPK and NF- κ B pathway activity can alleviate inflammatory responses, and new therapies and drugs have been developed against these signaling pathways [10,11].

Saposhnikoviae Radix, the dry roots of the plant *Saposhnikovia divaricata* (Turcz) schischk prepared at its vegetative growth period, are widely used in East Asia for antipyretic, analgesic, and anti-inflammatory purposes [12,13]. Chromones are among the main active components of Saposhnikoviae Radix [12]. Prim-o-glucosylcimifugin content is relatively high among all chromones [14], and it is demonstrated to have anti-inflammatory and analgesic effects [15]. Prim-o-glucosylcimifugin can be easily converted into its aglycone, cimifugin, *in vivo* [16,17]. Cimifugin suppresses allergic inflammation via regulating tight junctions [18]; however, it is still unclear whether cimifugin is an active component that exerts anti-inflammatory effects *in vivo*. In addition, it is not reported whether cimifugin affects the inflammatory factors and signaling pathways that are related with RA. In the present study we investigated the effect of cimifugin on the proliferation, migration, chemotaxis, release of inflammation-related factors, and inflammation-related signaling pathways of RAW264.7 cells using lipopolysaccharide (LPS) as the inducing reagent. In addition, the present study aimed to provide a theoretical basis for the prevention and treatment of RA.

Material and Methods

Cells

RAW264.7 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM high-glucose medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. For detection,

the medium was concentrated by centrifugation at 1000×g and 4°C for 10 min.

MTS assay

RAW264.7 cells (2×10⁵/well) were seeded onto 96-well plates in triplicate. After being cultured in the presence of 100 mg/L, 50 mg/L, 25 mg/L, 12.5 mg/L, 6.25 mg/L, or 0 mg/L cimifugin (A1271; Bellancom Chemistry, Beijing, China) for 72 h, the cells were subjected to viability test using the CellTiter 9 Aqueous One Solution Cell Proliferation Assay kit (CTB169; Promega, Fitchburg, WI, USA) following the manufacturer's manual. Each test was performed in triplicate.

Determination of nitric oxide (NO) content

The content of NO was determined by use of an NO detection kit (S0021, Beyotime, Shanghai, China) according to the manufacturer's manual and following a previously published method [19]. Each test was performed in triplicate.

Transwell assay

The QCM Laminin Migration Assay kit (ECM220, 5.0 μ m; Merck Millipore, Merck KGaA, Darmstadt, Germany) was used for Transwell assay. After being treated with 100 mg/L (high), 50 mg/L (medium) and 25 mg/L (low) cimifugin for 48 h, RAW264.7 cells were seeded onto the upper Transwell chamber at a density of 2×10⁵/well in 24-well plates. Because cell migration could be driven by serum, it was generally necessary to culture the cells without sera for a period of time to synchronize the cells. Therefore, the cells were cultured in serum-free DMEM high-glucose medium for 12 h to eliminate the influence of serum. To test migration ability, 500 μ L DMEM high-glucose medium supplemented with 10% fetal bovine serum was added into the lower Transwell chamber. To study chemotaxis, 500 μ L serum-free DMEM high-glucose medium containing 1 μ g/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) was added into the lower Transwell chamber. Then, the culture plates were incubated at 37°C and 5% CO₂ for 24 h. Afterwards, the medium in the chamber was discarded, and the chamber was washed with phosphate-buffered saline twice to eliminate remaining medium. The chamber was fixed with methanol for 30 min, and the cells in the upper chamber were wiped off. After the chamber was air-dried, it was stained for 15 min in 0.1% crystal violet solution, which was subsequently washed away. Following air-drying, the chamber was observed under a microscope. Five fields were randomly chosen for each chamber, and images were taken at a magnification of 200×. The number of cells that crossed the membrane was counted and used for the evaluation of migration and chemotaxis of cells. Each test was performed in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 cells were divided into a control group (no LPS was added), LPS group (treated with 1 µg/mL LPS for 24 h), high-dose group (1 µg/mL LPS+100 mg/L cimifugin for 24 h), medium-dose group (1 µg/mL LPS+50 mg/L cimifugin for 24 h), and low-dose group (1 µg/mL LPS+25 mg/L cimifugin for 24 h). Cell culture supernatants (100 µL/well) were added into ELISA plates for the determination of the concentrations of IL-6 (EMC004.96; Neobioscience Technology Company, Shenzhen, China), TNF-α (EMC102a.96; Neobioscience Technology Company, Shenzhen, China), IL-1β (EMC001b.96; Neobioscience Technology Company, Shenzhen, China), MIP-2 (EMC122.96; Neobioscience Technology Company, Shenzhen, China), MCP-1 (EMC113.96; Neobioscience Technology Company, Shenzhen, China), and IL-8 (EMC104.96; Neobioscience Technology Company, Shenzhen, China) following the respective manuals. Each sample was tested in triplicate.

Western blotting

RAW264.7 cells were divided into a control group (no LPS was added), LPS group (treated with 1 µg/mL LPS for 24 h), high-dose group (1 µg/mL LPS+100 mg/L cimifugin for 24 h), medium-dose group (1 µg/mL LPS+50 mg/L cimifugin for 24 h), and low-dose group (1 µg/mL LPS+25 mg/L cimifugin for 24 h). Total proteins were extracted using the E.Z.N.A Total DNA/RNA/Protein kit (Omega Bio-Tek Inc., Norcross, GA, USA) following the manufacturer's manual. The protein concentration was determined by using the bicinchoninic acid (BCA) protein concentration determination kit (P0010; Beyotime Biotechnology, Shanghai, China). Protein samples were then mixed with 5× sodium dodecyl sulfate loading buffer before denaturation in a boiling water bath for 5 min. Afterwards, the samples (20 µg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (100 V, 2 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-human P65 polyclonal primary antibody (ZS-372; ZSGB-BIO, Beijing, China), rabbit anti-human P50 polyclonal primary antibody (ZS-1140; ZSGB-BIO, Beijing, China), mouse anti-human p-IκB-α monoclonal antibody (ZS-8404; ZSGB-BIO, Beijing, China), rabbit anti-human P-P65 polyclonal primary antibody (sc-33020; Santa Cruz, Dallas, TX, USA), rabbit anti-human P-P50 polyclonal primary antibody (sc-33022; Santa Cruz, Dallas, TX, USA), rabbit anti-human IκB-α polyclonal primary antibody (sc-371; ZSGB-BIO, Beijing, China), rabbit anti-human ERK1/2 polyclonal primary antibody (ab115799; Abcam, Cambridge, UK), rabbit anti-human P-ERK1/2 monoclonal primary antibody (#4370; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-human P38 monoclonal primary antibody (#9212; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-human P-P38 (#4511; Cell Signaling Technology,

Danvers, MA, USA), and mouse anti-human GAPDH monoclonal antibody (TA-08; ZSGB-BIO, Beijing, China) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 5 times for 5 min each time, the membranes were incubated with goat anti-rabbit IgG secondary antibody (ZDR-5306; ZSGB-BIO, Beijing, China) or goat anti-mouse IgG (ZDR-5307; ZSGB-BIO, Beijing, China) for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 for 5 times for 5 min each time. Then, the membrane was developed using an enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative expression of target proteins was calculated against GAPDH. Each test was performed in triplicate.

Statistical analysis

The results were analyzed using SPSS 18.0 statistical software (IBM, Armonk, NY, USA). The data are expressed as means ±SEM. Data were tested for normality. In case of homogeneity of variance, multigroup measurement data were analyzed using one-way ANOVA and Dunnett test. In case of heterogeneity of variance, multigroup measurement data were analyzed using Kruskal-Wallis test followed by Tamhane's T2 or Dunnett's T3 method. The 2 groups of data were examined by Mann-Whitney method. $P<0.05$ indicated statistically significant differences.

Results

Cimifugin (0–100 mg/L) has no cytotoxicity for RAW264.7 cells.

To test the effect of cimifugin on the viability of RAW264.7 cells, MTS assay was performed. The data showed that the viability of RAW264.7 cells treated with 100 mg/L, 50 mg/L, 25 mg/L, 12.5 mg/L, 6.25 mg/L, or 0 mg/L cimifugin for 72 h were not different from each other ($P>0.05$) (Figure 1). The results suggest that cimifugin (0–100 mg/L) has no cytotoxicity for RAW264.7 cells.

LPS stimulation induces morphological differentiation of RAW264.7 cells, but intervention by cimifugin inhibits the activation effect by LPS

To examine the effect of cimifugin on RAW264.7 cells stimulated by LPS, the cells were observed under a microscope and NO content in cell culture supernatant was determined. Microscopy showed that some of the cells gradually became spindle-shaped after treatment with LPS, while treatment with cimifugin inhibited the morphological differentiation of the cells (Figure 2A). Detection of NO content in supernatant

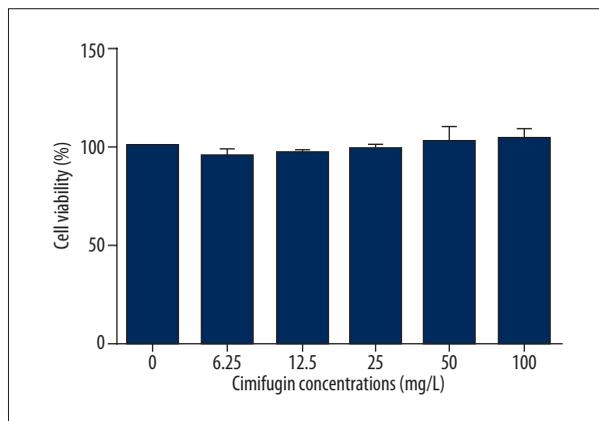


Figure 1. Effect of cimifugin on the viability of RAW264.7 cells. MTS assay was performed to determine cell viability after treatment with 100 mg/L, 50 mg/L, 25 mg/L, 12.5 mg/L, 6.25 mg/L, or 0 mg/L cimifugin for 72 h.

of cell culture showed that LPS stimulation significantly up-regulated the release of NO to a level 4 times that in the control group ($P<0.05$). By contrast, treatment with 100 mg/L or 50 mg/L cimifugin reduced the release of NO to 40–50% that in the LPS group ($P<0.05$) (Figure 2B). The results indicate that LPS stimulation induces morphological differentiation of

RAW264.7 cells, but intervention by cimifugin inhibits the activation effect by LPS.

Cimifugin decreases the migration and chemotaxis of RAW264.7 cells by inhibiting the release of migration- and chemotaxis-associated factors

To study how cimifugin affects the migration and chemotaxis of RAW264.7 cells induced by fetal bovine serum or LPS, Transwell assay was carried out. The data showed that cells induced by serum crossed the membrane successfully, but 100 mg/L and 50 mg/L cimifugin significantly reduced the number of cells that crossed the membrane to 33% and 50% of that in the control group, respectively ($P<0.05$) (Figure 3A). Similarly, cells induced by LPS crossed the membrane successfully, but 100 mg/L cimifugin significantly reduced the number of cells that crossed the membrane to 28% of that in the control group ($P<0.05$) (Figure 3B). To determine the concentrations of migration- and chemotaxis-associated factors in cell supernatants, ELISA was employed. The data showed that the concentrations of migration- and chemotaxis-associated cytokines, MIP-2, IL-8 and MCP-1, were at least doubled after LPS stimulation ($P<0.05$). In contrast, treatment with 100 mg/L and 50 mg/L cimifugin reduced their concentrations in cell supernatants to 50–70%

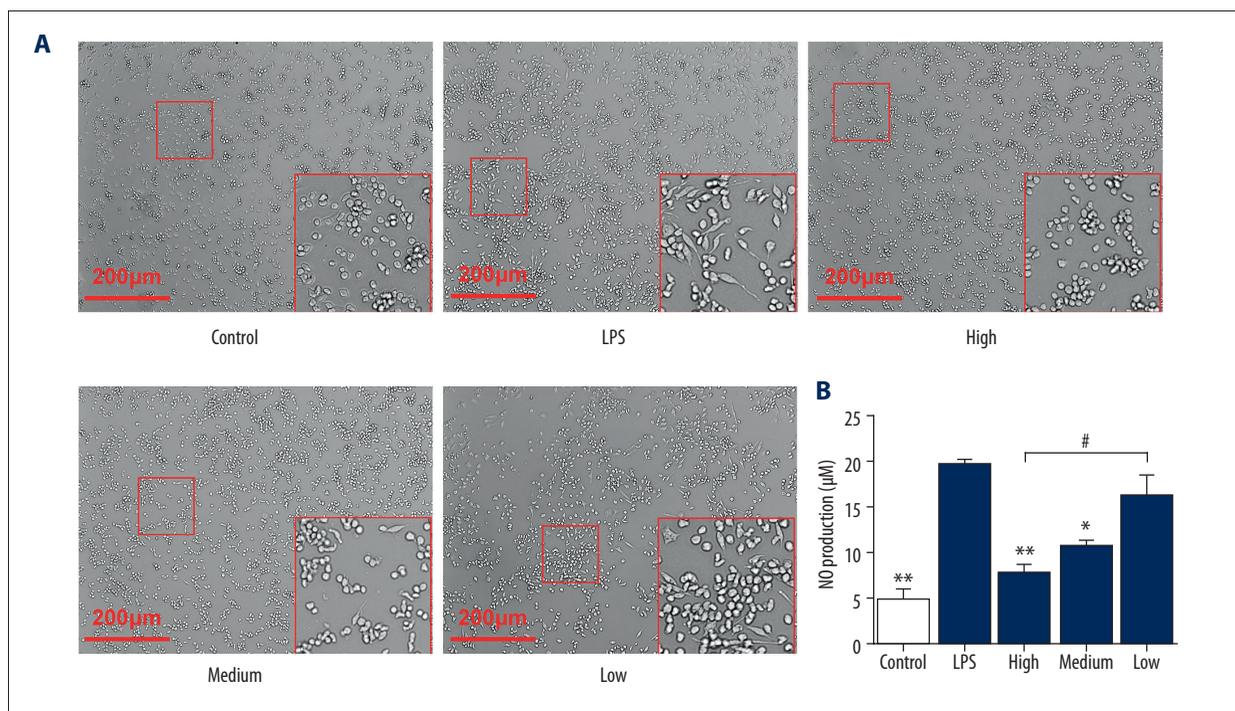


Figure 2. Effect of cimifugin on (A) morphology and (B) NO secretion of RAW264.7 cells. RAW264.7 cells were divided into a control group (no LPS was added), LPS group (treated with 1 µg/mL LPS for 24 h), high-dose group (1 µg/mL LPS+100 mg/L cimifugin for 24 h), medium-dose group (1 µg/mL LPS+50 mg/L cimifugin for 24 h), and low-dose group (1 µg/mL LPS+25 mg/L cimifugin for 24 h). Microscopy was used to observe cell morphology. NO content in cell supernatant was determined as described in Material and Methods. * $P<0.05$ and ** $P<0.01$ compared with LPS group. # $P<0.05$ compared among indicated groups.

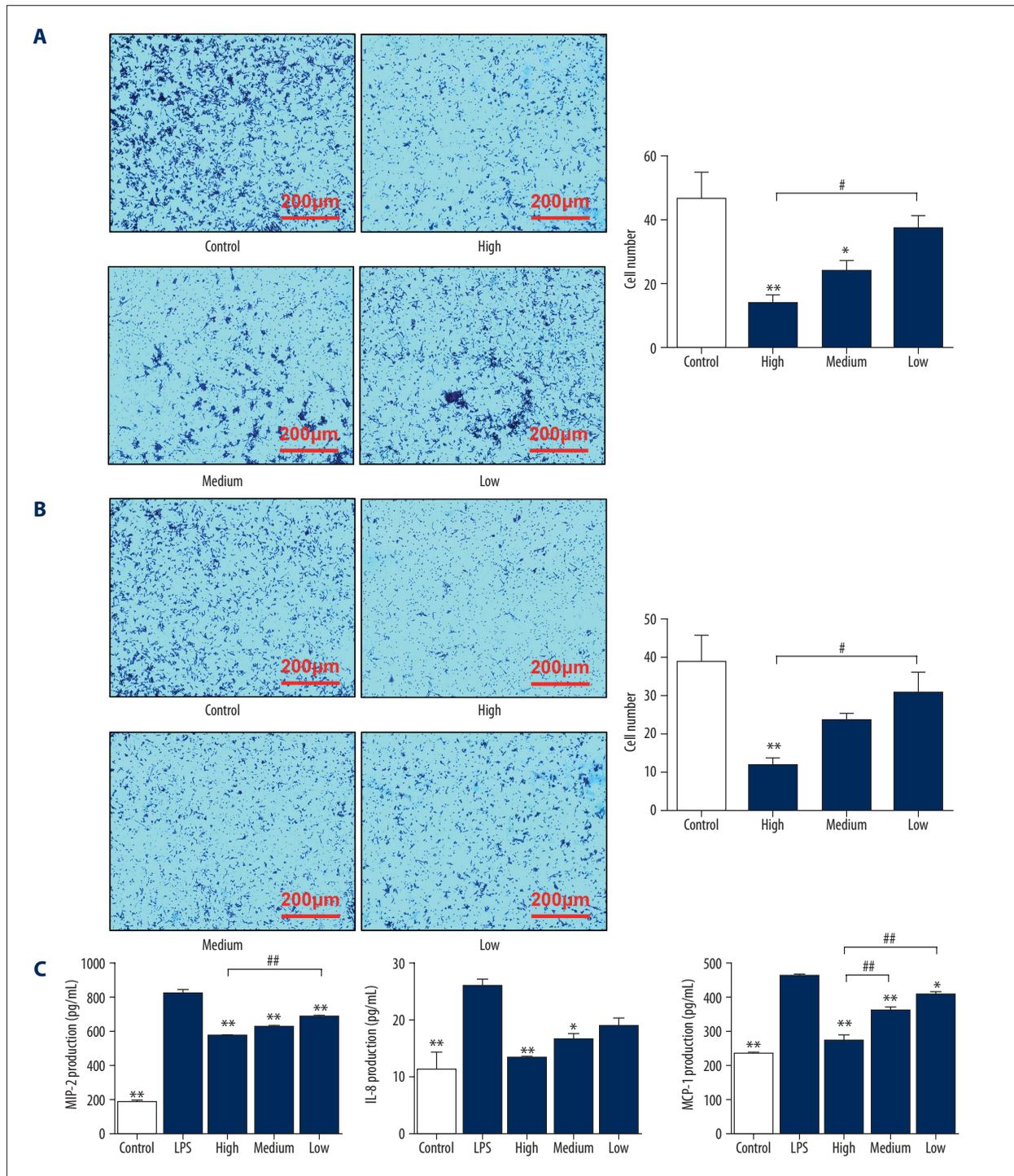


Figure 3. Effect of cimifugin on the migration and chemotaxis of RAW264.7 cells. RAW264.7 cells were divided into a control group (no LPS was added), LPS group (treated with 1 $\mu\text{g}/\text{mL}$ LPS for 24 h), high-dose group (1 $\mu\text{g}/\text{mL}$ LPS+100 mg/L cimifugin for 24 h), medium-dose group (1 $\mu\text{g}/\text{mL}$ LPS+50 mg/L cimifugin for 24 h), and low-dose group (1 $\mu\text{g}/\text{mL}$ LPS+25 mg/L cimifugin for 24 h). **(A, B)** Effect of cimifugin on the migration and chemotaxis of RAW264.7 cells after treatment with **(A)** fetal bovine serum or **(B)** LPS. Transwell assay was performed to determine the migration of cells. * $P<0.05$ and ** $P<0.01$ compared with control group. # $P<0.05$ compared among indicated groups. **(C)** Effect of cimifugin on the concentrations of migration- and chemotaxis-associated factors in cell culture supernatants. ELISA was used to determine the concentrations of the factors. * $P<0.05$ and ** $P<0.01$ compared with LPS group. # $P<0.05$ and ## $P<0.01$ compared among indicated groups.

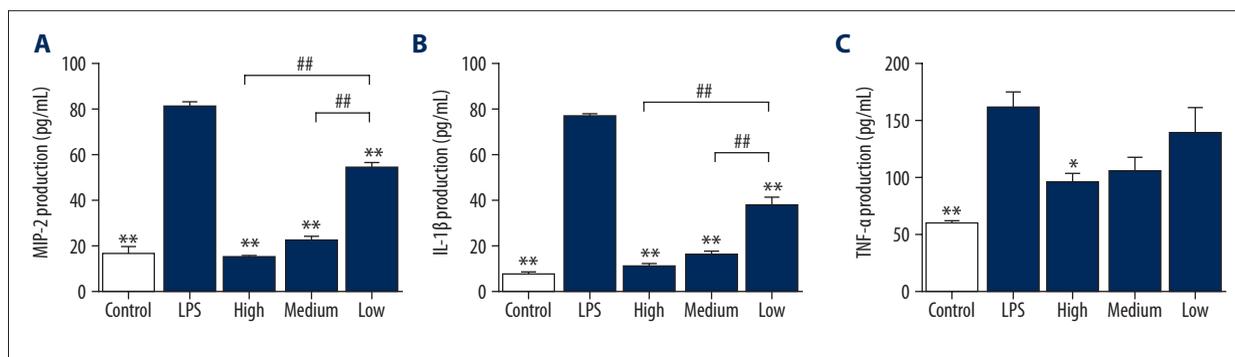


Figure 4. Effect of cimifugin on the contents of inflammatory factors in RAW264.7 cell culture supernatants. RAW264.7 cells were divided into a control group (no LPS was added), LPS group (treated with 1 μ g/mL LPS for 24 h), high-dose group (1 μ g/mL LPS+100 mg/L cimifugin for 24 h), medium-dose group (1 μ g/mL LPS+50 mg/L cimifugin for 24 h), and low-dose group (1 μ g/mL LPS+25 mg/L cimifugin for 24 h). (A) IL-6, (B) IL-1 β , and (C) TNF- α concentrations were determined by ELISA. * $P < 0.05$ and ** $P < 0.01$ compared with LPS group. ## $P < 0.01$ compared among indicated groups.

of that in the LPS group ($P < 0.05$) (Figure 3C). The results suggest that cimifugin decreases the migration and chemotaxis of RAW264.7 cells by inhibiting the release of migration- and chemotaxis-associated factors.

Cimifugin suppresses the release of inflammatory factors from RAW264.7 cells

To measure the contents of inflammatory factors in cell culture supernatants, ELISA was used. The data showed that the concentrations of IL-6, IL-1 β , and TNF- α in the supernatants of RAW264.7 cells stimulated with LPS were more than 3 times that in the control group ($P < 0.05$). However, treatment with 100 mg/L, 50 mg/L, and 25 mg/L cimifugin decreased the release of IL-6 and IL-1 β to less than 20% of that in the LPS group ($P < 0.05$), and treatment with 100 mg/L cimifugin reduced the concentration of TNF- α to 60% of that in LPS group ($P < 0.05$) (Figure 4). The results indicate that cimifugin suppresses the release of inflammatory factors from RAW264.7 cells.

Cimifugin inhibits the activities of MAPKs and NF- κ B signaling pathways

To understand the molecular mechanism by which cimifugin inhibits the release of cytokines from RAW264.7 cells induced by LPS, Western blotting was utilized. The data showed that relative expression of P-P65, P-I κ B α , P-ERK1/2, and P-P38 in RAW264.7 cells stimulated by LPS was 3 times that of the control group, while the expression of P-P50 phosphorylated protein was 1/3 that of the control group ($P < 0.05$) (Figure 5A, 5B). In contrast, treatment with 100 mg/L or 50 mg/L cimifugin reduced the expression of P-P65, P-I κ B α , P-ERK1/2, and P-P38 in RAW264.7 cells to 40% of that in the LPS group ($P < 0.05$), but at least doubled the expression of P-P50 phosphorylated protein compared with LPS group ($P < 0.05$) (Figure 5A, 5B).

The results suggest that cimifugin inhibits the activities of MAPKs and NF- κ B signaling pathways.

Discussion

Prim-o-glucosylcimifugin has anti-inflammatory effects by regulating MAPK and NF- κ B signaling pathways [20]. Cimifugin, which is the aglycone of prim-o-glucosylcimifugin, is shown to inhibit allergic inflammation [18]. In the present study, we first investigated the cytotoxicity of cimifugin on RAW264.7 cells. Many drugs or compounds have certain inhibitory effects on the proliferation of macrophages, and down-regulate relevant signaling pathways or reduce the release of inflammatory factors [21,22]. However, cimifugin (0–100 mg/L) showed no differences in proliferation compared with the control group, suggesting that cimifugin at doses smaller than 100 mg/L does not inhibit the proliferation of RAW264.7 cells.

RAW264.7 cells induced by LPS is a classical cell model for inflammation research [23,24]. The stimulation of LPS can induce M1-like macrophages, and the biomarkers activated by M1 include the upregulation of TNF- α and iNOS [25]. In addition, the process of inflammation is accompanied by the release of a series of cytokines [26]. Our results show that the shapes of RAW264.7 cells changed to spindle shapes, and the release of NO is increased, suggesting that the RAW264.7 cell model of inflammation was successfully constructed. However, the number of RAW264.7 cells with differentiated shapes was decreased after treatment with cimifugin. In addition, the release of NO in the supernatants of RAW264.7 cells after treatment with high- and medium-dose cimifugin was inhibited, suggesting that cimifugin inhibits the differentiation of RAW264.7 cells and may have anti-inflammation effects.

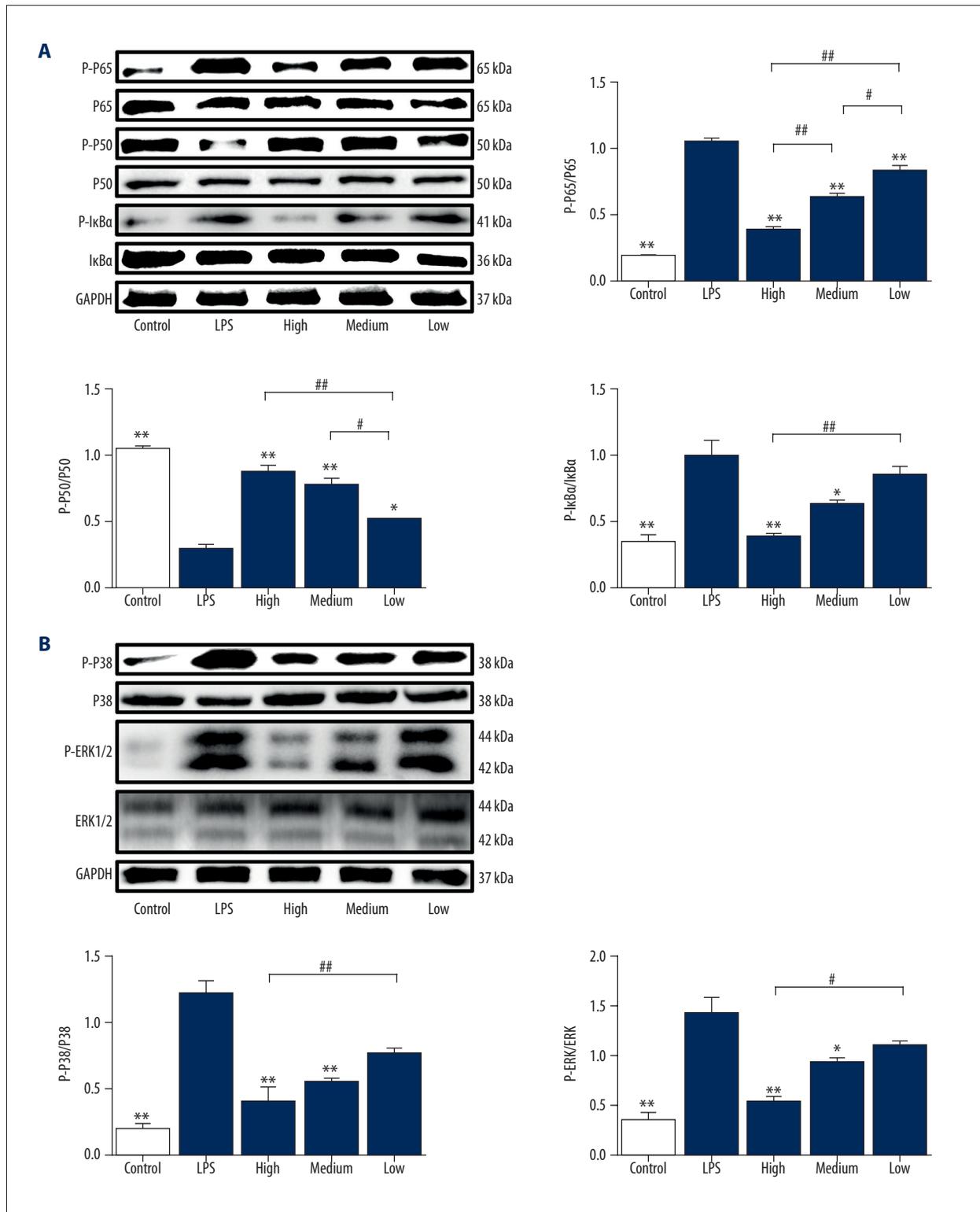


Figure 5. Effect of cimifugin on the activities of (A) MAPKs and (B) NF-κB signaling pathways. (A) Expression of P-P65, P65, P-P50, P50, P-IκBα, and IκBα proteins in MAPKs signaling pathway. (B) Expression of P-P38, P38, P-ERK1/2, and ERK1/2 proteins in NF-κB signaling pathway. Western blotting was used to measure protein expression. * P<0.05 and ** P<0.01 compared with LPS group. # P<0.05 and ## P<0.01 compared among indicated groups.

Subsequently, we used fetal bovine serum and LPS to induce RAW264.7 cells [27,28], and tested the effect of cimifugin on the migration and chemotaxis of RAW264.7 cells. The results show that treatment with high- or medium-dose cimifugin reduced the number of cells after inducing by fetal bovine serum. In addition, treatment with high-dose cimifugin reduced the number of cells after inducing by LPS. This suggests that cimifugin reduces the migration and chemotaxis of RAW264.7 cells. The contents of migration- and chemotaxis-associated factors MIP-2, MCP-1, and IL-8 [29–32] are reduced by cimifugin. This suggests that intervention by cimifugin reduces the accumulation of macrophages at inflammatory sites and alleviates inflammatory symptoms.

Release of inflammatory factors and activation of inflammatory signaling pathways after stimulation by LPS are important in anti-RA inflammatory drug research. In RA and RA model animals, proinflammatory cytokines TNF- α , IL-1 β , and IL-6 are highly expressed at lesion sites [33]. Cytokines play important roles in the development of RA, and are important factors in the destruction of joints and articular cartilages in patients with RA [34]. Our results in the present study show that NO release is reduced by treatment with cimifugin. In addition, the release of IL-6 and IL-1 β is also decreased with increased dosage of cimifugin, while the release of TNF- α is only decreased after treatment by high-dose cimifugin. These results suggest that cimifugin has inhibitory effects on the release of inflammatory factors.

MAPK and NF- κ B signaling pathways are closely related with the pathogenesis of RA and inflammatory responses, and can be activated by LPS induction. Stimulation by LPS can activate the NF- κ B signaling pathway [35,36]. Our results demonstrate that intervention by cimifugin reduces the expression of phosphorylated P65 and I κ B α proteins and elevates the expression of phosphorylated P50 protein. These results suggest that cimifugin inhibits inflammatory responses induced by LPS via the reduction of NF- κ B signaling pathway activity.

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Induction by LPS activates the phosphorylation of MAPKs in RAW264.7 cells [37–39] and increases the expression of inflammatory factors and chemotactic factors [40–42]. Studies show that NF- κ B also interacts with the MAPK signaling pathway [43,44]. In the present study, we found that cimifugin significantly reduced the expression of phosphorylated P38 and ERK proteins, indicating that cimifugin also blocks the ERK and P38 MAPK signaling pathway, and reduces inflammatory responses of RAW264.7 cells stimulated by LPS.

Interestingly, the present study shows that high- or medium-dose cimifugin has effects on migration and chemotaxis, release of inflammatory factors, and activation of inflammatory signaling pathways. However, the effect of low-dose cimifugin is relatively weak. We speculate that the reason may be that the pharmacological action of cimifugin has its own focus, or that the pharmacological action of cimifugin is dose-dependent.

In summary, the present study demonstrates that cimifugin inhibits NF- κ B and MAPK signaling pathways, reduces the release of inflammatory factors and chemotactic factors from RAW264.7 cells induced by LPS, weakens the migration and chemotaxis of RAW264.7 cells, and decreases the differentiation of RAW264.7 cells, thereby exerting anti-inflammatory effects.

Conclusions

Taken together, our results show that cimifugin inhibits the NF- κ B and MAPK signaling pathways, reduces the release of inflammatory factors and chemotactic factors from RAW264.7 cells induced by LPS, weakens the migration and chemotaxis of RAW264.7 cells, and decreases the differentiation of RAW264.7 cells, thereby exerting anti-inflammatory effects. Cimifugin may have potential pharmacological effects against RA.

Conflict of interest

None.

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