Current Medical Science
DOI https://doi.org/10.1007/s11596-019-2106-3
39(5):784-793,2019

Effectiveness of Huai Qi Huang Granules on Juvenile Collagen-induced Arthritis and Its Influence on Pyroptosis Pathway in Synovial Tissue*

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Summary: Huai Qi Huang (HQH) exerts great effects in clinic, such as anti-inflammation, immune-regulation, anti-cancer, and so on. However, the mechanism by which HQH protects juvenile idiopathic arthritis (JIA) is obscure. Thus, we explored deeply the protective mechanisms in juvenile collagen-induced arthritis (CIA) rat model. Pyroptosis is Gasdermin D (GSDMD)-dependent programmed cell death, involved in many diseases, such as sepsis. We investigated whether GSDMD-induced pyroptosis take part in mechanisms of juvenile CIA arthritis. Juvenile Wistar rats (3–4 weeks) were injected intradermally with fully emulsified bovine type II collagen and complete Freund's adjuvant to establish CIA rat models. Later, the CIA rats received oral administration of HQH (4.16 g/kg) once a day from the day 21 of modeling, with the treatment lasting for 28 days. Varieties of indicators were measured for evaluation of anti-inflammation effect of HQH, including hind paw swelling, arthritis scores, micro CT, and histopathological changes and the level of pro-inflammatory cytokines in the serum, including tumor necrosis factor alpha (TNF-α) and interleukin-18 (IL-18). The expression of GSDMD and caspase-1 in the joint synovial tissues was detected. The results demonstrated that the expression of the pyroptotic protein GSDMD and its upstream caspase-1 was significantly increased in the synovial tissues of CIA rats. The treatment of HQH ameliorated the symptoms in CIA rats, reduced levels of pro-inflammatory cytokines and hind paw swelling, down-regulated the expression of GDSMD and caspase-1. GSDMD-induced pyroptosis participated in the pathogenesis of CIA rats. The study supported that HQH can effectively improve joints inflammation of juvenile collagen-induced arthritis rats by inhibiting pyroptosis pathway in the joint synovial tissues.
Key words: Huai Qi Huang; juvenile collagen-induced arthritis; Gasdermin D; pyroptosis

Juvenile idiopathic arthritis (JIA) is a chronic, autoimmune inflammatory arthritis with multi-system involvement and is the most common pediatric rheumatic disease causing disability and blindness to children[1]. The pathogenesis of JIA is still unknown, though infection, immunology and heredity are linked to JIA pathogenesis. JIA is classified into systemic, oligo articular, polyarticular, psoriatic, enthesitis-related arthritis, and undifferentiated arthritis, and this classification is based on clinical features of arthritis. The prevalence of JIA range was between 3.8 to 400 cases/100 000 children, and an annual new incidence case is between 1.6 and 23 cases/100 000 children, systemic onset JIA (SoJIA) accounts for approximately 30%–40% of all JIA cases in Asia[2]. Currently, the incidence of JIA in the world is increasing, hence threatening children's life and health, bringing heavy mental and financial burden to society and families. SoJIA is considered as an auto-inflammatory disease and differs from other JIA type, due to the absence of specific autoantibodies or susceptibility to major histocompatibility complex (MHC) alleles, also because of its clinical presentation including fever and extra-articular manifestations like erythematous rash, serositis, hepatosplenomegaly[3, 4].

Autoinflammatory diseases are a group of heterogeneous diseases caused by defects in a number of molecules, such as inflammasomes, proteases, cytokine receptors or antagonists, and various enzymes, characterized by recurrent fever, local or systemic inflammation, and an absence of infectious agents, detectable autoantibody or antigen-specific autoactive T cells[4–6]. Canonical inflammasomes are multi-protein complexes composed of a NOD-like receptor (NLR)/an AIM-like receptor (ALR), the

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*This study was supported by the National Natural Science Foundation of China (No. 81270949).

adapter molecule apoptosis-associated speck-like protein that contains a CARD (ASC), and the effector protein caspase-1, including NLRP3, NLRP1b, NLRC4, AIM2[7, 8]. Caspase-1 is activated by various activated canonical inflammasomes, which trigger pyroptosis[9]. Gasdermin D (GSDMD) is an executor of pyroptosis, which is cleaved by activated inflammatory caspases-1 into Gasdermin N-terminal and Gasdeimin C-terminal. N-terminal can assemble membrane pores to induce cytolysis, resulting in programmed necrosis-cell death, while C-terminal inhibits pyroptosis through intramolecular association with the N-terminal domain[10–14]. Subsequently activated caspases-1 simultaneously promotes pro-inflammatory cytokines IL-1β and IL-18 precursors into a mature form, and a large number of them are released through the pores formed by GSDMD N-term, resulting in strong inflammatory reaction. Obvious pyroptosis has significant difference from necrosis and apoptosis[15]. GSDMD as the crucial executor of pyroptosis plays a key role in many kinds of diseases, such as infectious disease, immune disorders, and cancer[16–18], and is also involved in neurodegenerative disease, liver disease and cardiovascular diseases[19–21]. Despite of the important functions of GSDMD in immune and inflammatory diseases, it still remains unknown whether GSDMD-induced pyroptosis participates in the pathogenesis of JIA or rheumatoid arthritis (RA).

Huai Qi Huang (HQH) granules with the brand name Huan Er Jin are composed of ancient Chinese medicines of Huaier, Chinese wolfberry and polygonatum sibiricum. Pharmacology shows that Huaier has functions of anti-inflammatory, regulating the immune system, anti-allergy, improving microcirculation and promoting tissue repair. Huaier is a kind of medicinal fungus which has been used for more than 1600 years. Its extracts polysaccharide protein have significant effects on promoting apoptosis of cancer cells, enhancing chemosensitivity, reversing drug resistance and enhancing anti-tumor immunity[22, 23]. Polysaccharide protein (PS-T) is the main active ingredient of Huaier fungi and Huaier extract. Previous studies have confirmed that Huaier can significantly induce the production of Th1 cytokines such as IFN-α and IFN-γ in human and mouse. Huaier has a synergistic effect on IFN-α to promote the activity of NK cells, which in turn secretes cytokines such as IFN-γ and IL-2. It also can activate macrophages to produce IFN-α, IL-1 and so on. Under the synergistic action of these cytokines, the cellular immune response is obviously enhanced, and the anti-tumor and anti-virus effects are exerted. Different from exogenous cytokines, the Th1 cytokines such as IFN-α, γ, IL-2 induced by huaier are endogenous and have the features of low dose, multi-stimulation and combined action. Therefore, they have their own features compared with other polysaccharide drugs and are regarded as an ideal immune modulator[24].

Current treatments of JIA and RA include non-steroidal anti-inflammatory drugs, disease-modifying anti-rheumatic drugs, and biological agents which put into use in recent years . Though these drugs can quickly improve the condition and alleviate symptoms, there are many adverse reactions of glucocorticoids when used for a long time, rapid recurrence after stopping the drug, and side effects of the expensive biological agents are still unclear. Traditional Chinese medicine has good tolerance and can reduce recurrence after long-term maintenance in treatment of JIA or RA. The anti-inflammatory and anti-arthritic activities of many Chinese herbs have been validated in experimental models of arthritis and tested in clinical trials in RA patients[25]. At present, there is no report of HQH granule in the treatment of immune arthritis, and there is no basis for direct treatment of arthritis with HQH granule. Based on the above situation, according to the features of Huaier and the pathogenesis of JIA, this study explored the treatment effect of HQH granules on juvenile collagen-induced arthritis and its underlying mechanism.

1 MATERIALS AND METHODS

1.1 Chemicals and Reagents

HQH granules were obtained from Qidong Gaitianli Medicine Co., Ltd. (China). Prednisone acetate tablets were purchased from Tianjin Lisheng Pharmaceutical Co., Ltd. (China). Bovine collagen type II (CII) and Complete Freund's adjuvant were purchased from Chondrex, Inc. (USA). The electronic caliper was purchased from Lujiang Tools Manufacturing Co., Ltd. (China). The small animal living micro-cCT (SkyScan 1176) instrument was purchased from Bruker (Germany) and provided by the Public Technology Platform of Tongji Medical College, Huazhong University of Science and Technology. The IL-18 enzyme-linked immunosorbent assay kit (ab213909) was supplied by Abcam (UK). TNF-α ELISA Kit (ERC102a) is acquired from Neobioscience Biotechnology Co., Ltd. (China). Gasdermin-D polyclonal antibody (abs143289) was purchased from Absin Bioscience Biotechnology Co., Ltd. (China). Caspase-1 (ab108362) monoclonal antibody was acquired from Abcam (UK).

1.2 Animals

A total of 50 male Wistar rats (2–3 weeks, 45–60 g) were purchased from Hubei Experimental Animal Research Center (animal certificate number: scxk2015-0018), raised in the animal room of Tongji Medical College. During the experiment, according to the standard animal experiments protocol, rats were housed four to five per cage, under specific pathogen-free (SPF) conditions, at a constant temperature of 22–24°C, humidity 60%–65%, fed freely with food

and water at standard laboratory, and kept under a 12 h dark/light cycle. The experiment was started after a week of adaptive feeding.

1.3 Induction of Juvenile Collagen-Induced Arthritis (CIA)

Bovine CII (2 mg/mL, dissolved in 0.05 mol/L glacial acetic acid) and an equal amount of Complete Freund's adjuvant (4 mg/mL, heat-killed mycobacterium tuberculosis dissolved in Eph) mixed into emulsifier, using ultrasound until the CII/CFA emulsion remained unscattered on the water. The emulsion cooled at ice until use. CIA model rats were intracutaneously injected with 0.25 mL CII/CFA emulsion at 2 cm from the base of the tail on day 1. On day 7 after the primary immunization, the rats were boosted with 0.15 mL CII-CFA emulsion at different sites from the initial injection on the tail. The control group (C) rats were injected with the same volume of physiological saline at the same site of the tails[26].

1.4 Drug and Group Intervention

After the initial immunization, the weight and ankle joint thickness were measured every 3 days, and the activity and general condition were observed. At the same time, the arthritis index (AI) was measured on the model of CIA twice a week. Briefly, the thickness of two hind paws was measured twice using digital vernier calipers at the same site of rats by the same person always, and the average of two values for hind paws was measured as the paw thickness. The severity of arthritis in each hind paw was monitored and scored on a scale of 0–5, where 0: no redness or swelling; 1: slight swelling in the ankle or redness in the foot; 2: progressive swelling, inflammation and redness from the ankle to the midfoot; 3: swelling and inflammation of the entire foot, not including the toes; 4: swelling and inflammation of the entire foot, including the toes; and 5: swelling and inflammation of the entire foot, with loss of mobility[27]. The total score of the two hind paws can be up to 10 points. More than 6 points of rats at 21 days of initial immunization indicate successfully modeling (modeling success rate: 75%). To explore the effect of HQH granules and their synergy with prednisone, we randomly divided rats with AI more than 6 on the 21st day into 4 groups (n=6 in each group): CIA model (M) group , prednisone (P) group, HQH (H) group, prednisone and HQH (HP) group. According to human and rat drug dose conversion coefficient of 6.25, each rat in the experimental groups was intragastrically administered with 4.16 mg/kg prednisone solution and 4.16 g/kg HQH suspension solution every day for four weeks, while the rats of control group were intragastrically administered with the same amount of normal saline.

1.5 Ankle Joint Micro-CT

After four weeks of intervention, 10% chloral hydrate (0.4 mL/kg) was injected intraperitoneally. Then, one of hind limbs was harvested and fixed with 4% paraformaldehyde. Two ankle joints were randomly taken from each group, and erosion and destruction of the bone were observed by Small live animal Micro-CT (resolution: 18 μm).

1.6 Ankle Joint and Knee Join Synovial Tissue Histological Examinations

One of hind paws of every rat was fixed for 48 h using 4% paraformaldehyde, then decalcified with 10% EDTA decalcifying solution, and decalcifying liquid was replaced every 3 days for 2 to 3 months until there was no resistance to acupuncture ankle joint. Tissues were embedded by paraffin, sectioned (4 μm), stained by hematoxylin-eosin. For semi-quantitative histological evaluation, the stained tissue sections were examined by two independent observers blindly and graded on a scale of 0–4, where 0: no detectable abnormalities, 1: some inflammatory cell infiltration in the synovial membrane with no significant fibrosis or cartilage erosion, 2: joint sections with extensive inflammatory cell infiltration and thickening of the synovial membrane, 3: joint sections with severe lesion, fibrosis and presence of inflammatory cells, while the most severe lesion with significant fibrosis, involvement of the articular cartilage, the influx of inflammatory cells in the joint space and extensive synovitis was graded as 4[28]. Measurements were carried out using ordinary upright microscope (n=6, objective lens magnification ×40). Three knee joint synovial tissues randomly from each group were fixed with 4% paraformaldehyde, and the remaining joints were preserved at –80°C. After 48 h of fixation, they were stained with hematoxylin-eosin and synovial cell proliferation, hypertrophy, inflammatory cell infiltration and vascular proliferation observed.

1.7 Enzyme-linked Immunosorbent Assay

Blood was collected from the abdominal aorta and centrifuged at 4000 r/min for 5 min. The serum was stored at –80°C for cytokine analysis. The concentrations of IL-18 and TNF-α in the serum were analyzed according to the procedure of the ELISA kit.

1.8 Immunohistochemistry

The expression of GSDMD in synovial tissue was assessed by immunohistochemistry. Rat knee synovial tissues were sectioned (4 μm), embedded by paraffin, dewaxed, repaired, blocked, and the sections were incubated with primary antibodies containing GSDMD (1:500) at 4°C overnight. The secondary antibody was incubated for 50 min at room temperature. Then, DAB was used for color development, and the color of positive cells was brownish yellow. The nuclei were stained with hematoxylin at room temperature, and its color was blue. Finally, the acquisition of images was from an optical microscope. The area ratio of positive stained areas was analyzed and calculated using ImageJ-win64 software. Three images (magnification,

×40) were randomly selected from every tissue of each group (n=3), and the results were the average of the ratio of the positive areas.

1.9 Western Blotting

Synovial tissues frozen at –80°C were lysed through the grinder and sonication in the ice bath after adding cell lysis buffer RIPA and enzyme inhibitor. After centrifugation for 15 min (12 000 r/min, 4°C), the supernatant was protein solution. The concentration was measured by BCA Protein Assay kit (Thermo Fisher Scientific, USA). All protein samples were adjusted to the same concentration of 2.5 μg/μL. After adding loading buffer solution, the protein was fully denatured at 100°C for 5 min. Western blotting steps are as follows: loading, electrophoresis, transmembrane, blocking (5% milk), incubating with primary antibody (5% BSA dilution, antibody dilution ratio 1:1000) overnight at 4°C, washing membrane (0.1% TBST), incubating with secondary antibody at room temperature for 1 h, washing membrane again. Then color was developed with the ECL detection system and an image of the immune response strip acquired. The results were the ratio of gray value of the target protein to the reference.

1.10 Statistical Analysis

All statistical analyses were performed by SPSS 24.0 software (IBM, USA). Results were expressed as mean±standard deviation ($\bar{x}\pm s$). One-way analysis of variance (ANOVA) was used to analyze the difference among five groups. And P value less than 0.05 was considered as statistically significant. All the statistical analysis graphs were performed using graph pad prism 7.0 software (Graph Pad Software, Inc., USA).

2 RESULTS

2.1 General Conditions and Changes

Ulcers and nodules appeared in the injection site of tail about 5 days after the initial immunization. Most of the ulcers were tended to heal about 10 days after the initial immunization and all the nodules disappeared about 21 days after the initial immunization. In the model group, part of the rats' ulcers (n=2) in the tail root unhealed and gradually extended (fig. 1C). Compared with the control group, diet, fur gloss, activity and resistance in the CIA model group were poor. Weight was not increased as fast as the control group (P<0.05) (fig. 1A). Subsequently, claudication and extension disorder of ankle joints occurred in the CIA model group about 28 days after the initial immunization. After the treatment, the general conditions, joint extension disorder and lameness were generally relieved in the three treatment groups. The body weight of the three treatment groups and the model group was significantly different from that of the control group (P<0.05). However, there was no significant difference in the body weight between the three treatment groups and the model group (P>0.05).

2.2 Swelling Degree and AI of the Hind Limb Joints (fig. 2)

The ankle joint began to swell on the 12th day, and gradually extended to the metatarsophalangeal joint, reaching to a peak around the 18th day. The skin of hind limbs and toes was swollen and skin temperature of the

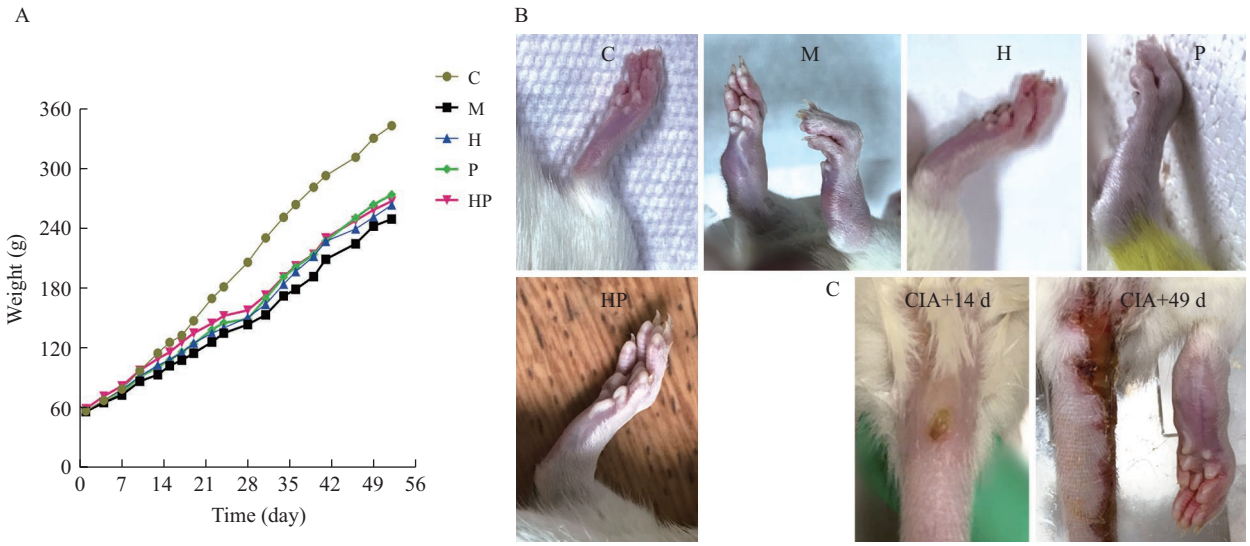


Fig. 1 The weight growing of each group

A: the initial immunization on the 7th day and the second immunization on the 14th day. In HQH, P and HP groups, the development and progression of CIA rats were inhibited. B: the typical ulcer of CIA rats on the 14th day and severe ulcer of CIA rats on the 49th day

CIA: collagen-induced rheumatoid arthritis; C: control group; M: CIA model group; P: prednisone group; H: Huang Qi Huang group, HP: Huang Qi Huang and prednisone group

foot pads was elevated. After treatment for 1 week, the swelling of the joints in the P and the HP groups began to relieve. In the H group, the joint swelling began to regress about 2 weeks after the administration. On the first day of intervention (on the 21st day), there was no significant difference in AI between the treatment groups and the model group (P>0.05). After treatment for 4 weeks, the hind limb swelling and AI in the P group and the HP group were significantly lower than those in the M group (P<0.001). The swelling degree of H group was lower than that of the M group (P<0.05).

2.3 Inflammation Evaluation and Mechanisms Exploration

2.3.1 Bone Destruction in Rats (fig. 3) Two of the hind paws of each group were further examined by small live animal Micro-CT. Images of normal rat joints showed intact joint structure as well as normal bone surfaces. In the CIA rat model group, the ankle joint and metatarsophalangeal joint were obviously eroded, and the joint space was narrowed and even fused. In the three intervention groups, significant protection against bone destruction and preservation of the architecture of the affected hind joints appeared.

2.3.2 Evaluation of Inflammation of Ankle Joints and Synovial Tissue of Knee Joints (fig. 4) Sections of joints and synovial tissues were stained with hematoxylin and eosin (H&E) for a general histological evaluation. Representative synovial tissues (fig. 4A) and ankle joints (fig. 4B) of CIA rats were assessed by histological examination. The structure of ankle joints and synovial tissues of normal group was clear and smooth. However, we could observe intensive infiltration of inflammatory cells, pannus formation, a great number of synovial cells and fibrous tissue proliferation, and severe cartilage and bone destruction in the CIA model group. Compared with H and P groups, HP improved the above conditions more significantly. In the H, P and HP groups, the histological score of ankle joints was markedly decreased after four-week treatment compared to CIA rats (P<0.05) (fig. 4C).

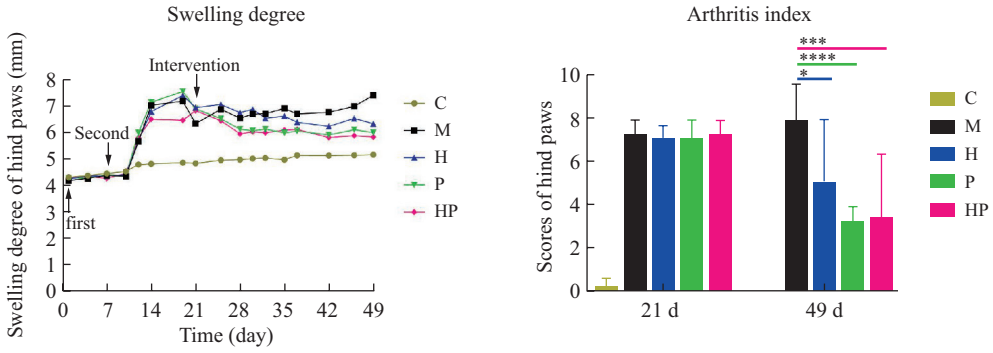


Fig. 2 The changes of swelling degree of hing paws in each group (swelling degree)
AI of every group on the 21st day (21 d, the first day of intervention) and 49th day (49 d, treatment for four weeks). $^{****}P$<0.0001, $^{***}P$<0.001, $^{*}P$<0.05 *vs.* CIA model group
C: control group; M: CIA model group; P: prednisone group; H: Huang Qi Huang group, HP: Huang Qi Huang and prednisone group

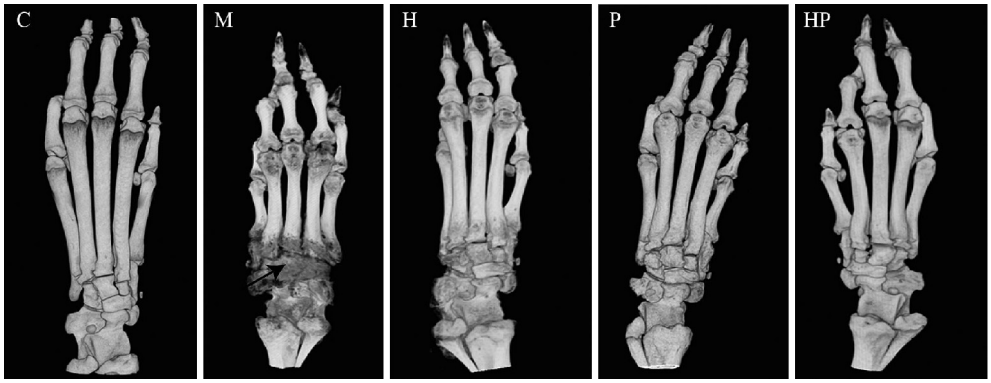


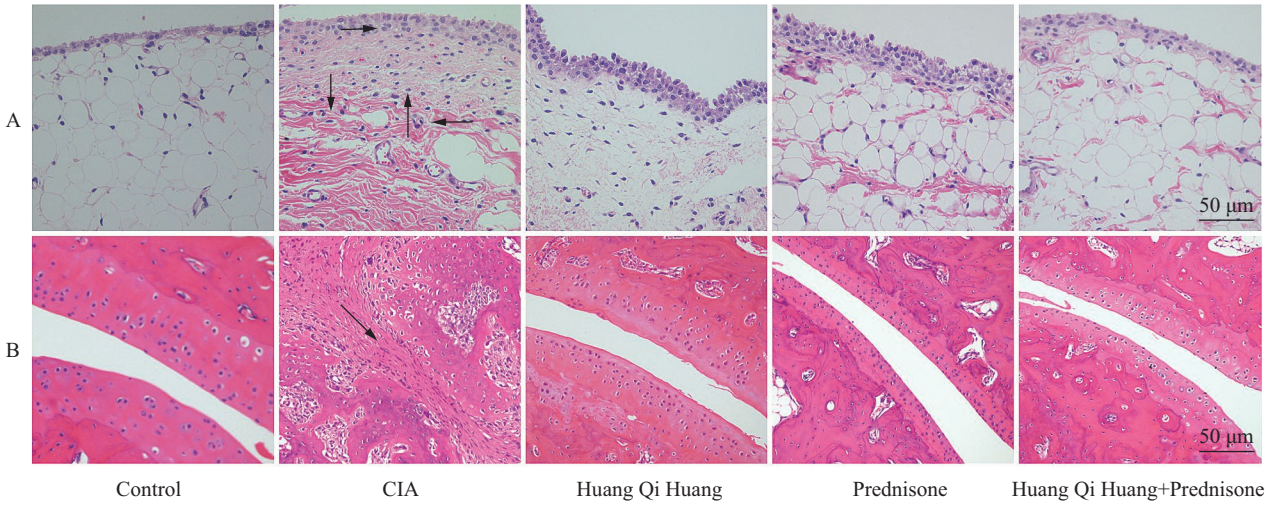
Fig. 3 Small live animal Micro-CT radiographs of the hind paws from five groups
The radiological changes were observed 28 days after oral treatment. From the images, severe destruction and bone erosion in a representative arthritic hind paw (M) were observed. Black arrow showed significant destruction of tarsometatarsal joint in CIA model group. These radiological changes were significantly improved in the H, P and HP groups.
C: control group; M: CIA model group; P: prednisone group; H: Huang Qi Huang group; HP: Huang Qi Huang and prednisone group

2.3.3 Pro-inflammatory cytokines in serum (fig. 5)

Serum pro-inflammatory cytokines including IL-18 and TNF-α in CIA rats were significantly increased when compared with the normal control group. Compared with the model group, IL-18 and TNF-α in the three treatment groups were significantly decreased (P<0.01).

2.3.4 Expression of GSDMD in Synovial Tissues of CIA Rats (fig. 6) GSDMD is a crucial executor of pyroptosis. We meant to explore whether pyroptosis pathway was involved in the pathogenesis of CIA in rats and whether HQH might improve the clinical symptoms by regulating the pyroptosis pathway. Using immunohistochemistry, we found the expression of GSDMD markedly increased in inflamed synovial tissues from CIA rats. Moreover, the expression of GSDMD was distinctly inhibited by treatment with HQH. The scores of the three groups were significantly lower than those of the CIA model group (all P<0.0001).

2.3.5 Expression of Caspase-1 and GSDMD in Synovial Tissues (fig. 7) To further investigate whether GSDMD-induced cell death pyroptosis participates in the pathogenesis of CIA in rats and if HQH exerts its effects on arthritis through this



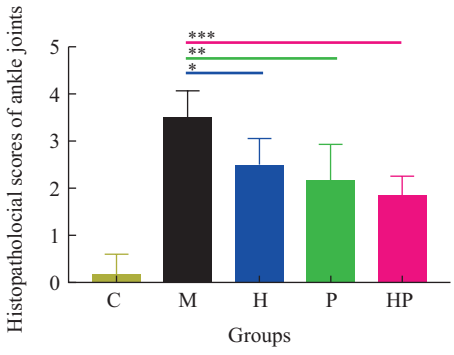


Fig. 4 The representative histological pathology sections changes of synovial tissues in knee joints (A, ×40) and ankle joints (B, ×20) and histopathological scores of ankle joints

A: In CIA model group, the right arrow indicates the thickened synovial cells, the left arrow indicates the proliferation of small vessels, the upward arrow indicates the infiltration of inflammatory cells, and the downward arrow indicates the proliferation of large amounts of fibrous tissue. B: The oblique arrow shows structural disorders of ankle and foot joints, serious destruction of articular cartilage and bone tissue, marked thickening of synovial layer, narrowing or even fusion of articular cavity, and infiltration of a large number of inflammatory cells. $^{***}P$<0.001, $^{**}P$<0.01, $^{*}P$<0.05. C: control group; M: CIA model group; P: prednisone group; H: Huang Qi Huang group; HP: Huang Qi Huang and prednisone group

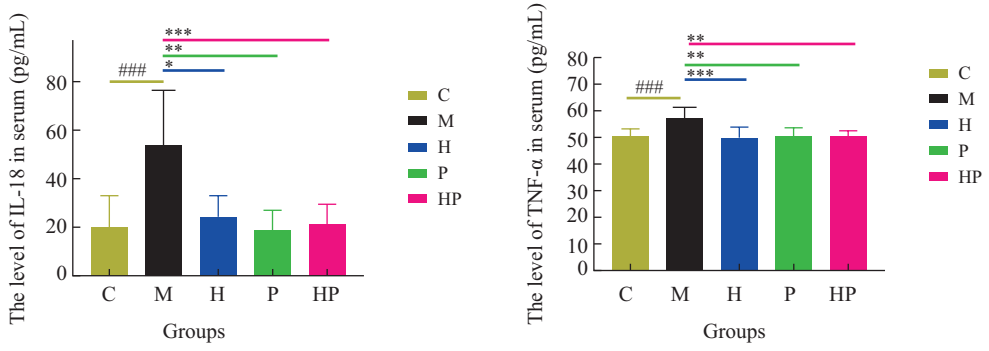


Fig. 5 Serum IL-18 and TNF-α levels in each group (mean±standard deviation, $\bar{x}\pm s$, n=6, pg/mL)

The CIA model group was compared with the normal control group ($^{###}P$<0.001, $^{##}P$<0.01). After treatment for four weeks, the three treatment groups were compared with the CIA model group ($^{**}P$<0.01, $^{***}P$<0.001).

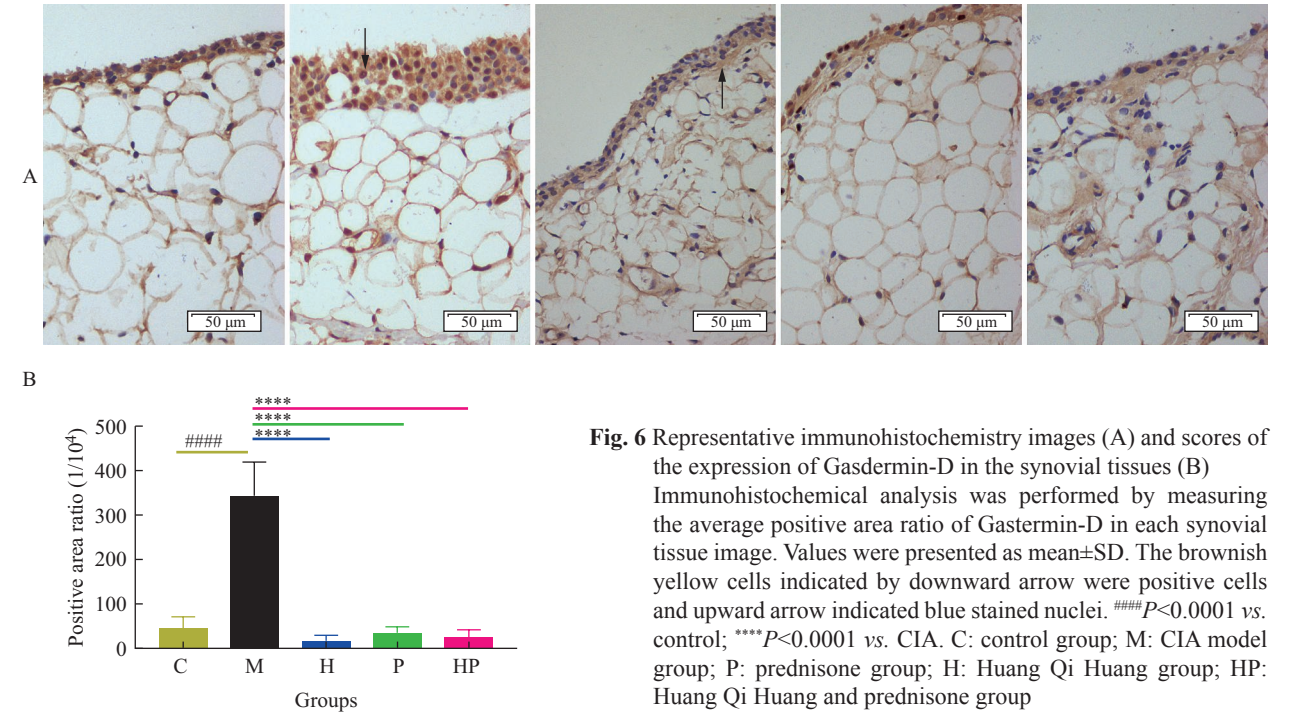


Fig. 6 Representative immunohistochemistry images (A) and scores of the expression of Gasdermin-D in the synovial tissues (B) Immunohistochemical analysis was performed by measuring the average positive area ratio of Gastermin-D in each synovial tissue image. Values were presented as mean±SD. The brownish yellow cells indicated by downward arrow were positive cells and upward arrow indicated blue stained nuclei. $^{\#\#\#\#}P<0.0001$ *vs.* control; $^{****}P<0.0001$ *vs.* CIA. C: control group; M: CIA model group; P: prednisone group; H: Huang Qi Huang group; HP: Huang Qi Huang and prednisone group

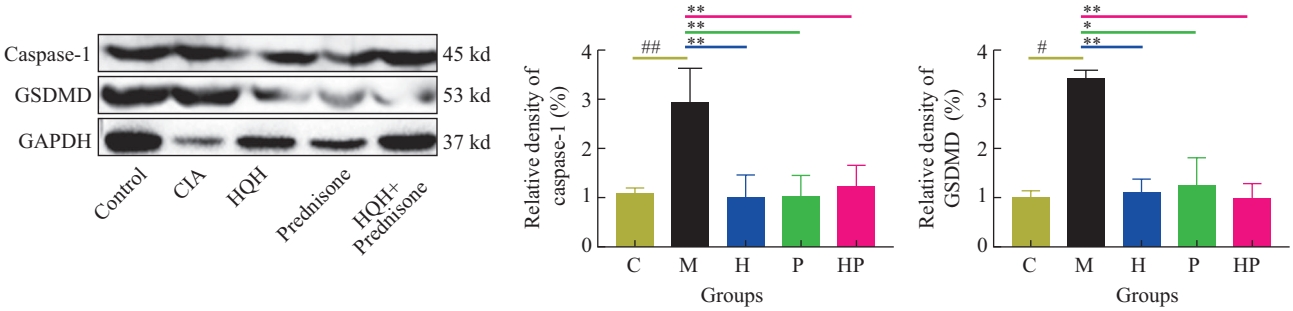


Fig. 7 The expression of GSDMD and caspase-1 in synovial tissue by Western blotting and gray value ratio of GSDMD and caspase-1 to GAPDHexpression in synovial tissue of each group
$^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* control; $^{*}P<0.05$, $^{**}P<0.01$ *vs.* CIA. C: control group; M: CIA model group; P: prednisone group; H: Huang Qi Huang group, HP: Huang Qi Huang and prednisone group

mechanism, we examined the expression of GSDMD and caspase-1 in synovial tissue by Western blotting. The results showed that the expression levels of GSDMD and caspase-1 in the CIA model group were significantly higher than those in the control group. Moreover, we found that the expression levels of GSDMD and caspase-1 in the synovial tissues of three treatment groups were decreased ($P<0.05$).

3 DISCUSSION

The studies about the role of the immune system in the pathogenesis of JIA in the past 10 years has been further developed. Current research on the pathogenesis of JIA focuses on immune cells and cytokines. IL-18 is produced primarily by macrophages, and its production is regarded as the beginning of innate immune system activation. As a member of the IL-1 superfamily, IL-18 is stored in inflammasomes and is the most effective cytokine that regulates the activity of natural killer cells (NK cells)[29]. The level of IL-18 in serum and synovial fluid in children with SoJIA was positively correlated with C-reactive protein, joint involvement, and IL-6 levels. High levels of serum IL-18 indicate SoJIA disease activity, and SoJIA is stable with a mild to moderate increase in IL-18 levels[30]. Although IL-18 was elevated in the serum of all children with JIA, the elevation of IL-18 was more obvious in children with SoJIA[31]. Some studies have found that children with SoJIA with elevated IL-6 levels may be associated with more joint involvement, while macrophage activation syndrome may develop in children with elevated IL-18[32]. TNF-α plays a key role in the immune pathogenesis and inflammation of JIA and is a key inflammatory factor in JIA. The increased TNF-α in serum and synovial fluid promotes systemic and local

inflammatory responses[33]. TNF-α not only participates in the synovial inflammatory response, but also induces joint destruction. Anti-TNF-α biological agents can not only rapidly reduce inflammation, control the symptoms of arthritis, effectively alleviate the disease, but also prevent the destruction of joint structure by specifically antagonizing TNF-α[34]. The cytokines IL-18 and TNF-α are abnormally elevated during disease activity in JIA patients[35]. At this experiment, the pro-inflammatory cytokines IL-18 and TNF-α were also significantly elevated in the CIA rats.

Pyroptosis is a GSDMD-dependent programmed cell death mediated by caspase-1, which is activated by various inflammasome complexes (including NLRP3, NLRC4, nlrp1, AIM2) in innate immunity, and subsequently the pro-inflammatory cytokines IL-1β and IL-18 are activated and released[36–38]. A20myel-KO (myeloid-cell-specific deletion of the RA-susceptibility gene) mice can trigger a spontaneous erosive polyarthritis that resembles RA in patients[39]. At the first experimental model to study the role of inflammasomes in RA pathology, the deletion of NLRP3 and caspase-1 markedly protected against RA-associated inflammation and cartilage destruction in A20 myel-KO mice[40]. In a study of analyzing gene expression of NLRP3 inflammasome components in RA patients treated with infliximab (anti-TNF-α monoclonal antibody), NLRP3 inflammasome components (including ASC, full-length NLRP3, short-length NLRP3 and caspase-1) were significantly higher in RA patients than in controls, and single nucleotide polymorphisms (SNPs) at the NLRP3 were associated with RA susceptibility and anti-TNF therapy[41, 42]. In this experiment, arthritis was induced in juvenile Wistar rats (3–4 weeks old) by immunogenic bovine type II collagen and complete emulsifier of Freund's adjuvant. In the juvenile rats, autoimmune reaction to collagen induced secondary hind limb arthritis with systemic inflammatory response. Moreover, the expression levels of GSDMD and caspase-1 were significantly increased in synovial tissue of CIA rats.

The main active ingredients of the traditional Chinese medicine HQH granules are polysaccharide protein extracted by hot water from fungi of Huaier and Chinese wolfberry. Huaier is a binding protein composed of 6 monosaccharides and 18 amino acids which have the functions of enhancing immunity, anti-oxidation and anti-tumor[22]. At present, the Huaier extract and its compound HQH granules have attracted great attention and increased its use in the field of anti-tumor and regulating immune function in China[23, 43–46]. In recent years, it has been found that HQH granules also protect podocytes, reduce the level of protein in urine, and contribute to the recovery of glomerular function in kidney diseases[47–49]. In this study, after four weeks treatment with Chinese traditional medicine HQH and prednisone, the arthritis scores, the average degree of swelling of hind limbs, the micro-CT of hind limbs and the synovial and ankle joint HE staining indicated that they significantly relieved the joint swelling and inflammatory reaction, markedly alleviated the bone destruction and erosion of articular cartilage and bone tissue, and reduced the inflammatory cell infiltration of synovial tissue and inhibited proliferation of synovial cells, and hyperplasia of fibrous tissue and blood vessel. The results showed that HQH granules reduced joint and synovial tissue inflammation action. What's more, the expression of GSDMD and caspase-1 in synovial tissue and pro-inflammatory cytokine IL-18 and TNF-α also markedly decreased through the treatment.

We conjectured that it can reduce the expression of GSDMD and caspase-1 in the synovial tissues and the levels of IL-18 and TNF-α in serum to exert anti-inflammatory effects. Also combination of HQH with prednisone had synergic effect by markedly reducing inflammation. This ultimately may provide an alternative intervention approach for some diseases involving GSDMD-induced pyroptosis, such as infectious diseases, and sepsis[50].

In conclusion, we found that GSDMD-induced pyroptosis participates in collagen-induced arthritis in rats and inferred that HQH can ameliorate collagen-induced RA in rats, which might be partially attributed to inhibiting the activation of the GSDMD-induced pyroptosis pathway. The study is useful because it may play a role in developing novel therapeutic strategies for JIA and RA.

Conflict of Interest Statement

The authors declare that there is no conflict of interest relevant to this article.

REFERENCES

1 Fellas A, Hawke F, Santos D, *et al*. Prevalence, presentation and treatment of lower limb pathologies in juvenile idiopathic arthritis: A narrative review. J Paediatr Child Health, 2017,53(9):836-840
2 Cimaz R. Systemic-onset juvenile idiopathic arthritis. Autoimmun Rev, 2016,15(9):931-934
3 Nigrovic PA. Autoinflammation and autoimmunity in systemic juvenile idiopathic arthritis. Proc Natl Acad Sci USA, 2015,112(52):15 785-15 786
4 Aksentijevich I, Mcdermott MF. Lessons from characterization and treatment of the autoinflammatory syndromes. Curr Opin Rheumatol, 2017,29(2):187-194
5 Cush J J. Autoinflammatory syndromes. Dermatol Clin, 2013,31(3):471
6 Pepper RJ, Lachmann HJ. Autoinflammatory syndromes in children. Indian J Pediatr, 2016,83(3):242-247
7 Yang C, Chiang B. Inflammasomes and human autoimmunity: A comprehensive review. J Autoimmun, 2015,61:1-8
8 Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. Cell, 2014,157(5):1013-1022

9 Shi J, Zhao Y, Wang K, *et al*. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature, 2015,526(7575):660-665
10 Ding J, Wang K, Liu W, *et al*. Pore-forming activity and structural autoinhibition of the gasdermin family. Nature, 2016,535(7610):111
11 Yang J, Liu Z, Wang C, *et al*. Mechanism of gasdermin D recognition by inflammatory caspases and their inhibition by a gasdermin D-derived peptide inhibitor. Proc Natl Acad Sci USA, 2018,115(26):6792-6797
12 Liu X, Zhang Z, Ruan J, *et al*. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature, 2016,535(7610):153
13 Shi J, Gao W, Shao F. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. Trends Biochem Sci, 2017,42(4):245-254
14 Liu Z, Wang C, Rathkey JK, *et al*. Structures of the Gasdermin D C-Terminal Domains Reveal Mechanisms of Autoinhibition. Structure, 2018,26(5):778
15 He WT, Wan H, Hu L, *et al*. Gasdermin D is an executor of pyroptosis and required for interleukin-1beta secretion. Cell Res, 2015,25(12):1285-1298
16 Gao Y, Zhai J, Chai Y. Recent Advances in the Molecular Mechanisms Underlying Pyroptosis in Sepsis. Mediat Inflamm, 2018(5823823)
17 Kanneganti A, Malireddi RKS, Saavedra PHV, *et al*. GSDMD is critical for autoinflammatory pathology in a mouse model of Familial Mediterranean Fever. J Exp Med, 2018,215(6):1519-1529
18 Wang WJ, Chen D, Jiang MZ, *et al*. Downregulation of gasdermin D promotes gastric cancer proliferation by regulating cell cycle-related proteins. J Digest Dis, 2018,19(2):74-83
19 Mckenzie BA, Mamik MK, Saito LB, *et al*. Caspase-1 inhibition prevents glial inflammasome activation and pyroptosis in models of multiple sclerosis. Proc Natl Acad Sci USA, 2018,115(26):E6065-E6074
20 Mitra S, Exline M, Habyarimana F, *et al*. Microparticulate Caspase 1 Regulates Gasdermin D and Pulmonary Vascular Endothelial Cell Injury. Am J Resp Cell Mol, 2018,59(1):56-64
21 Xu B, Jiang M, Chu Y, *et al*. Gasdermin D plays a key role as a pyroptosis executor of non-alcoholic steatohepatitis in humans and mice. J Hepatol, 2018,68(4):773-782
22 Han J, Lin M, Zhou D, *et al*. Huang Qi Huai Granules Induce Apoptosis in Acute Lymphoblastic Leukemia Cells through the Akt/FoxO1 Pathway. Cell Physiol Biochem, 2016,38(5):1803-1814
23 Song X, Li Y, Zhang H, *et al*. The anticancer effect of Huaier. Oncol Rep, 2015,34(1):12-21
24 Zhang S, Cui L. Research progress of huaier and its compound preparation Huai Qi Huang granules. Yixue Zongshu (Chinese), 2015,21(1):114-116
25 Moudgil KD, Berman BM. Traditional Chinese medicine: potential for clinical treatment of rheumatoid arthritis. Expert Rev Clin Immu, 2014,10(7): 819-822
26 Shan J, Peng L, Qian W, *et al*. Integrated Serum and Fecal Metabolomics Study of Collagen-Induced Arthritis Rats and the Therapeutic Effects of the Zushima Tablet. Front Pharmacol, 2018,9:891
27 Hsu Y, Hsieh P, Chang M. Interleukin-19 blockade attenuates collagen-induced arthritis in rats. Rheumatology, 2012,51(3):434-442
28 Ansari MM, Neha, Khan HA. Effect of cadmium chloride exposure during the induction of collagen induced arthritis. Chem Biol Interact, 2015,238:55-65
29 Shimizu M, Nakagishi Y, Inoue N, *et al*. Interleukin-18 for predicting the development of macrophage activation syndrome in systemic juvenile idiopathic arthritis. Clin Immunol, 2015,160(2):277-281
30 Shimizu M, Nakagishi Y, Kasai K, *et al*. Tocilizumab masks the clinical symptoms of systemic juvenile idiopathic arthritis-associated macrophage activation syndrome: the diagnostic significance of interleukin-18 and interleukin-6. Cytokine, 2012,58(2):287-294
31 de Jager W, Vastert SJ, Beekman JM, *et al*. Defective phosphorylation of interleukin-18 receptor beta causes impaired natural killer cell function in systemic-onset juvenile idiopathic arthritis. Arthritis Rheumatol, 2009,60(9):2782-2793
32 Shimizu M, Nakagishi Y, Kasai K, *et al*. Tocilizumab masks the clinical symptoms of systemic juvenile idiopathic arthritis-associated macrophage activation syndrome: the diagnostic significance of interleukin-18 and interleukin-6. Cytokine, 2012,58(2):287-294
33 Scardapane A, Ferrante R, Nozzi M, *et al*. TNF-α gene polymorphisms and juvenile idiopathic arthritis: Influence on disease outcome and therapeutic response. Semin Arthritis Rheu, 2015,45(1):35-41
34 Kaminiarczyk-Pyzalka D, Adamczak K, Mikos H, *et al*. Proinflammatory cytokines in monitoring the course of disease and effectiveness of treatment with etanercept (ETN) of children with oligo- and polyarticular juvenile idiopathic arthritis (JIA). Clin Lab, 2014,60(9):1481-1490
35 Takahara T, Shimizu M, Nakagishi Y, *et al*. Serum IL-18 as a potential specific marker for differentiating systemic juvenile idiopathic arthritis from incomplete Kawasaki disease. Rheumatol Int, 2015,35(1):81-84
36 Shi J, Gao W, Shao F. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. Trends Biochem Sci, 2017,42(4): 245-254
37 Russo HM, Rathkey J, Boyd-Tressler A, *et al*. Active Caspase-1 Induces Plasma Membrane Pores That Precede Pyroptotic Lysis and Are Blocked by Lanthanides. J Immunol, 2016,197(4):1353-1367
38 Vanaja SK, Rathinam VAK, Fitzgerald KA. Mechanisms of inflammasome activation: recent advances and novel insights. Trends Cell Biol, 2015,25(5):308-315
39 Matmati M, Jacques P, Maelfait J, *et al*. A20 (TNFAIP3) deficiency in myeloid cells triggers erosive polyarthritis resembling rheumatoid arthritis. Nat Genet, 2011,43(9):129-908
40 Vande Walle L, Van Opdenbosch N, Jacques P, *et al*. Negative regulation of the NLRP3 inflammasome by A20 protects against arthritis. Nature, 2014,512(7512):69
41 Mathews RJ, Robinson JI, Battellino M, *et al*. Evidence of NLRP3-inflammasome activation in rheumatoid arthritis (RA); genetic variants within the NLRP3 -inflammasome complex in relation to susceptibility to RA and response to anti-TNF treatment. Ann Rheum Dis, 2014,73(6):1202-1210
42 Jenko B, Praprotnik S, Tomsic M, *et al*. NLRP3 and CARD8 Polymorphisms Influence Higher Disease

Activity in Rheumatoid Arthritis. J Med Biochem, 2016,35(3):319-323
43 Sun Y, Sun T, Wang F, *et al*. A polysaccharide from the fungi of Huaier exhibits anti-tumor potential and immunomodulatory effects, Carbohyd Polym, 2013,92(1):577-582
44 Wu T, Chen W, Liu S, *et al*. Huaier suppresses proliferation and induces apoptosis in human pulmonary cancer cells via upregulation of miR-26b-5p. Febs Lett, 2014,588(12):2107-2114
45 Li C, Wu X, Zhang H, *et al*. A Huaier polysaccharide inhibits hepatocellular carcinoma growth and metastasis. Tumor Biol, 2015,36(3):1739-1745
46 Xie H, Xu Z, Tang J, *et al*. Effect of Huaier on the proliferation and apoptosis of human gastric cancer cells through modulation of the PI3K/AKT signaling pathway. Exp Ther Med, 2015,10(3):1212-1218
47 Li T, Mao J, Huang L, *et al*. Huaiqihuang may protect from proteinuria by resisting MPC5 podocyte damage via targeting p-ERK/CHOP pathway. Bosnian J Basic Med, 2016,16(3):193-200
48 Sun W, Zhu Z, Yu J, *et al*. Effects of Chinese herbal medicine Huaiqihuang Granule on nephrin and podocin expressions in renal tissues of rats with adriamycin-induced nephrosis. Zhong Xi Yi Jie He Xue Bao (Chinese), 2011,9(5):546-552
49 Zhu C, Huang S, Ding G, *et al*. Protective effects of Huang Qi Huai granules on adriamycin nephrosis in rats. Pediatr Nephrol, 2011,26(6):905-913
50 Ma Y, Jiang J, Gao Y, *et al*. Research progress of the relationship between pyroptosis and disease. Am J Transl Res, 2018,10(7):2213-2219

(Received Mar. 6, 2019; revised Jun 20, 2019)