

Effect of Scutellarin inhibits collagen-induced arthritis through TLR4/NF- κ B-mediated inflammation

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Abstract. Scutellarin is the major effective constituent of the commonly used Chinese medicine *Erigeron breviscapus*. It has been applied in the clinic to treat various diseases, and is characterized by high content, a stable source, controllable quality, high efficiency and low toxicity. In addition, its potential pharmacological effects have been increasingly identified and elucidated. The present study was performed to examine the role of scutellarin on collagen-induced arthritis (CIA). Mice were injected subcutaneously with bovine collagen type II and administered scutellarin for 2 weeks by gavage 20 mg/kg/day. ELISA kits were used to measure the levels of interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF- α), oxidative stress markers [superoxide dismutase (SOD) and malondialdehyde (MDA)] and caspase-3/-9 activity. Bax, Bcl-2, toll like receptor 4 (TLR4) and nuclear factor (NF)- κ B protein expression was analyzed using western immunoblot analyses. The present study demonstrated that scutellarin prevented CIA, and inhibited the expression of inflammation factors, IL-1 β , IL-6 and TNF- α . In addition, scutellarin reduced the levels of oxidative stress markers, SOD and MDA, as well as intercellular adhesion molecule-1 and monocyte chemoattractant protein 1 in CIA mice. Caspase-3/-9, Bax/Bcl-2, TLR4 and NF- κ B protein expression were reduced in CIA mice following scutellarin treatment. The results of the current study suggest a novel effect of scutellarin involving the inhibition of TLR4/NF- κ B-mediated inflammation.

Introduction

Arthritis refers to a kind of chronic inflammatory disease occurring in human joints as well as the peripheral tissues, which is one of the most common diseases affecting human

health (1). Generally, arthritis will induce clinical responses such as swelling, pain, dysfunction or joint deformity in joints of patients. Moreover, it may further result in progressive joint deformity, thus severely affecting the quality of life of patients. Incidence of arthritis shows no obvious regional characteristics, and it can be seen in any region and any ethnic group in the world (2). However, morbidity of arthritis shows certain difference among different ethnic groups and races (3). The most major feature of arthritis is synovitis and hypertrophic synovium (swelling) induced by production of autoantibodies or systemic dysfunction (3). Multiple diseases, such as cardiovascular disease, lung disease, mental disorder or bone disease can induce production of autoantibodies or systemic dysfunction (4).

Arthritis is associated with a wide variety of types, including rheumatic arthritis, rheumatoid arthritis, osteoarthritis, gouty arthritis, ankylosing spondylitis, reactive arthritis, infectious arthritis, traumatic arthritis and enteropathic arthritis (5). TLRs are expressed in all kinds of immune cells, among which, TLR4 is mainly expressed on surface of mononuclear macrophage and dendritic cell (6). TLR4 can selectively recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, flagellin and microbial nucleic acid, as well as damage-associated molecules patterns (DAMPs) such as endogenous molecule hyaluronic acid released by tissue and cell injury and necrosis, high mobility group box-1 and heat shock protein (3). Consequently, it can trigger MyD88-dependent and MyD88-independent pathways, activates NF- κ B, results in the release of inflammatory cytokines and chemokines like TNF- α and IL-1 β , and induces inflammation (3,6). It has been verified that rheumatoid arthritis (RA) patients are associated with massive expression of inflammatory cytokines such as TNF- α and IL-1 β in serum and synovial joints. As the major pro-inflammatory cytokines, TNF- α and IL-1 β play core roles in RA joint synovial lesions and participate in multiple pathological processes of RA. Meanwhile, they induce synovial fibroblast proliferation and production of multiple cytokines, induce production of metalloprotease, and take part in bone destruction (7).

Scutellarin (Fig. 1) mainly exists in Chinese medicine *Erigeron breviscapus*, which is the major effective constituent of *Erigeron breviscapus* and its extract breviscapine (8). It has been confirmed in modern research to possess extensive

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pharmacological effects (8). The protective effects of scutellarin on heart and cerebral vessels have attracted extensive attention from the medical field. Scutellarin has the therapeutic effects of anti-inflammation, anti-oxidation and anti-apoptosis (9). The present study was performed to examine the role of scutellarin on collagen-induced arthritis (CIA).

Materials and methods

Animals, CIA and drug administration. Eight-week-old male DBA/1J mice (20–22 g weight, male) from Jackson Laboratory (Bar Harbor, Maine, USA) were housed under specific-pathogen-free (SPF) condition. Mice were randomized into three groups ($n=8$ each group). Sham group, CIA model group, scutellarin group. Mice were injected subcutaneously (s.c.) with bovine collagen type II (4 mg/ml, 0.05 ml; Chondrex, Inc., Redmond, WA, USA). After 2 weeks, mice were administered by gavage 20 mg/kg/day of scutellarin for 2 weeks. Study is approved by Shandong Provincial Hospital affiliated to Shandong University.

Hematoxylin and eosin staining. Mice from each group were sacrificed after treatment with scutellarin and the hind-limbs were collected. Hind samples were fixed in 10% buffered formalin and decalcified in 15% EDTA before paraffin section for 3 days. Hind samples were embedded with paraffin, and cut into 6- μ m sections on vibratome. Then, samples were stained with hematoxylin and eosin assay and measured using an Olympus microscope (Olympus Optical, Tokyo, Japan).

Cell factor detection. Blood was collected from the mouse hearts after treatment with scutellarin and centrifuged at 2,000 \times g for 1 h. Serum was used to measure interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), oxidative stress [superoxide dismutase (SOD) and MDA] using ELISA kits by ELISA reader (Sunrise, Tecan Inc., Switzerland) at 450 nm. Caspase-3/-9 activity were measured ELISA kits by ELISA reader (Sunrise, Tecan Inc.) at 405 nm.

Western immunoblot analyses. Mice from each group were sacrificed after treatment with scutellarin and the hind-limbs were collected. Proteins were extracted using a RIPA assay (Beyotime, Nanjing, China) and protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime). Soluble protein (50 μ g) was separated by 8–12% gradient SDS/PAGE and then transferred onto polyvinylidene fluoride membranes (PVDF; Millipore, Billerica, MA, USA). PVDF membranes were incubated with Bax, Bcl-2, TLR4, NF- κ B and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight after blocked with Tris-buffered saline (pH 8.0) containing 5% BSA and 0.5% Tween-20. Membranes were incubated with HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology) at 37°C for 1 h. The bands were probed with Chemiluminescent HRP Substrate (Millipore) and quantitated by densitometry with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All quantitative values are given as the mean \pm SD for 3 times. All data were analyzed by one-way

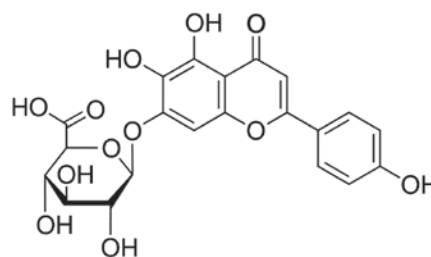


Figure 1. The chemical structure of scutellarin.

analysis of variance (ANOVA) followed by the least significant difference (LSD) post-hoc test. A value of $P<0.05$ was considered statistically significant.

Results

Scutellarin prevents CIA. Firstly, CIA-induced mice were treatment with scutellarin, we measured cartilage degeneration scores and vertical episode count. As showed in Fig. 2, in CIA-induced model mice, there was some bone inattention, and these amounts were higher than those of control mice. However, scutellarin significantly inhibited these CIA-induced bone inattention in CIA mice. Meanwhile, we also found that cartilage degeneration scores was significantly increased in CIA-induced model mice, compared with control group (Fig. 3A).

As showed in Figs. 2 and 3, significant increase of bone inattention and cartilage degeneration scores, and inhibition of vertical episode count in CIA-induced model mice, compared with control group. Then, scutellarin significantly inhibited CIA-induced bone inattention and cartilage degeneration scores, and increased vertical episode count in CIA-induced mice, compared with CIA-induced model group (Figs. 2 and 3).

Scutellarin inhibited inflammation factors. Next, we analyzed the changes of inflammation factors in CIA-induced model mice treated by scutellarin. Fig. 4 showed that the increases of IL-1 β , IL-6 and TNF- α levels in CIA-induced model mice were notably observed, compared with control group. Scutellarin significantly reduced CIA-induced IL-1 β , IL-6 and TNF- α levels in CIA mice, compared with CIA-induced model group (Fig. 4).

Scutellarin inhibited oxidative stress. To confirm the protective effect of scutellarin on oxidative stress, we examined SOD and MDA levels. The inhibition of SOD level and increase of MDA level in CIA-induced model mice were notably observed, compared with control group (Fig. 5). Treatment with scutellarin significantly increased the inhibition of SOD level and decreased increase of MDA level in CIA-induced mice, compared with CIA-induced model group (Fig. 5).

Scutellarin inhibited ICAM-1 and MCP-1 levels. We evaluated ICAM-1 expression and MCP-1 levels in CIA mice by scutellarin. As showed in Fig. 6, ICAM-1 protein expression and MCP-1 levels were observably higher in CIA model group than those of sham group. Treatment with scutellarin significantly inhibited ICAM-1 protein expression and MCP-1 levels in CIA mice, compared with CIA-induced model group.

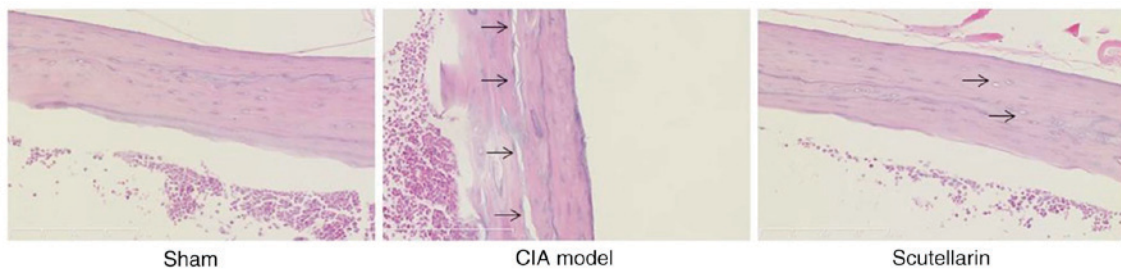


Figure 2. Scutellarin prevents bone ination of CIA animals, illustrated on hematoxylin & eosin stained sections. CIA, collagen-induced arthritis. Scutellarin, 20 mg/kg of scutellarin for 2 week. All quantitative values are given as the mean \pm SD for 3 times. Sham, sham group (n=8); CIA model, CIA model group (n=8); scutellarin, treatment with scutellarin group (n=8).

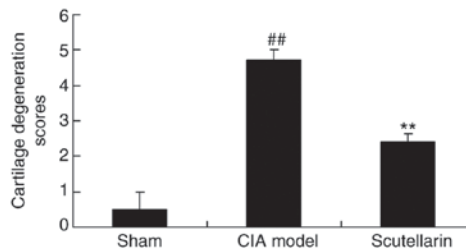


Figure 3. Scutellarin protects against cartilage degradation evaluated by cartilage degeneration scores. Bone ination and cartilage degeneration scores (A). CIA, collagen-induced arthritis. Scutellarin, 20 mg/kg of Scutellarin for 2 week. All quantitative values are given as the mean \pm SD for 3 times. Sham, sham group (n=8); CIA model, CIA model group (n=8); scutellarin, treatment with scutellarin group (n=8). ^{##}P<0.01 vs. sham group; ^{**}P<0.01 vs. CIA model group.

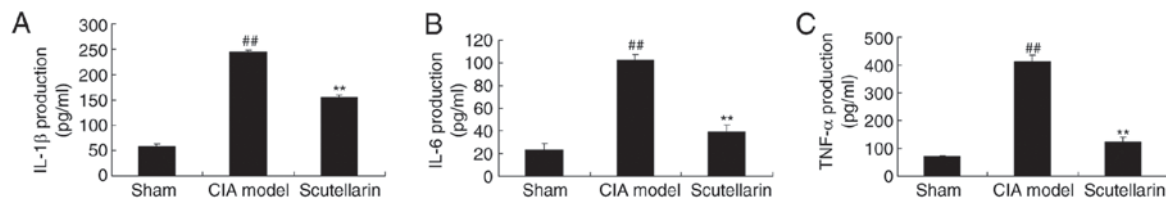


Figure 4. Scutellarin inhibited inflammation factors. Scutellarin inhibited IL-1 β (A), IL-6 (B) and TNF- α (C) levels using ELISA kits. CIA, collagen-induced arthritis. Scutellarin, 20 mg/kg of scutellarin for 2 week. All quantitative values are given as the mean \pm SD for 3 times. Sham, sham group (n=8); CIA model, CIA model group (n=8); scutellarin, treatment with scutellarin group (n=8). ^{##}P<0.01 vs. sham group; ^{**}P<0.01 vs. CIA model group.

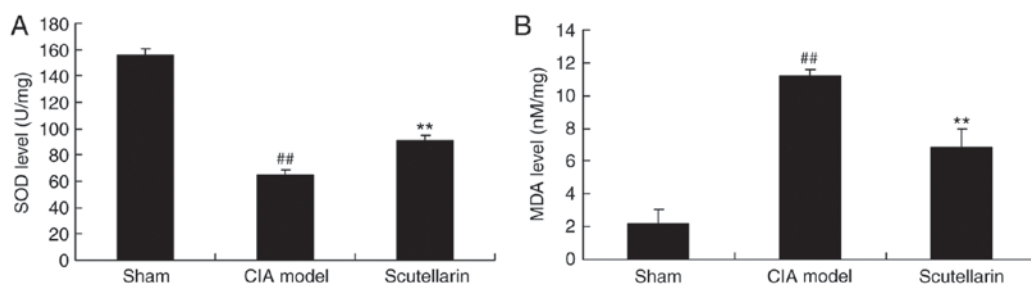


Figure 5. Scutellarin inhibited oxidative stress. Scutellarin inhibited SOD (A) and MDA (B) levels using ELISA kits. CIA, collagen-induced arthritis. Scutellarin, 20 mg/kg of Scutellarin for 2 week. All quantitative values are given as the mean \pm SD for 3 times. Sham, sham group (n=8); CIA model, CIA model group (n=8); Scutellarin, treatment with scutellarin group (n=8). ^{##}P<0.01 vs. sham group; ^{**}P<0.01 vs. CIA model group.

Scutellarin inhibited apoptosis. To further confirm whether the anti-apoptosis effects of scutellarin in CIA mice, caspase-3/-9 and Bax protein expression were measured. There were significant increases of caspase-3/-9 and Bax protein expression in CIA model mice, compared with control group (Fig. 7). Scutellarin significantly reduced caspase-3/-9 levels and Bax protein expression in CIA mice, compared with CIA-induced model group (Fig. 7).

Scutellarin inhibited TLR4 and NF- κ B protein expression. We next evaluated the anti-inflammation mechanism of scutellarin in CIA mice, TLR4 and NF- κ B protein expression were measured. As showed in Fig. 8, TLR4 and NF- κ B protein expression were significantly induced in CIA model mice, compared with control group. The CIA-induced TLR4 and NF- κ B protein expression were significantly suppressed by scutellarin, compared with CIA-induced model group (Fig. 8).

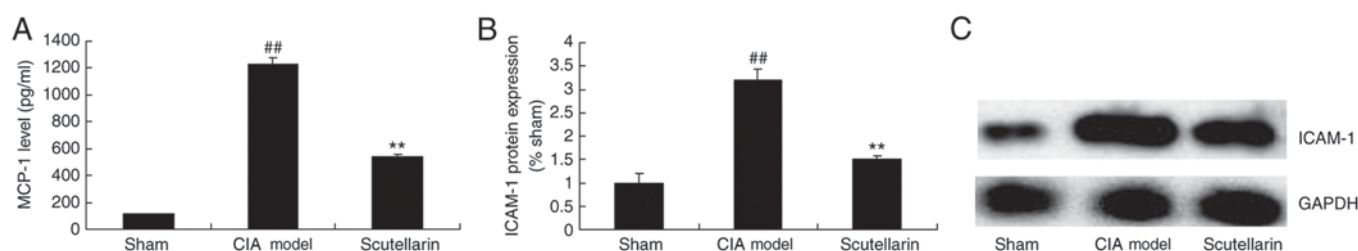


Figure 6. Scutellarin inhibited ICAM-1 and MCP-1 levels. Scutellarin inhibited MCP-1 levels (A) and ICAM-1 protein expression using statistical analysis (B) and western blot analysis of ICAM-1 protein expression (C). CIA, collagen-induced arthritis. Scutellarin, 20 mg/kg of scutellarin. All quantitative values are given as the mean \pm SD for 3 times. Sham, sham group (n=8); CIA model, CIA model group (n=8); scutellarin, treatment with scutellarin group (n=8). ^{##}P<0.01 vs. sham group; ^{**}P<0.01 vs. CIA model group.

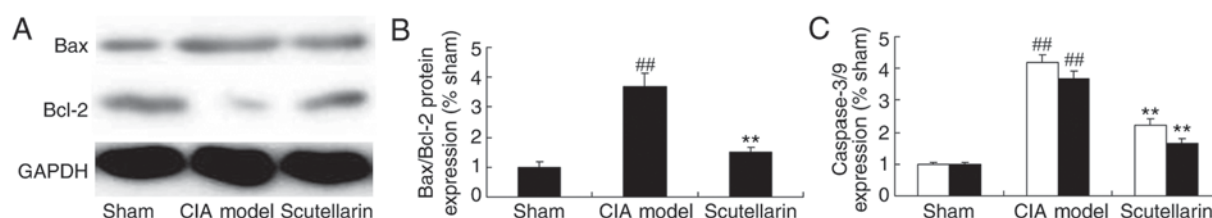


Figure 7. Scutellarin inhibited apoptosis. Scutellarin inhibited Bax protein expression using western blot analysis (A) and statistical analysis of Bax protein expression (B), caspase-3 and -9 (C). CIA, collagen-induced arthritis. Scutellarin, 20 mg/kg of scutellarin for 2 week. All quantitative values are given as the mean \pm SD for 3 times. Sham, sham group (n=8); CIA model, CIA model group (n=8); scutellarin, treatment with scutellarin group (n=8). ^{##}P<0.01 vs. sham group; ^{**}P<0.01 vs. CIA model group.

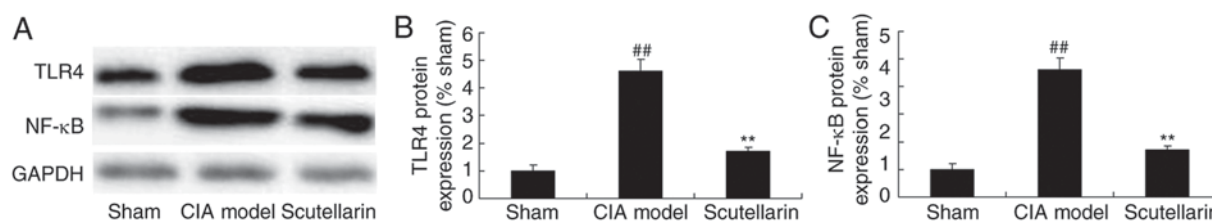


Figure 8. Scutellarin inhibited TLR4 and NF-κB protein expression. Scutellarin inhibited TLR4 and NF-κB protein expression using western blot analysis (A) and statistical analysis of TLR4 and NF-κB protein expression (B and C). CIA, collagen-induced arthritis. Scutellarin, 20 mg/kg of scutellarin for 2 week. All quantitative values are given as the mean \pm SD for 3 times. Sham, sham group (n=8); CIA model, CIA model group (n=8); scutellarin, treatment with scutellarin group (n=8). ^{##}P<0.01 vs. sham group; ^{**}P<0.01 vs. CIA model group.

Discussion

Arthritis is one of the most common chronic arthritis, which is a systemic disease characterized by synovitis that may lead to human joint deformity and even loss of function (4). The major causes of such disease include genetic factor, as well as bacterial and viral infection (4). Female aged 40 to 60 years are the populations with the highest morbidity, which is 2 to 3 times of that in male (4). Meanwhile, it is also an allergic disease, with migrating aching pain in joints and muscles being its major clinical feature (10). Large joints such as knee, angle, shoulder, elbow and wrist are the major involved joints of arthritis. Besides, arthritis will metastasize among all joints. Few patients may even develop arthritis involving multiple joints (10). However, the most dangerous effect of such disease is that it is associated with repeated attack and the involvement of heart, thus inducing myocarditis and even valve disorders (11). In the present study, we demonstrate that scutellarin significantly inhibited CIA-induced bone inanition and cartilage degeneration scores, and increased vertical episode count in CIA-induced mice.

Production and elimination of reactive oxygen species (ROS) maintains at a dynamic balance in normal body internal environment (12). Damaged antioxidant system of the body will result in excessive aggregation of ROS and its relevant metabolites. As a result, it will lead to tissue injury, cognitive defect, emotional disorder and multiple other diseases (13). It is indicated in research that oxidative stress is positively correlated with RA. Elevated serum lipid peroxidation reactant levels, while decreased superoxide dismutase level and abnormal antioxidant activity can be seen in RA (14). In this study, scutellarin significantly increased the inhibition of SOD level and decreased increase of MDA level and caspase-3/-9 and Bax protein expression in CIA-induced mice. Wang *et al* reported that scutellarin protects cardiomyocyte ischemia-reperfusion injury by reducing ROS production and oxidative stress (15). These results showed that scutellarin inhibited oxidative stress and bone cell apoptosis in CIA, which may be a good research direction in further study.

Apoptosis of chondrocyte is significantly increased during arthritis, which leads to destroyed dynamic balance between

its proliferation and proliferation (16). The chondrocytes are decreased substantially, accompanying with gradual degradation of extracellular matrix. This has eventually led to degradation and disappearance of articular cartilage (17). Apoptosis of chondrocyte is the programmed death of chondrocyte under certain pathophysiological conditions, which is also the result that multiple internal and external biological signals act on chondrocyte together (18). As can be found in clinical research, levels of inflammatory cytokines such as IL-1 β and TNF- α are markedly elevated in synovial fluid during the pathogenesis of arthritis. Moreover, the increased degree is partly correlated with severity of cartilage destruction (19). Findings from our study revealed Scutellarin significantly reduced CIA-induced IL-1 β , IL-6 and TNF- α levels in CIA mice. Yuan *et al* suggested that scutellarin inhibited microglia-mediated neuroinflammation in cerebral ischemia (8). Hence in this study, we demonstrated that scutellarin reduced inflammation in CIA, and the underlying mechanism needs to be analyzed.

Two signal transduction methods are available for the TLRs family, which are MyD88-dependent and MyD88-independent signal transduction pathways (7). MyD88 is a key adaptor protein in the TLRs signal transduction pathways, which mainly contains three domains (20). Its C-terminal is the TIR domain that can bind with TIR domains of TLR and IL-1R. Its N-terminal is the death domain, which is responsible for recruiting downstream signal molecules with death domain into the downstream signal transduction. The middle part is the short adaptor domain (20). Deletion of MyD88 death domain may lead to limited recruitment of downstream IRAK, thus playing a negative regulatory role in TLR4 and IL-1R signals (21). In addition, TRIF, TIRAP/MAL, TRAM and SARM are also the important adaptor proteins in TLRs signal transduction pathways (22). When an extracellular signal enters the cell, TIR domain will bind with specific intracellular adaptor, thus initiating a series of intracellular cascade reactions and activating corresponding signal transduction pathways (22). In line with these results, the present study found that scutellarin significantly suppressed CIA-induced TLR4 protein expression in CIA mice.

In resting cells, NF- κ B dimer binds with inhibitor- κ B (κ I-B), and covers the nuclear localization signal of NF- κ B. This has rendered the retention of the complex formed by NF- κ B and κ I-B in the cytoplasm (23). IKK complex is composed by the catalytic subunits IKK α and IKK β , as well as the regulatory subunit IKK γ . It can result in phosphorylation and ubiquitination of κ I-B, thus leading to the release of κ I-B from κ I-B/NF- κ B complex (17,24). Thereby, NF- κ B is activated and nuclear translocation is developed, which induces expression of a series of specific genes. Meanwhile, this contributes to producing primary pro-inflammatory cytokines such as TNF- α , IL-1, IL-6 and IL-8, and completes the signal transduction process of inflammation (25). Our results showed that scutellarin significantly suppressed CIA-induced NF- κ B protein expression in CIA mice. Chen *et al* revealed that scutellarin exhibits anti-inflammatory and anti-apoptotic properties in hypertensive rats through suppression of TLR4, NF- κ B p65 protein expression (9). Our result showed that scutellarin suppressed to TLR4/NF- κ B signaling pathway

to reduce inflammatory in CIA. Certainly, TLR4/NF- κ B signaling pathway is most common signaling pathway for inflammation, and scutellarin may regulate other inflammation signaling pathway, such as NLRP3, NLRP6, STATs signaling pathway, which will study in further research.

In conclusion, the present study demonstrated that the effect of scutellarin inhibits CIA, and it-induced oxidative stress, apoptosis and inflammation through TLR4/NF- κ B-mediated inflammation. In this study, we not adjust TLR4 or NF- κ B to explore function of TLR4/NF- κ B signal pathway in effects of scutellarin on CIA, which is a limitations of the study. We will utilize TLR4 or NF- κ B inhibitor to analyze the function of TLR4/NF- κ B signal pathway in effects of scutellarin on CIA in further study. Certainly, scutellarin maybe affects other inflammation signal pathway, such as inflammasome, NLRP3, NLRP6, and so on. Our results may provide an experimental basis for the possible new drug of scutellarin against CIA in clinical applications.

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