

Gambogic acid suppresses inflammation in rheumatoid arthritis rats via PI3K/Akt/mTOR signaling pathway

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Abstract. Gamboge is the dried resin secreted by the *Garcinia maingayi* gambogic tree and is a substance that may be used to treat a variety of diseases, exhibits anti-tumor and detoxification effects and prevents bleeding. The primary active constituent is gambogic acid. The present study aimed to investigate the anti-inflammatory effects of gambogic acid in rheumatoid arthritis (RA) rats and to elucidate the mechanisms by which these effects occur. The swelling degree, the clinical arthritic scoring and pain threshold measurements were used to evaluate the effects of gambogic acid on RA. ELISA kits and western blot analysis were used to investigate inflammatory processes and the expression of RA-associated proteins, respectively. The present results demonstrated that gambogic acid significantly inhibited the degree of right foot swelling, increased pain thresholds and reduced clinical arthritic scores of RA rats. Treatment with gambogic acid suppressed the activities of interleukin (IL)-1 β and IL-6, promoted the protein expression of phosphorylated (p)-Akt serine/threonine kinase (Akt), p-mammalian target protein of rapamycin (mTOR) and inhibited hypoxia-inducible factor-1 α and vascular endothelial growth factor expression in RA rats. The results of the present study therefore suggest that the anti-inflammatory effects of gambogic acid in RA rats occur via regulation of the phosphoinositide 3-kinase/Akt/mTOR signaling pathway.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease with etiology that remains to be fully elucidated. At

present, it is widely recognized that RA is an autoimmune disease. The disease is often presented in facet joints, including hands, wrists and feet with symmetric distribution (1). Patients at an early stage of the disease, often present with pain in red, swelled and heated joints and functional barriers, whereas patients at an advanced stage of the disease present ankylosis or malformation at varying degrees, accompanied with atrophy of bone muscle, bone necrosis and numerous disabling symptoms (2). The specific pathogenesis of the disease is not clear and may be associated with multiple factors, including social differences, geographical conditions, internal secretion alterations, vocational status, nutrition and metabolic disturbance and bacterial and viral infections (3). RA as of yet has no effective treatment and its therapeutic regimen primarily involves relieving pain and inflammation (4).

Arthrocele at early stage results from synovium congestion, edema and exudation of articular cavity, in addition to periarticular tissue edema (5). In rheumatic synovitis, fenestra expansion of synovial endothelial cells and stimulation of inflammatory mediators, including histamine, bradykinin, prostaglandin E₂, free radicals and neurosecretion of substance P result in hemangiectasis, increase in capillary permeability and exudation, which lead to synovial membrane and periarticular tissue edema (6). Collagen and protein polysaccharide degradation are mediated by cytokines interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF)- α , chondrocyte swelling and degeneration are mediated by nitric oxide (NO) and NO-induced oxygenase expression in cartilage (5,7). Adhesion of hemameba, T cells, cell endothelial cells and fibrinogen mediated by adhesion molecules promote formation of a fibrin clot, resulting in disturbed blood vessel function and a subsequent synovium inflammatory response (8). Growth hormone inhibiting factor, TNF- α and IL increase in synovium results in increasing T cell expression with autoantigen reactivity, resulting in arthritic symptoms (9).

The phosphoinositide 3-kinase (PI3K), Akt serine/threonine kinase (Akt) signaling pathway is an important intracellular transduction pathway and has previously been demonstrated to be associated with the development of RA (10). The active state of the signaling pathway is tightly regulated by the negative regulatory factor phosphatase and tensin homolog (PTEN), and the regulatory factors TNF- α , transforming growth factor

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(TGF)- β and TNF-related apoptosis-inducing ligand (11). In the synovial cells of RA patients, the signaling pathway is maintained in the abnormal activated state, resulting in high expression of downstream anti-apoptosis genes and a subsequent impact on multiple downstream effector molecules (12). Furthermore, it is important in the synovial cell proliferation and apoptotic imbalance of RA patient joints. Excessively-grown synovial cells infiltrate and grow in the cartilage articularis and bone tissues, resulting in joint deformity and dysfunction. Therefore, the inhibition of the PI3K-AKT signal pathway may negatively regulate proteins, reverse excessive proliferation of RA synoviocytes, and may provide a novel target for curing the disease (13).

The primary constituents of gamboge are composed of 70-80% resin, 15-25% gum and include gambogic acid, neogambogic acid, allogambogic acid, morellin, isomorellin, morellic acid and isomorellic constituents. Gambogic acid is the primary effective constituent (14). The pharmacological and pharmacokinetic properties of preparation of gambogic acid have previously been studied (15). The anti-tumor effects of gambogic acid involve induction of tumor cell apoptosis, restraining the cell cycle and impacting oncogenes, cancer suppressor genes and expression of associated proteins (16). The present study therefore aimed to investigate the anti-inflammatory effects of gambogic acid in RA rats and to reveal the potential signaling pathways involved.

Materials and methods

Experimental rat model. All study protocols employed were in accordance with the guidelines of the Animal Care and Use Committee of Baodi District People's Hospital of Tianjin City (Tianjin, China). The study was approved by the Ethics Committee of Sichuan Provincial People Hospital (Chengtu, China). A total of 30 male Sprague-Dawley rats (age, 8-10 weeks; weight, 250-300 g) were obtained from Laboratory Animal Center of Tianjin Medical University (Tianjin, China) and maintained in individual cages under standard conditions (temperature, 22-23°C; humidity, 55-60%; 12-h light/dark cycle), and provided with free access to food and water.

Grouping and model establishment. The experimental rats were randomly divided into 5 groups (6 rats/group): Sham, RA model, gambogic acid (1 mg/kg/day), gambogic acid (5 mg/kg/day) and gambogic acid (10 mg/kg/day) groups for 3 weeks. The chemical structure of gambogic acid ($\geq 95\%$, high performance liquid chromatography) is indicated in Fig. 1 and was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). In the RA model, gambogic acid (1 mg/kg/day), gambogic acid (5 mg/kg/day) and gambogic acid (10 mg/kg/day) groups, rats were anesthetized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital (Sigma-Aldrich; Merck KGaA). A total of 10 mg/ml Freund's complete adjuvant (Sigma-Aldrich; Merck KGaA) was subcutaneously injected into the 2nd and 3rd toes of the right foot. The right ankle then demonstrated acute inflammatory swelling.

Swelling degree measurement. The posterior right limb was inserted in 20 ml water, and the volume of displaced water

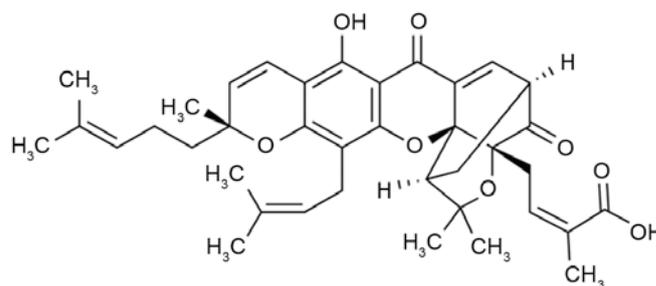


Figure 1. The constitutional formula of gambogic acid.

was measured as representative of foot volume. The swelling degree was measured as follows: (Foot volume 7 days following RA induction-foot volume prior to RA induction)/(foot volume prior to RA induction) $\times 100\%$.

Clinical arthritic scoring measurement. The rats were observed according to a macroscopic scoring system, as follows: 0, no sign of arthritis; 1-5, 2 joints involved; 6-10, >2 joints involved; 11-15; severe arthritis of the entire paw and digits. Clinical arthritic scoring was performed three times in each session.

Pain threshold measurement. Pain threshold measurement was conducted as previously described (17). The pressure pain threshold (g) was detected using an electronic pressure pain detector (Somedic AB, Sösdala, Sweden). Pain threshold measurement was conducted three times in each session.

Inflammatory effects measurement. Following sacrifice of rats using 100 mg/kg sodium pentobarbital (Sigma-Aldrich; Merck KGaA), blood was extracted from the inferior vena cava (3 ml) and centrifuged at 3,000 \times g for 10 min at 4°C. The supernatant was used to analyze IL-1 β (cat. no. ER008-96) and IL-6 (cat. no. ER003-96) activities with ELISA kits, according to the manufacturer's protocol (Genetimes Technology, Inc., Shanghai, China).

Western blotting. The synovium was digested with radioimmunoprecipitation assay lysis buffer (Roche Diagnostics, Basel, Switzerland) at 4°C for 30 min and centrifuged at 12,000 \times g for 10 min at 4°C. The supernatant was used to analyze the protein content using a bicinchoninic acid kit (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amounts of extracted protein samples (50 μ g) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked using 5% skim milk powder in TBS containing 0.1% Tween-20 at 37°C for 1 h and incubated with the following primary antibodies at 4°C overnight: Anti-Akt (1:300; cat. no. sc-8312), anti-phosphorylated (p)-Akt (1:500; cat. no. sc-7985-R), anti-mammalian target protein of rapamycin (mTOR; 1:300; cat. no. sc-8319), anti-p-mTOR (1:500; cat. no. sc-101738), anti-vascular endothelial growth factor (VEGF; 1:300; cat. no. sc-13083), anti-hypoxia-inducible factor-1 α (HIF-1 α ; 1:300; cat. no. sc-10790) and anti-GAPDH (1:300; cat. no. sc-367714), all obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Membranes were then

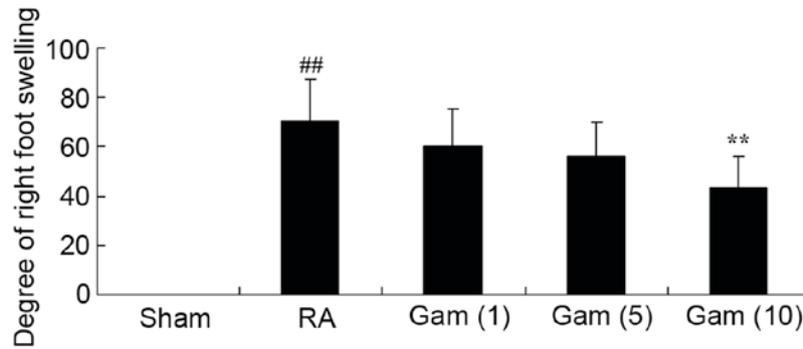


Figure 2. Effects of gambogic acid on degree of right foot swelling. Administration of differing doses of gambogic acid decreased right foot swelling in a model of RA. ^{##}P<0.01 vs. sham group; ^{**}P<0.01 vs. RA group. Sham, sham group; RA, rheumatoid arthritis model group; Gam (1), gambogic acid (1 mg/kg/day) group; Gam (5), gambogic acid (5 mg/kg/day) group; Gam (10), gambogic acid (10 mg/kg/day) group.

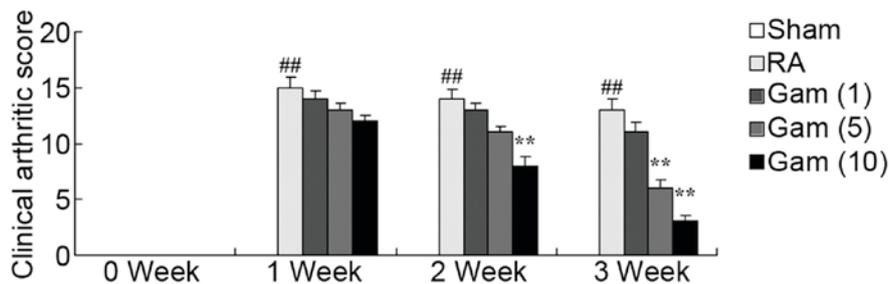


Figure 3. Effects of gambogic acid on clinical arthritic score of RA rats. Administration of differing doses of gambogic acid decreased clinical arthritic score in RA model rats. ^{##}P<0.01 vs. sham group; ^{**}P<0.01 vs. RA group. Sham, sham group; RA, rheumatoid arthritis model group; Gam (1), gambogic acid (1 mg/kg/day) group; Gam (5), gambogic acid (5 mg/kg/day) group; Gam (10), gambogic acid (10 mg/kg/day) group.

incubated with goat anti-rabbit immunoglobulin G (1:2,000; cat. no. 14708; Cell Signaling Technology, Inc., Danvers, MA, USA) at 37°C for 1 h. Protein bands were visualized using enhanced chemiluminescence (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Blots were semi-quantified using ImageJ software version 1.41 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation of 3 independent experiments. One-way analysis of variance followed by the Tukey's post hoc test was used to determine the differences among groups using SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Gambogic acid decreases degree of right foot swelling. The constitutional formula of gambogic acid is presented in Fig. 1. The present study firstly examined the potential effects of gambogic acid on the degree of right foot swelling in RA rats. The degree of right foot swelling was increased in the RA model group compared with sham group (Fig. 2). The results demonstrated that treatment with gambogic acid at 10 mg/kg/day significantly inhibited the degree of right foot swelling in RA rats (Fig. 2).

Gambogic acid decreases clinical arthritic score of RA rats. The potential effects of gambogic acid on the clinical arthritic

score of RA rats was next investigated. As presented in Fig. 3, the clinical arthritic score of the RA rat model group significantly increased over the period of 3 weeks, compared with sham group. However, administration of 10 mg/kg/day gambogic acid significantly reduced the clinical arthritic score of RA rats, over this time period (Fig. 3).

Gambogic acid increases pain threshold of the RA rat model. To elucidate the potential effects of gambogic acid on the RA rat model, the pain threshold of the RA rat model was measured. As presented in Fig. 4, compared with sham group, the pain threshold of the RA rat model was decreased. Treatment with gambogic acid significantly increased the pain threshold of the RA rats (Fig. 4).

Gambogic acid decreases inflammation of the RA rat model. To elucidate the anti-inflammatory effect of gambogic acid in the RA rat model, IL-1 β and IL-6 activities were analyzed using ELISA kits. Notably, RA significantly promoted IL-1 β and IL-6 activities in the RA rat model, compared with sham group (Fig. 5). Following treatment with gambogic acid, the promotion of IL-1 β and IL-6 activities in RA rats was significantly suppressed (Fig. 5).

Gambogic acid suppresses inhibition of the Akt signaling pathway in the RA rat model. p-Akt expression was examined from tissue via western blotting, in order to analyze the potential effects of gambogic acid on RA. The p-Akt protein expression was significantly inhibited in the RA rat model,

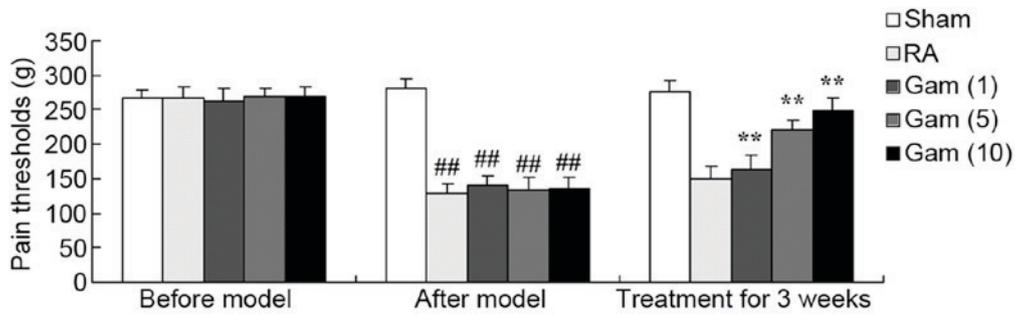


Figure 4. Effects of gambogic acid on pain threshold of the RA rat model. Administration of differing doses of gambogic acid increased the pain threshold in RA model rats. ^{##} $P < 0.01$ vs. Sham group; ^{**} $P < 0.01$ vs. RA group. Sham, sham group; RA, rheumatoid arthritis model group; Gam (1), gambogic acid (1 mg/kg/day) group; Gam (5), gambogic acid (5 mg/kg/day) group; Gam (10), gambogic acid (10 mg/kg/day) group.

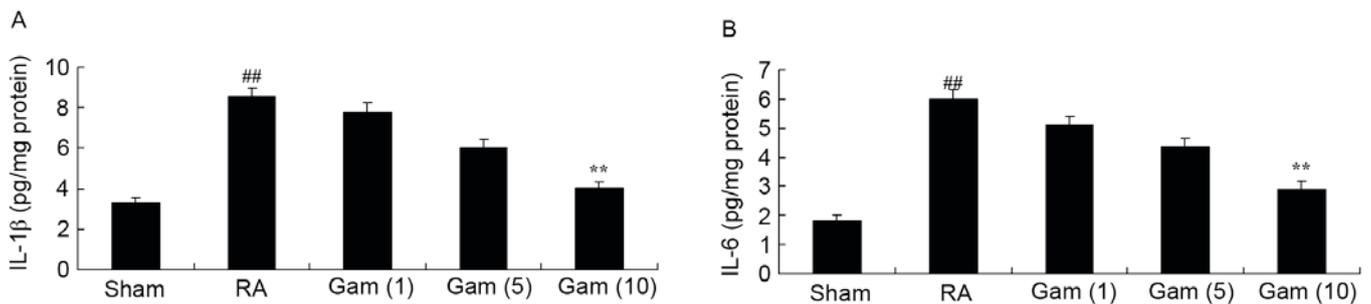


Figure 5. Effects of gambogic acid on inflammation in the RA rat model. The effects of differing doses of gambogic acid were investigated on (A) IL-1 β and (B) IL-6 activities in the RA rat model. ^{##} $P < 0.01$ vs. Sham group; ^{**} $P < 0.01$ vs. RA group. IL, interleukin; Sham, sham group; RA, rheumatoid arthritis model group; Gam (1), gambogic acid (1 mg/kg/day) group; Gam (5), gambogic acid (5 mg/kg/day) group; Gam (10), gambogic acid (10 mg/kg/day) group.

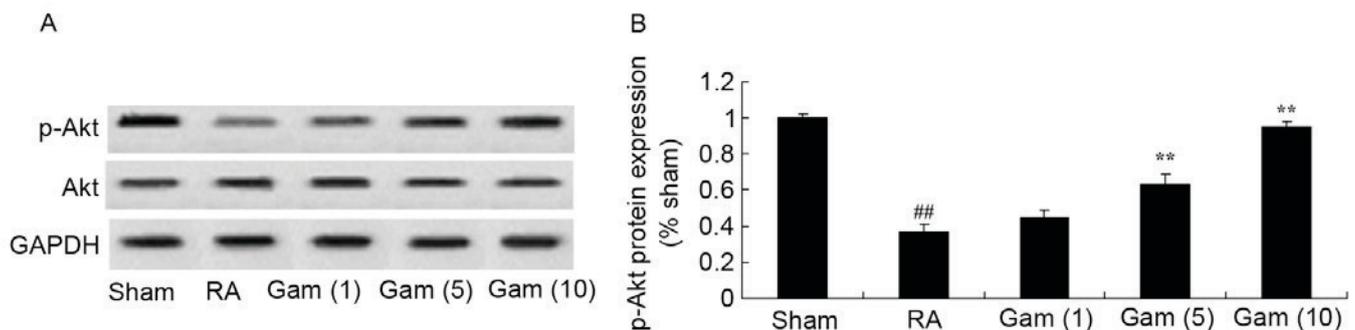


Figure 6. Effects of gambogic acid on Akt signaling pathway of the RA rat model. (A) Representative image and (B) statistical analysis of western blotting of the effects of gambogic acid on p-Akt protein expression in the RA rat model. ^{##} $P < 0.01$ vs. sham group; ^{**} $P < 0.01$ vs. RA group. p, phosphorylated; Akt, Akt serine/threonine kinase; Sham, sham group; RA, rheumatoid arthritis model group; Gam (1), gambogic acid (1 mg/kg/day) group; Gam (5), gambogic acid (5 mg/kg/day) group; Gam (10), gambogic acid (10 mg/kg/day) group.

compared with the sham group (Fig. 6). Notably, gambogic acid significantly suppressed the inhibition of p-Akt protein expression in RA rats (Fig. 6).

Gambogic acid activates mTOR signaling pathway in the RA rat model. To explore if gambogic acid activates the mTOR signaling pathway in RA rats, the models were treated with differing doses. The p-mTOR protein expression levels in the RA rat model group were decreased compared with sham group (Fig. 7). However, treatment with gambogic acid significantly activated the mTOR signaling pathway via increased p-mTOR protein expression in RA rats (Fig. 7).

Gambogic acid inhibits VEGF signaling pathway in the RA rat model. To examine the role of the VEGF signaling pathway in the potential effects of gambogic acid on RA, VEGF protein expression was measured using western blotting. As presented in Fig. 8, the activation of VEGF protein expression was significantly increased in the RA model group, compared with sham group. Gambogic acid significantly suppressed the activation of VEGF protein expression in RA rats (Fig. 8).

Gambogic acid inhibits HIF-1 α signaling pathway in the RA rat model. To investigate the role of the HIF-1 α signaling pathway in mediating the potential effects of gambogic acid on RA, HIF-1 α protein expression was measured via western

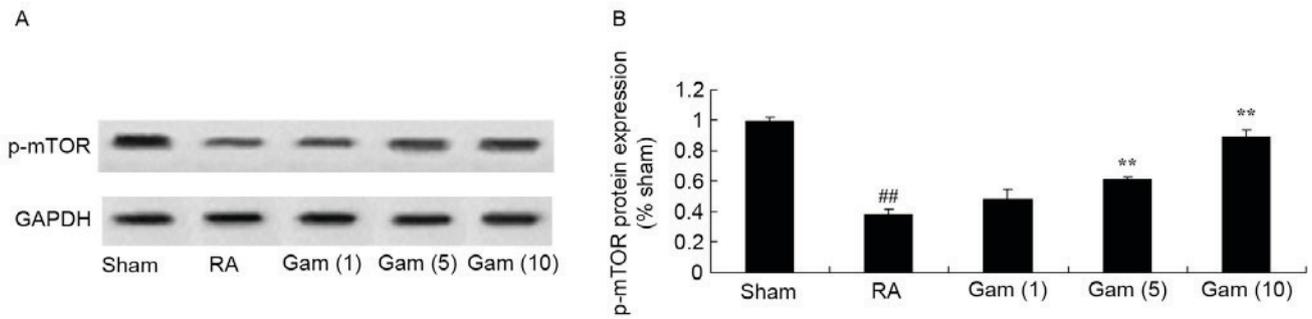


Figure 7. Effects of gambogic acid on mTOR signaling pathway of the RA rat model. (A) Representative image and (B) statistical analysis of western blotting of the effects of gambogic acid on p-mTOR protein expression in the RA rat model. ##P<0.01 vs. Sham group; **P<0.01 vs. RA group. p, phosphorylated; mTOR, mammalian target protein of rapamycin; Sham, sham group; RA, rheumatoid arthritis model group; Gam (1), gambogic acid (1 mg/kg/day) group; Gam (5), gambogic acid (5 mg/kg/day) group; Gam (10), gambogic acid (10 mg/kg/day) group.

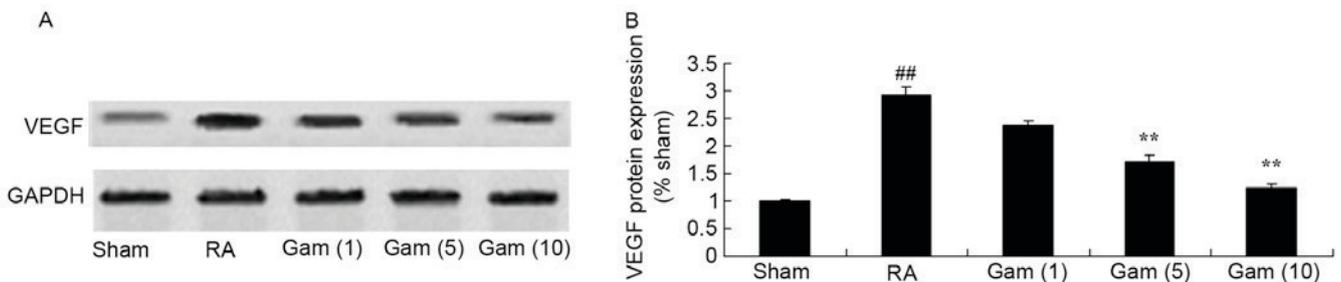


Figure 8. Effects of gambogic acid on VEGF signaling pathway of the RA rat model. (A) Representative image and (B) statistical analysis of western blotting of the effects of gambogic acid on VEGF protein expression in the RA rat model. ##P<0.01 vs. Sham group; **P<0.01 vs. RA group. VEGF, vascular endothelial growth factor; Sham, sham group; RA, rheumatoid arthritis model group; Gam (1), gambogic acid (1 mg/kg/day) group; Gam (5), gambogic acid (5 mg/kg/day) group; Gam (10), gambogic acid (10 mg/kg/day) group.

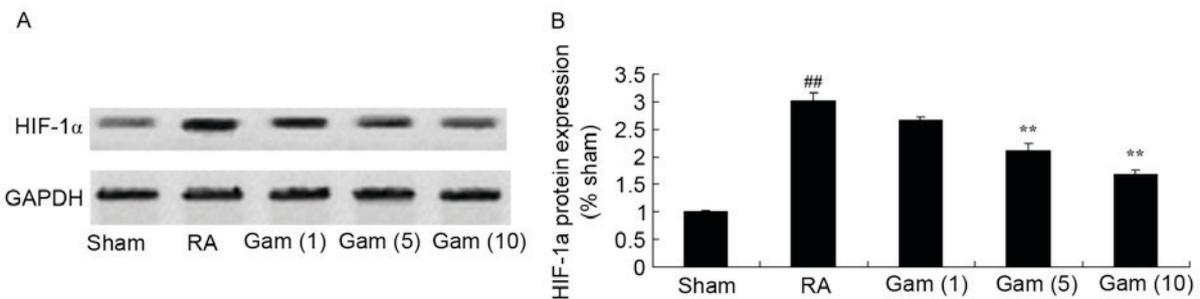


Figure 9. Effects of gambogic acid on HIF-1α signaling pathway of the RA rat model. (A) Representative image and (B) statistical analysis of western blotting of the effects of gambogic acid on HIF-1α protein expression in the RA rat model. ##P<0.01 vs. Sham group; **P<0.01 vs. RA group. HIF-1α, hypoxia-inducible factor-1α; Sham, sham group; RA, rheumatoid arthritis model group; Gam (1), gambogic acid (1 mg/kg/day) group; Gam (5), gambogic acid (5 mg/kg/day) group; Gam (10), gambogic acid (10 mg/kg/day) group.

blotting. There was a significant increase in HIF-1α protein expression in the RA rat model group, compared with sham group (Fig. 9). Correspondingly, treatment with gambogic acid significantly suppressed the activation of HIF-1α protein expression in RA rats (Fig. 9).

Discussion

RA is a systemic autoimmune disease with a primary manifestation of corrosive arthritis. The predominant pathological alterations are chronic inflammation of synovial tissues (18). Lesions violate multiple joints of body, generally beginning with facet joints, including hands and feet, present symmetry,

and may additionally result in systematic diseases, including rheumatoid, vasculitis and pericarditis (19). Mortality rates are positively correlated with age. The disability rate of the disease is high, having a strong impact on physical and psychological health and daily quality of life (20). At present, anti-inflammatory drugs, slow acting anti-rheumatic drugs, glucocorticoids and biological agents are used as therapeutic agents in clinics, however long-term application of these drugs may result in serious gastrointestinal symptoms and damage of the liver and kidney (21,22). In the present study, gambogic acid significantly inhibited the degree of right foot swelling, reduced the clinical arthritic score of RA and increased the pain threshold of the RA rats. These results suggest that

gambogic acid may act as a potential future therapeutic reagent in the treatment of RA.

At the later stages of RA, cartilago articularis and bone tissues of patients are seriously corroded and ultimately result in damage and functional loss of the entire joint structure (9). RA affects ~1% of the total population worldwide (23). It is reported that inflammatory cells and chemotactic factors are important in the development and progression of RA and autoimmune diseases. TNF- α , IL-1 β and IL-6 are important in mediating RA-associated mortality, the synovial inflammatory response and the process of osteoclasia (21). At present, inhibitors of IL-1 β and IL-6 have already been used for clinical research and therapy of RA and exhibit beneficial effects (24). The findings of the present study revealed that gambogic acid significantly suppressed the promotion of IL-1 β and IL-6 activities in RA rats.

PI3K/Akt signaling regulates proliferation and differentiation of B lymphocytes by activating mTOR (12). mTOR is the target molecule of sirolimus and is vital in the regulation of cell growth and proliferation. mTOR forms two types of complexes (C) with different functions, including mTORC1 and mTORC2 (11). mTORC1 is sensitive to sirolimus, whereas mTORC2 is not sensitive to sirolimus. mTORC1 reinforces protein synthesis via phosphorylation of 4E-binding protein 1 and ribosomal protein S6 kinase B1. mTOR signaling impacts the cell cycle, cell growth and cell proliferation (25). In numerous human cancers, various important tumor-inhibiting factors (PTEN, tuberous sclerosis 1/2, liver kinase B1) in the mTOR signaling pathway are deficient. Cell mutation and gene amplification in PI3CA (P110 α subtype of PI3K), in addition to an Akt activated mutation, result in increases in cell proliferation (13). The present study demonstrated that gambogic acid significantly prevented the decrease in p-Akt and p-mTOR protein expression induced by RA in the rat models. Activation of the PI3K/Akt/mTOR signaling pathway was important in the effect of gambogic acid on RA. Liu *et al* (14) suggested that gambogic acid induces G0/G1 cell cycle arrest via the PI3K/Akt/mTOR signaling pathway.

Vascular endothelial growth factor is additionally termed vascular permeability enhancement factor (26). It participates in human embryonic development, wound healing, hair growth and diabetic retinopathy, with pathological and physiological roles. The molecular weight of VEGF is ~46 kDa. It is a dimer glycoprotein molecule comprised of two monomers combined, and has two important physiological functions (27). Pro-blood vessel endothelium proliferation is the leading function of VEGF. It is an endothelial cell mitogen and promotes fission of endothelial cells in order to promote neovascularization. The VEGF receptor is located in vascular endothelial cells (28). Therefore, the function of VEGF is concentrated and has stronger specificity (27). VEGF may additionally increase permeability of blood capillaries and is the blood vessel anti-reflection molecule that exhibits the strongest biological effect, >5 million times more potent compared with histamine substances (28). The results of the present study suggested that gambogic acid significantly suppressed the activation of VEGF protein expression, blocking activation of the VEGF signaling pathway in RA rats. Lu *et al* (16) suggested that gambogic acid may be a

structurally novel angiogenesis inhibitor via suppressing VEGF production.

HIF-1 α is an important regulatory factor of histiocyte anoxia. RA intra-articular anoxia results in an upregulation of HIF-1 α expression in synovial tissues and regulates multiple signaling pathways, including inflammation, angiogenesis, cell invasion and proliferation, and intensifies the occurrence and development of RA (29). In the pathological state, HIF-1 α expression is abnormal. The RA intra-articular environment is an anaerobic environment which leads to high expression of HIF-1 α in synovial tissues (30). High expression of HIF-1 α may promote RA in synovial tissues, secrete chemotactic factors, recruit monocytes, T and B lymphocytes, reinforce differentiation of T helper 17 cells directly and participate in the overall inflammatory response of RA (31). Furthermore, HIF-1 α may additionally promote VEGF expression and the subsequent generation of blood vessels (31). In the present study, gambogic acid significantly suppressed the activation of HIF-1 α protein expression in RA rats. Lu *et al* (16) demonstrated that gambogic acid inhibited angiogenesis via suppression of the HIF-1 α pathway. These findings suggested that gambogic acid downregulates HIF-1 α , in treatment of RA.

In conclusion, the present study demonstrated that gambogic acid significantly inhibited the degree of right foot swelling, increased pain threshold, reduced clinical arthritic score and suppressed inflammation in RA rats, via regulation of the Akt, mTOR, VEGF and HIF-1 α signaling pathways. Therefore, gambogic acid may act as a potential future therapeutic reagent in the treatment for RA.

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