

Therapeutic Effect of Anwulignan on Knee Osteoarthritis and Its Mechanism through Cell Pyroptosis Pathway in Rats

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Abstract

Anwulignan (AN) is a monomer compound in the lignans of *Schisandra chinensis*. In this study, the effect of AN on knee osteoarthritis (KOA) in male SD rats was observed and its potential mechanisms were explored. The results showed that AN could significantly increase the number of standing times of rats within the same time, reduce the degree of knee joint swelling in rats, and alleviate the damage of cartilage tissue. AN could increase the activity of superoxide dismutase (SOD), and decrease the contents of malondialdehyde (MDA) and reactive oxygen species (ROS) in the synovial tissue of rats, and reduce the levels of inflammatory factors such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β and IL-18, prostaglandin E2 (PGE2) and nitric oxide (NO) in the serum and synovial tissue of rats. AN could play an antioxidant role by regulating the nuclear factor erythroid E2-related factor 2 (Nrf2) signaling pathway-related proteins, and play anti-inflammatory and anti-pyoptosis roles by regulating pyroptosis pathway-related proteins, including the nuclear factor-kappa gene binding (NF- κ B) signaling pathway-related proteins and NOD-like receptor family pyrin domain containing 3 (NLRP3). AN could significantly increase the expression level of CollagenII protein in KOA rats and decrease the expression levels of matrix metalloproteinase1 (MMP1) and matrix metalloproteinase13 (MMP13) proteins. The results of immunofluorescence analysis further confirmed the protective effect of AN against KOA in rats, which may be related to the regulation of Nrf2/HO-1 signaling pathway to play an antioxidant role and the regulation of TLR4/NLRP3/Caspase-1 signaling pathway-mediated anti-pyoptosis effect.

Introduction

Knee osteoarthritis (KOA) is a degenerative joint disease characterized by cartilage degeneration, subchondral osteoporosis and synovitis¹, with the main clinical manifestations of joint swelling, pain and limited mobility². In addition, the reduction of extracellular matrix (ECM) is also considered an important factor in the cartilage damage in KOA³. KOA is more common in middle-aged and elderly people⁴, seriously affecting the health and quality of life of people⁵. With the acceleration of population aging in recent years, the prevalence of KOA among elderly people aged 65 and above is relatively high, ranking the 11th among disabilities in the world⁶. Cytokines, matrix metalloproteinases (MMPs), genes, signaling pathways, etc. are believed to be closely related to the occurrence of KOA⁷. Furthermore, factors such as aging, trauma, obesity, and changes in biomechanics and biological rhythm can also lead to an imbalance in cartilage metabolism homeostasis, resulting in cartilage damage⁸, and various pathogenic factors can promote or inhibit each other, forming its complex pathogenesis⁹. At present, the main treatment method for KOA is drug intervention¹⁰, such as glucocorticoids and cyclooxygenase inhibitors, but their therapeutic effects are still limited, and some patients may experience adverse drug reactions induced by these drugs¹¹. Therefore, it is necessary to search for new drug therapies for KOA¹².

It has been found that the onset of KOA in patients is associated with cell apoptosis¹³. Pyroptosis is a Caspase-1-dependent programmed cell death accompanied by the release of inflammatory mediators

caused by inflammasomes¹⁴. Pro-inflammatory factors such as IL-1 β and IL-18 will be produced in synovial macrophage pyroptosis, and IL-1 β and IL-18 can enhance the catabolism of cartilage, activate the expression of MMPs in cartilage by inhibiting the production of Collagen II¹⁵, and promote the degradation of extracellular matrix to cause the damage of cartilage and exacerbate the inflammation of synovium¹⁶, leading to the occurrence and progression of KOA. Other studies have shown that in pro-inflammatory reaction, the level of ROS increases, and the inhibition of the Nrf2/HO-1 signaling pathway by activating the NF- κ B pathway can further promote the production of inflammatory factors, indicating that there is an interaction between the Nrf2/HO-1 signaling pathway and the NF- κ B signaling pathway¹⁷. Nrf2 may regulate the inflammatory response in the process of KOA lesions through the NF- κ B-mediated NLRP3 inflammasome-related pyroptosis of synovial cells¹⁸.

Anwulignan (AN) is a highly representative lignan active monomer compound extracted from *Schisandra chinensis*, a traditional Chinese medicine¹⁹. Our previous studies have indicated that AN has strong antioxidant and anti-inflammatory activities in fatigue animal models²⁰ and D-galactose-induced aging mouse models²¹ been no relevant report on the protective effect of AN against KOA so far.

In this study, a rat KOA model was established by injecting papain into the knee joint cavity of rats, and the protective effect and mechanism of AN against the KOA in rats were investigated, in order to provide an experimental basis for the research and development of anti-knee osteoarthritis drugs.

Results

Effects of AN on the body weight and behavior of rats

The weight of rats in all group showed an upward trend overall, and there was a significant difference in it between CON group and MOD group ($P < 0.05$). As shown in Fig. 2A, compared with those in CON group, the movement trajectory and number of standing of rats in MOD group were significantly reduced ($P < 0.01$); Compared with those in MOD group, the movement trajectory and number of stands of rats in AN and IND groups were significantly increased ($P < 0.05$ or $P < 0.01$). There was no significant difference in the body weight of rats among groups treated with the different agents, of which rats in 4mg/kg AN (AN-H) group showed the most significant increase in the number of stands ($P < 0.01$), as shown in Fig. 2B and C).

Effects of AN on the knee joint swelling and knee joint diameter in rats

After administering the different doses of AN, it was observed that the swelling of the knee joint in rats was alleviated to varying degrees. Compared with that in CON group, the knee joint diameter of rats was significantly increased in MOD group ($P < 0.01$), and compared with that in MOD group, the knee joint diameter of rats in AN and IND groups was significantly decreased ($P < 0.05$ or $P < 0.01$), in

which the decrease in the knee joint diameter of rats was most significant in 4 mg/kg AN group ($P < 0.01$), as shown in Fig. 3A and E.

Effects of AN on histopathological changes in rats with KOA

As shown in Fig. 3B, in CON group, there was no significant pathological change in the cartilage tissue of rats, the surface of the cartilage was smooth and continuous, the layers were clear and closely arranged, and the cells were evenly distributed with complete tidal lines; In MOD group, the morphological structure of cartilage tissue was irregular, the cartilage surface cells were severely damaged and arranged in disorder, the tidal line was incomplete, showing a significant pathological damage of articular cartilage tissue in osteoarthritis, and the thickness of articular cartilage was decreased, the staining of cartilage matrix was significantly reduced, and the trabecula in the cancellous layer of bone were sparse; Compared with that in MOD group, the pathological damage of articular cartilage tissue in AN and IND groups was significantly alleviated, and the thickness of joint cartilage was significantly increased, suggesting that AN could alleviate the KOA induced by papain. The Masson staining results (Fig. 3C) showed that compared with that in CON group, collagen fibers in the knee joint cartilage were significantly decreased in MOD group, while compared with those in MOD group, collagen fibers in the knee joint cartilage were significantly increased in AN and IND groups. In addition, based on HE staining, the Mankin scoring on the cartilage injury in pathology was performed, and the results showed that the score of rats in MOD group was significantly increased compared with that in CON group ($P < 0.01$), the score of rats in AN and IND group was significantly decreased compared with that in MOD group, ($P < 0.05$ or $P < 0.01$), in which the pathological damage of cartilage tissue of rats in 4mg/kg AN group was most significantly alleviated ($P < 0.01$) (Fig. 3D).

Effects of AN on oxidative stress-related indicators in the knee synovial tissue

Papain can induce a synovial damage associated with oxidative stress in synovial tissue²⁷. In this study, the activity of SOD, and the content of MDA and ROS in the cartilage tissue of rats were measured. As shown in Fig. 4A, compared with that in CON group, the activity of SOD in the synovial tissue of rats was significantly decreased ($P < 0.01$), while the the content of MDA and ROS was significantly increased ($P < 0.01$) in MOD group; As shown in Fig. 4B and C, compared with that in MOD group, the activity of SOD was significantly increased ($P < 0.01$) and the content of MDA and ROS was significantly decreased ($P < 0.01$) in AN and IND groups, in which the activity of SOD was most significantly increased, and the content of MDA and ROS was most significantly decreased ($P < 0.01$) in 4mg/kg AN group.

Effects of AN on the levels of inflammatory factors in the serum and knee synovial tissue of rats

Compared with those in CON group, the levels of inflammatory factors TNF- α , IL-6, PGE2 and NO in the serum and synovial tissue of rats were significantly increased in MOD group ($P < 0.01$); Compared with those in MOD group, the levels of inflammatory factors TNF- α , IL-6, PGE2 and NO in the serum and

synovial tissue of rats were significantly decreased in AN and IND groups ($P < 0.05$ or $P < 0.01$), in which the decrease in the level of inflammatory factors was most significant in 4 mg/kg AN group ($P < 0.01$), as shown in Fig. 5.

Effects of AN on the expression levels of Nrf2/HO-1 signaling pathway-related proteins in the knee joint cartilage tissue of rats

The Nrf2/HO-1 signaling pathway is involved in oxidative stress response and can interact with the NF- κ B signaling pathway in it. The the expression levels of Nrf2/HO-1 signaling pathway-related proteins in the knee joint cartilage tissue were detected in this study. As shown in Figures 6A and B, compared with that in CON group, the p-Nrf2/Nrf2 ratio and HO-1 protein expression level were respectively decreased ($P < 0.01$), while the Keap1 protein expression level was significantly increased ($P < 0.01$) in MOD group; Compared with that in MOD group, the p-Nrf2/Nrf2 ratio and HO-1 protein expression level was significantly increased ($P < 0.05$ or $P < 0.01$), while the Keap1 protein expression level was significantly decreased ($P < 0.05$ or $P < 0.01$) in AN and IND groups, in which the increase in the level of p-Nrf2/Nrf2 ratio and HO-1 protein expressions was most significant ($P < 0.01$), while the decrease in the level of Keap1 protein expression was most significant ($P < 0.01$) in 4mg/kg AN group, indicating that AN may play an antioxidant role in rats with KOA, and AN can alleviate the papain-induced knee osteoarthritis by activating the Nrf2/HO-1 signaling pathway.

Effects of AN on the expression levels of NF- κ B signaling pathway-related proteins in the knee joint cartilage tissue of rats

NF- κ B signaling pathway is a typical inflammatory response regulatory pathway in the body, and the expression level of NF- κ B p65 protein and the changes in its upstream signaling molecules TLR4 and Myd88 can be used to evaluate inflammatory responses. In this study, Western blot was used to analyze the expression of NF- κ B p65, p-NF- κ B p65, TLR4 and Myd88 proteins in the articular cartilage tissue of rats. Compared with those in CON group, the ratio of p-NF- κ B p65/NF- κ B p65 and the expression levels of TLR4 and Myd88 proteins were significantly increased in MOD group ($P < 0.05$ or $P < 0.01$); Compared with those in MOD group, the ratio of p-NF- κ B p65/NF- κ B p65 and the expression levels of TLR4 and Myd88 proteins were significantly decreased in AN and IND groups ($P < 0.05$ or $P < 0.01$), in which the decreases in the ratio of p-NF- κ B p65/NF- κ B p65 and the expression levels of TLR4 and Myd88 proteins were most significant in 4 mg/kg An group ($P < 0.01$), as shown in Fig. 7A and B. These results were also confirmed by immunofluorescence (Fig. 7C), indicating that inhibiting the NF- κ B p65 nuclear translocation and the expression of TLR4 and Myd88 can play a anti-inflammatory role, and AN may regulate the NF- κ B signaling pathway and then exert its anti-inflammatory effect to alleviate the KOA in rats.

Effects of AN on the expression levels of pyroptosis signaling pathway-related proteins and IL-1 β and IL-18 in knee joint cartilage tissue cells of rats

Apoptosis plays an important role in KOA, and some pro-inflammatory factors such as IL-1 β and IL-18 produced in synovial macrophage apoptosis can directly cause synovitis and participate in inducing the degradation of cartilage matrix²⁸, promoting the pathological process of KOA. The Western blot results (Fig. 8A and B) showed that compared with those in CON group, the expression levels of NLRP3, ASC, Caspase-1, GSDMD, GSDMD-N, 1L-1 β and IL-18 proteins in knee joint cartilage tissue cells of rats were significantly increased in MOD group ($P < 0.05$ or $P < 0.01$); Compared with those in MOD group, the expression levels of NLRP3, ASC, Caspase-1, GSDMD, GSDMD-N, 1L-1 β and IL-18 proteins in knee joint cartilage tissue cells of rats were significantly decreased in AN and IND groups ($P < 0.05$ or $P < 0.01$), in which the decrease in the expression level of NLRP3, ASC, caspase-1, GSDMD, GSDMD-N, 1L-1 β and IL-18 proteins was most significant in 4mg/kg AN ($P < 0.01$). As shown in Fig. 8C and D, compared with those in CON group, IL-1 β and IL-18 contents in knee joint cartilage tissue cells of rats were significantly increased in MOD group ($P < 0.01$), and compared with those in MOD group, the contents of IL-1 β and IL-18 were significantly decreased in AN and IND groups ($P < 0.05$ or $P < 0.01$), in which the decrease in the content of IL-1 β and IL-18 in 4mg/kg AN group was most significant ($P < 0.01$). The immunofluorescence results (Fig. 8E) showed that AN could significantly alleviate the chondrocyte pyroptosis induced by papain in rats.

Effects of AN on the expression levels of extracellular matrix-related proteins in the knee joint cartilage tissue of rats

Pro-inflammatory factors can activate the expression of matrix metalloproteinases in cartilage by inhibiting the production of CollagenII, and then promote the degradation of extracellular matrix in cartilage cells, leading to the development of KOA²⁹. The Western blot results (Fig. 9A and B) showed that compared with those in CON group, the expression levels of CollagenII protein were significantly decreased ($P < 0.01$), while those of MMP1 and MMP13 proteins were significantly increased ($P < 0.01$) in MOD group; Compared with those in MOD group, the expression levels of CollagenII protein were significantly increased ($P < 0.01$), while those of MMP1 and MMP13 proteins were significantly decreased ($P < 0.05$ or $P < 0.01$) in AN and IND groups, in which the increase in the content of CollagenII protein was most significant ($P < .01$), while the decrease in the level of MMP1 and MMP13 proteins was most significant ($P < 0.01$). The immunofluorescence results also confirmed that AN could alleviate the degradation of extracellular matrix induced by papain in the knee joint cartilage tissue of rats (Fig. 9C).

Discussion

In order to evaluate the therapeutic effect of AN on KOA, a KOA rat model was established, and the model rats were treated with low, medium, and high doses of AN³⁰. The results indicated that AN could significantly increase the number of autonomous activities and standing times in rats, inhibit the degradation of extracellular matrix³¹ the increase in Mankin score, indicating that AN can alleviate the papain-induced cartilage damage in rats. In addition, AN could improve a series of biochemical indicators related to KOA in papain-induced rat KOA model. AN could decrease the levels of ROS, MDA,

TNF- α , IL-6, IL-1 β , IL-18, NO and PGE2, but increase the activity of SOD in the knee joint cartilage tissue to alleviate the papain-induced knee osteoarthritis in rats³², which are consistent with the results of previous study on the papain-induced knee osteoarthritis in rats³³, and there were almost differences among the treatment groups in each experiment and the effects in AN-H group were most significant, indicating that the effects of AN were dose-dependent.

It has been shown that cell apoptosis is associated with the pathological progression of KOA, and during the activation process of cell apoptosis, there are varying degrees of differences in some biochemical indicators and protein expression levels related to it, in which inflammasomes are a type of intracellular pattern recognition receptors (PRRs) to play important regulatory roles in immunity and inflammation. The activation of inflammasome is the cause of cell pyroptosis³⁴, and NLRP3 inflammasome is the most important and representative one³⁵. The inhibition of NLRP3 may prevent Pro-Caspase-1 from being processed into mature caspase-1³⁶, thereby reducing the expression level of Caspase-1 and GSDMD proteins, as well as the inflammatory factors IL-1 β and IL-18. IL-1 β . The production of IL-18 enhances cartilage catabolism and promotes the degradation of cartilage matrix by inhibiting the production of CollagenII, leading to the occurrence and progression of KOA³⁷. In this study, AN could inhibit the production of NLRP3 inflammasomes, reduce the expression level of inflammatory factors, and improve the degradation of cartilage matrix caused by the reduction of CollagenII, suggesting that AN may improve the papain-induced KOA by inhibiting cell apoptosis in rats.

NF- κ B can not only promote the secretion and activation of IL-1 β and IL-18 precursors, but also are important activators upstream of NLRP3 inflammasomes, promoting the synthesis of related inflammasomes by inducing the expression of NLRP3. The expression of NF- κ B is significantly upregulated in KOA. The results showed that AN could reduce the degradation of cartilage matrix by inhibiting the activation of NF- κ B, and also inhibit the transfer of p65 into the nucleus, indicating that it can inhibit the occurrence of chondrocyte pyroptosis. The TLR4/NF- κ B signaling pathway has been confirmed to be involved in the occurrence and progression of KOA in the initiation phase of the classic cell apoptosis pathway mediated by Caspase-1³⁸, in which the activation of its downstream NF- κ B signaling pathway mainly by activating the MyD88-dependent NF- κ B pathway can promote the expression of related factors in cells. TLR4, closely related to the pathogenesis of KOA, is highly expressed and involved in the destruction of cartilage in KOA³⁹, so the inhibition of TLR4/NF- κ B signaling pathway may play a therapeutic role in KOA. It was confirmed in this study that AN could significantly reduce the expression of TLR4/NF- κ B signaling pathway-related proteins, indicating that it may inhibit the occurrence and progression of KOA in rats

Oxidative stress and inflammation are closely related in some pathological processes and ROS plays an important role in KOA, and the antioxidant system in articular cartilage is insufficient to clear the excess ROS when an excessive ROS is produced, which can induce an oxidative stress⁴⁰. Activating the Nrf2 signaling pathway can upregulate the expression of HO-1 to activate the antioxidant capacity of cells. There are some evidences to suggest that activating the Nrf2/HO-1 signaling pathway can mitigate

inflammatory responses by inhibiting the NF- κ B pathway, while inhibiting the Nrf2/HO-1 signaling pathway can promote the production of cytokines by activating the NF- κ B pathway⁴¹, indicating that there may be an interaction between the Nrf2/HO-1 signaling pathway and the NF- κ B signaling pathway, and Nrf2 may mediate the process of cell pyroptosis through NF- κ B. To confirm this hypothesis, Nrf2/HO signaling pathway-related proteins were tested in this study. It was found that AN could downregulate the expression of Keap1 and upregulate the expression of p-Nrf2, Nrf2 and HO-1 in the cartilage tissue of rats, and in addition, it was confirmed in our previous study that AN could play a similar role in the liver tissue, brain tissue and intestinal tissue after intestinal ischemia-reperfusion in D-galactose-treated mice, suggesting that the activation of Nrf2/HO-1 signaling pathway may be a co-antioxidant mechanism of AN⁴²(Fig. 10).

Conclusion

AN can reduce the level of oxidative indicators and inflammatory factors in KOA, and inhibit the reduction of CollagenII and the accumulation of MMP1 and MMP13, the degeneration of joint cartilage and the occurrence of synovitis. The mechanism may be related to its regulating the Nrf2/HO-1 pathway to exert its antioxidant effect and the TLR4/NLRP3/Caspase1 pathway to exert its anti-inflammatory and anti-pyrolytic effects. This study indicates that AN has a potential to be used to treat KOA, which may provide a reference for new treatment options for KOA patients.

Materials and Methods

Experimental animals

Forty eight SD rats (male, 6-8weeks, 220-250g) were purchased from Changchun Yisi Experimental Animal Co., Ltd. (Changchun, China [SCXK (Ji) -2020 0002]). All animal experiments were approved by the Animal Ethics Committee of Beihua University, and conducted in accordance with the "Guides for the Care and Use of Laboratory Animals" issued by the State Council of China. Rats are raised in a pathogen-free laboratory at 22–25°C, in a humidity of 40–50% and a light/dark cycle of 12 hours, and with a free access to food and water.

Experimental drugs and reagents

AN (Chengdu Pufeide Biotechnology Co., Ltd., Chengdu, China); IND (Shanghai Jinbuhuan Lankao Pharmaceutical Co., Ltd., Shanghai, China); Ulatan (Shanghai Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); Papain (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China); L-cysteine (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China); Superoxide dismutase (SOD) and malondialdehyde (MDA) detection kit (Nanjing Chengjian Biotechnology Research Institute, Nanjing, China); Reactive oxygen species (ROS), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), prostaglandin E2 (PGE2), nitric oxide (NO), interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) detection kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China); Nuclear factor E2 related factor 2

(Nrf2), phosphorylated nuclear factor E2-related factor 2 (p-Nrf2), cytoplasmic protein chaperone 1 (Kelch-like ECH-associated protein 1, Keap1), heme oxygenase-1 (HO-1), Toll-like receptor 4 (TLR4), myeloid differentiation primary response 88 (Myd88), nuclear factor- κ B (Nuclear factor κ -gene binding NF- κ B) p65, phosphorylated nuclear factor κ -gene binding NF- κ B p65, NOD-like receptor thermal protein domain-associated protein 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, gasdermin D (GSDMD), gasdermin D-N-terminal (GSDMD-N), interleukin-1 β (IL-1 β), interleukin-18 (IL-18), collagen II, matrix metalloproteinase-1 (MMP1), matrix metalloproteinase-13 (MMP13), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (ABclonal Biotechnology, USA).

Establishment of KOA rat model and administration of rats

SD rats were anesthetized by the intraperitoneal injection of 20% urethane solution (0.5mL/kg), and then fixed in a supine position on an operation table. The local hair on the skin of the rats' knee joint was scraped off, and the skin was disinfected with iodophor, and 0.2 mL of papain solution with a mass fraction of 4% (4% papain mixed with 0.03 mol/L L-cysteine in the ratio of 1:1) was injected into the right knee joint cavity, once a day for 7 days. This study is reported in accordance with ARRIVE guidelines.

The rats were randomly divided into six groups, 8 rats in each group. Rats in the blank control group (CON) were injected with an equal volume of normal saline into the right knee joint cavity, once a day for 7 days, while those in the other groups were injected with 0.2 mL papain solution into the right knee joint cavity in the same way for establishing a rat KOA model. At the same time, rats in CON group and the model group (MOD) were gavaged with an equal volume of carboxymethyl cellulose sodium, those in 1 mg/kg AN (low-dose AN, AN-L), 2 mg/kg AN (medium-dose AN, AN-M) and 4 mg/kg AN (high-dose AN, AN-H) groups were gavaged with 1, 2 and 4 mg/kg AN, respectively, and those in the indomethacin (IND) group were gavaged with 16 mg/kg IND. After the last administration, blood samples of all rats were collected from the abdominal aorta of rats, and then the cartilage and synovial tissue in the rat knee joint were taken. The experimental protocol is shown in Fig. 1.

Observation on changes in the daily body weight of rats

During the experiment, changes in the body weight of all rats were measured and recorded daily, and the body weight curve was drawn.

Monitoring of the movement trajectory and number of stands of rats

On the 7th day after the completion of modeling and administration, the movement trajectory of rats in each group was observed and the number of their stands was counted at the same time using an OFT-100 rat opening activity experimental system.

Observation on the degree of swelling by measuring the diameter of knee joint

The swelling degree of knee joint of rats was observed by measuring the diameter of the right knee joint of rats in each group using an electronic caliper²².

Immunohistochemical analysis

The knee joint tissue of rats was fixed in a 10% formaldehyde solution for 48 hours first, then the knee joint tissue was decalcified by putting it in a 5% nitric acid formaldehyde decalcification solution for 29 hours, dehydrated in gradient ethanol, embedded in paraffin, and cut into 4 mm thick sections. The sections were stained with H&E or Masson for evaluating the cartilage tissue damage. Histopathological changes in the cartilage tissue were examined under an optical microscope and scored according to the Mankin scoring criteria: cartilage structure (0–5), chondrocytes (0–3), matrix staining (0–4), and tidal line integrity (0–1). Mankin's score was the sum of scores in the different group. The score range was from 0 to 13, and the tissue with a higher score was considered one with a severe joint degeneration²³.

Detection of biochemical indicators related to oxidative stress and inflammation

The knee joint synovial tissue was mixed with pre-cooled PBS in the ratio of 1:9 to prepare a synovial tissue homogenate. The homogenate was centrifuged at 3500 rpm and 4 °C for 15 minutes to obtain the homogenate supernatant. The SOD activity and ROS, MDA, TNF- α , IL-6, IL-1 β , IL-18, PGE2 and NO contents in the supernatant were measured according to the instructions of the reagent kits²⁴. The collected blood samples in a 2-mL centrifuge tube was centrifuged at 3500 rpm and 4 °C for 10 minutes to obtain the serum, and TNF- α , IL-6, IL-1 β , IL-18, PGE2 and NO contents in the serum of rats in each group were detected according to the instructions of the reagent kits²⁵.

Western Blot analysis

The knee joint cartilage tissue of three mice from each group was added into the lysis buffer, incubated on ice for 1 hour. The lysis buffer contained phosphatase inhibitors and protease inhibitors. Then, the supernatant of tissue lysis solution was obtained by centrifuging it at 12000 rpm for 10 minutes. The total protein in the supernatant was determined by BCA method, then the SDS-PAGE gel electrophoresis on the proteins was conducted, and the proteins were transferred onto PVDF membranes for 2 hours. Then, the membranes were washed with Tris buffer saline/Tween 20 (TBST) for 5 minutes, blocked with the TBST buffer containing 5% skimmed milk powder at room temperature for 1.5 hours, and incubated with the rabbit primary antibodies of Nrf2 (1:1000), p-Nrf2 (1:1000), HO-1 (1:1000), Keap1 (1:1000), TLR4 (1:1000), myd88 (1:1000), NF- κ B p65 (1:1000), p-NF- κ B p65 (1:1000), NLRP3 (1:1000), ASC (1:1000), caspase-1 (1:1000), GSDMD (1:1000), GSDMD-N (1:1000), IL-1 β (1:1000), IL-18 (1:1000), collagen II (1:10000), MMP1 (1:1000), MMP13 (1:1000) and GAPDH (1:10000) at 4°C overnight. Next day, the membranes were washed with TBS-T buffer (15 minutes $\times$ 3), then incubated with the rabbit secondary antibody (1:5000) at room temperature for 1 hour, and washed with TBS-T buffer (10 minutes $\times$ 3) and then ECL developer was used for color development.

Immunofluorometric assay

The sections were dewaxed with xylene, repaired with citric acid antigen and blocked with 5% goat serum for 2 hours, and incubated with the primary antibodies of NF- κ B p65, caspase-1 and MMP13 (1:200) at 4 °C overnight. The sections were rinsed with PBS three times, and incubated with the fluorescent secondary antibody (1:5000) at 37 °C in dark for 1 hour. The sections were rinsed with PBS three times, then incubated with DAPI in dark for 5 minutes for the staining of nuclei, and mounted with glycerol. The images were captured and the NF- κ B p65 nuclear translocation and the expression of caspase-1 and MMP13 were observed using a fluorescence microscope²⁶.

Statistical analysis

The statistical analysis was conducted using SPSS 20.0 software, and all data was represented by Mean $\pm$ SD. The comparison of two sample sizes between groups was conducted using one-way ANOVA test, and a value with $P < 0.05$ indicated a statistical significance.

Declarations

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Author contributions

S.N. and H.L. designed the study. S.N.,W.J. and C.Z. performed the experiments.S.N wrote the manuscript.S.N.,W.J. and H.C. annlyzed the data.C.W.,J.S.,C.Z. and H.L. revised the manuscript critically for important intellectual content.H.L. is the corresponding author. All authors contributed to and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

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Figures

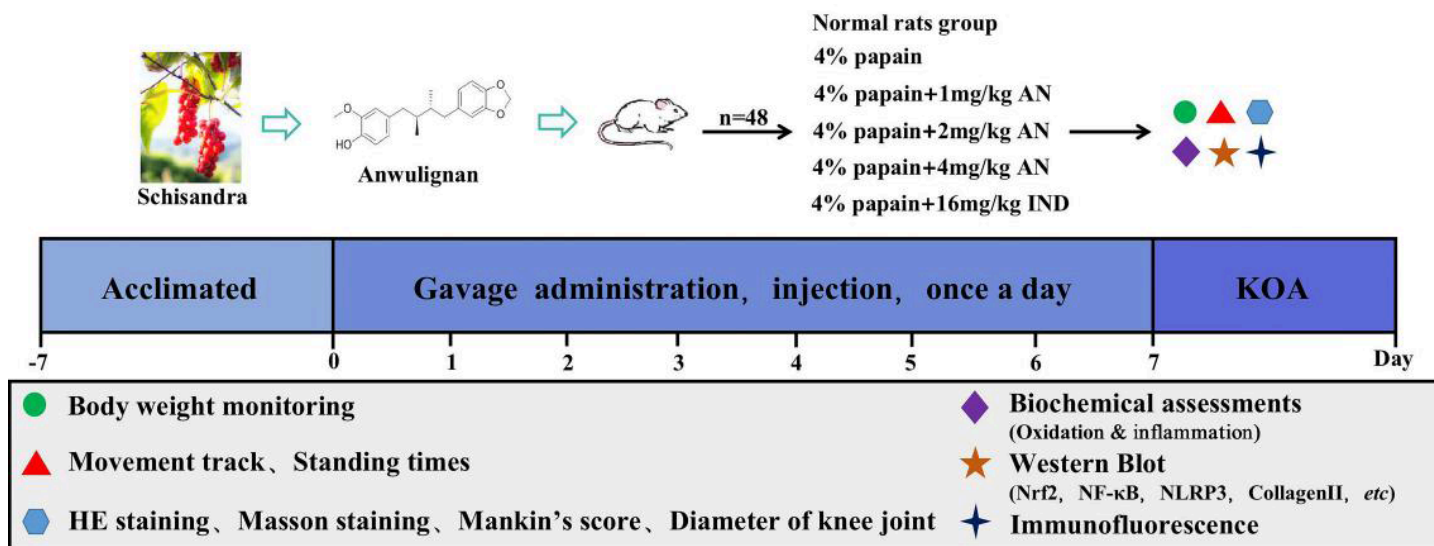


Figure 1

Experimental protocol

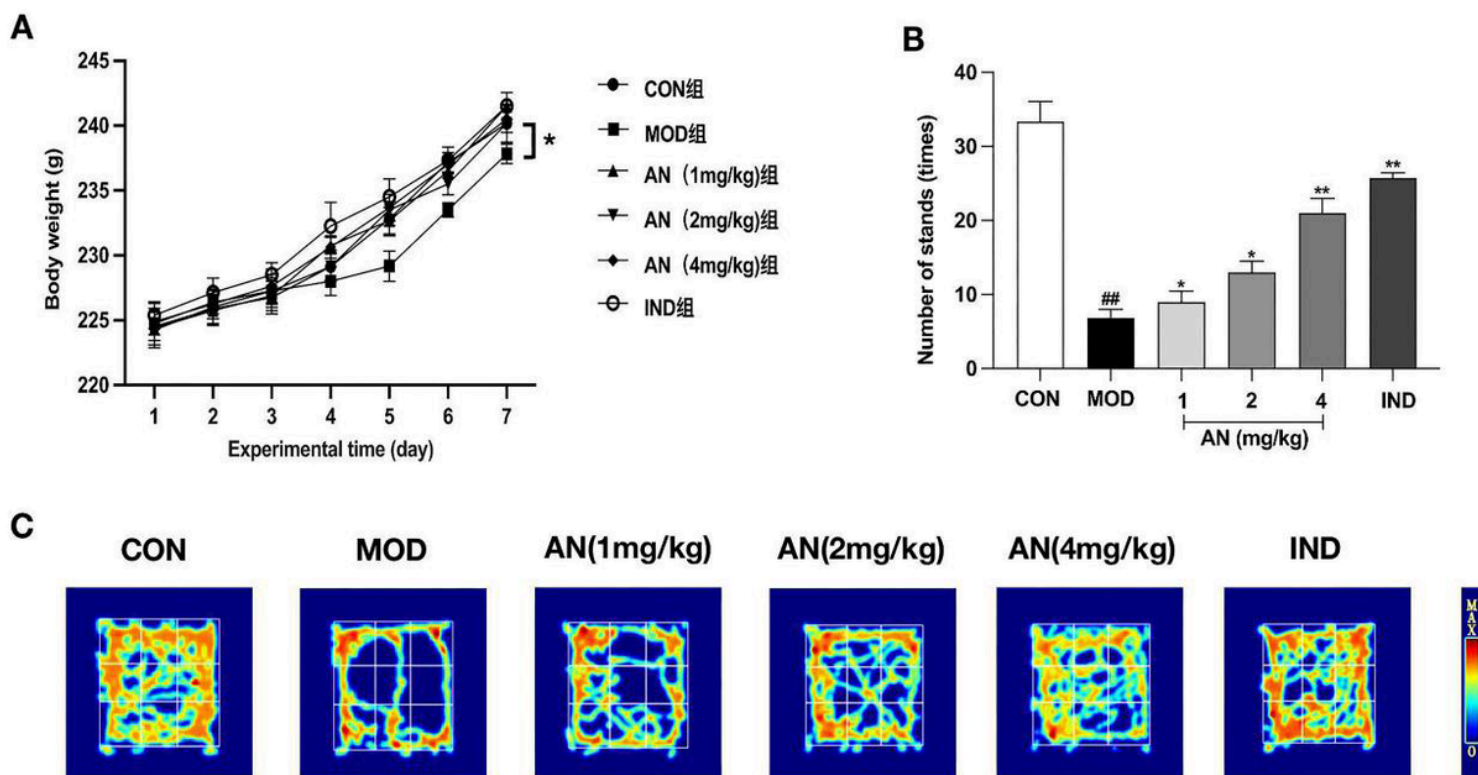


Figure 2

Effects of AN on the body weight, the movement trajectory and the number of stands of rats (Mean $\pm$ SD, $n = 8$). (A) Body weight; (B) Number of stands; (C) Movement trajectory. Compared with CON group, $^{\#}P < 0.05$, $^{##}P < 0.01$; Compared with MOD group, $^{*}P < 0.05$, $^{**}P < 0.01$.

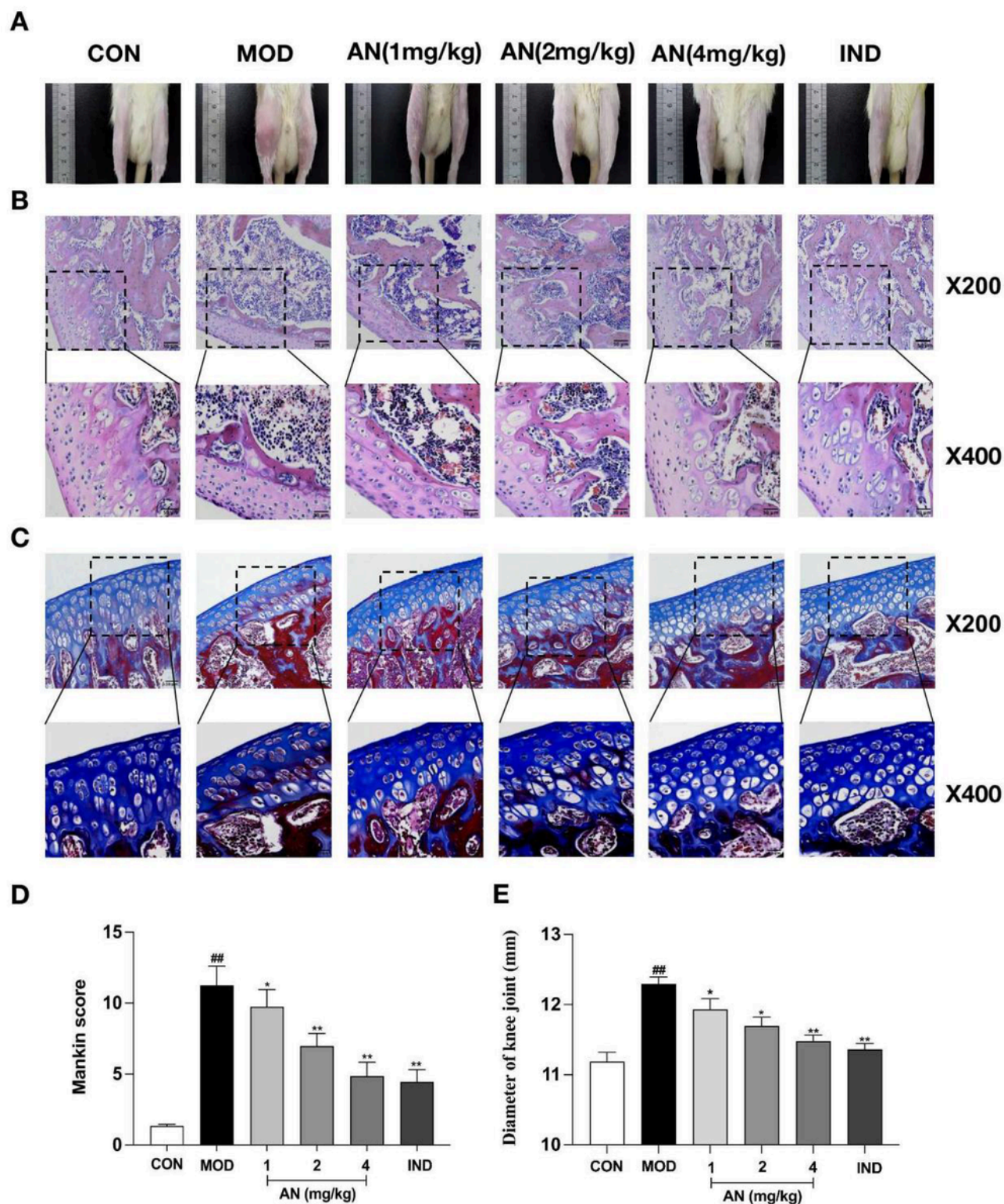


Figure 3

Effects of AN on the degree of knee joint swelling, knee joint diameter, histopathological damage and Mankin score in rats (Mean $\pm$ SD, n = 3). A. Degree of swelling; B. HE staining ($\times 200$, $\times 400$); C. Masson staining ($\times 200$, $\times 400$); D. Mankin score; E. Knee joint diameter. Compared with CON group, $^{\#}P < 0.05$, $^{##}P < 0.01$; Compared with MOD group, $^{*}P < 0.05$, $^{**}P < 0.01$.

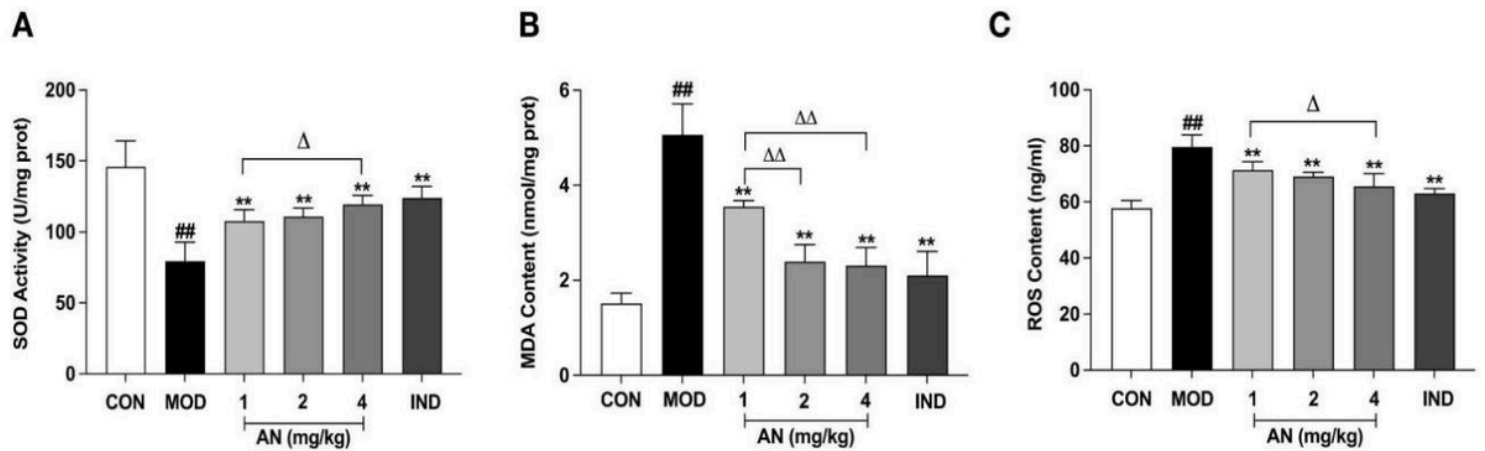


Figure 4

Effects of AN on oxidative stress-related indicators in knee joint synovial tissue (Mean $\pm$ SD, n = 8). A. SOD activity; B. MDA content; C. ROS content. Compared with CON group, $^{\#}P < 0.05$, $^{##}P < 0.01$; Compared with MOD group, $^{*}P < 0.05$, $^{**}P < 0.01$. Comparison among administration groups, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$.

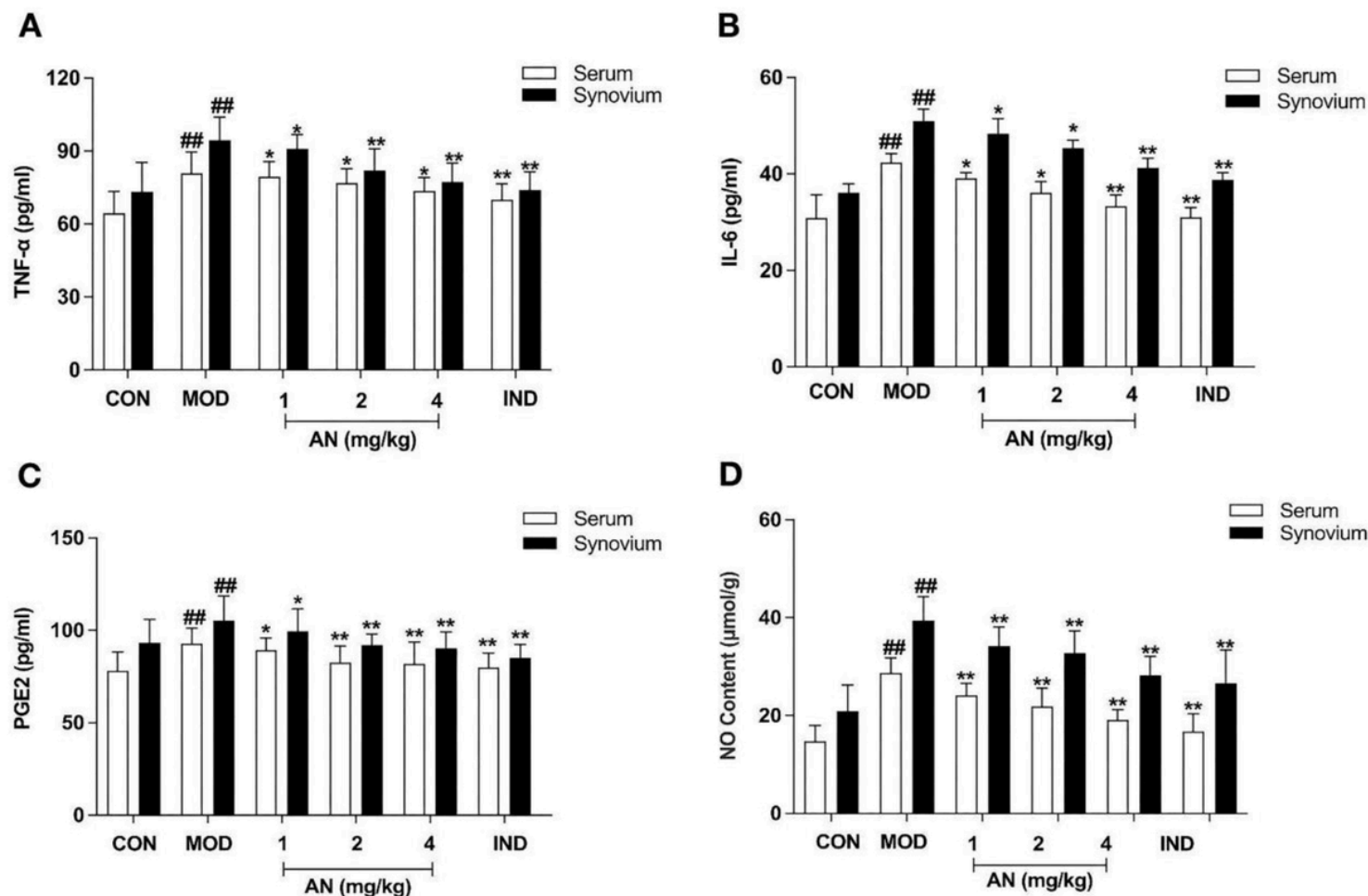


Figure 5

Effects of AN on inflammatory factors TNF- α , IL-6, PGE2 and NO levels in the serum and knee synovial tissue (Mean $\pm$ SD, $n = 8$). A. TNF- α ; B. IL-6; C. PGE2; D. NO. Compared with CON group, $^{\#}P < 0.05$, $^{##}P < 0.01$; Compared with MOD group, $^*P < 0.05$, $^{**}P < 0.01$.

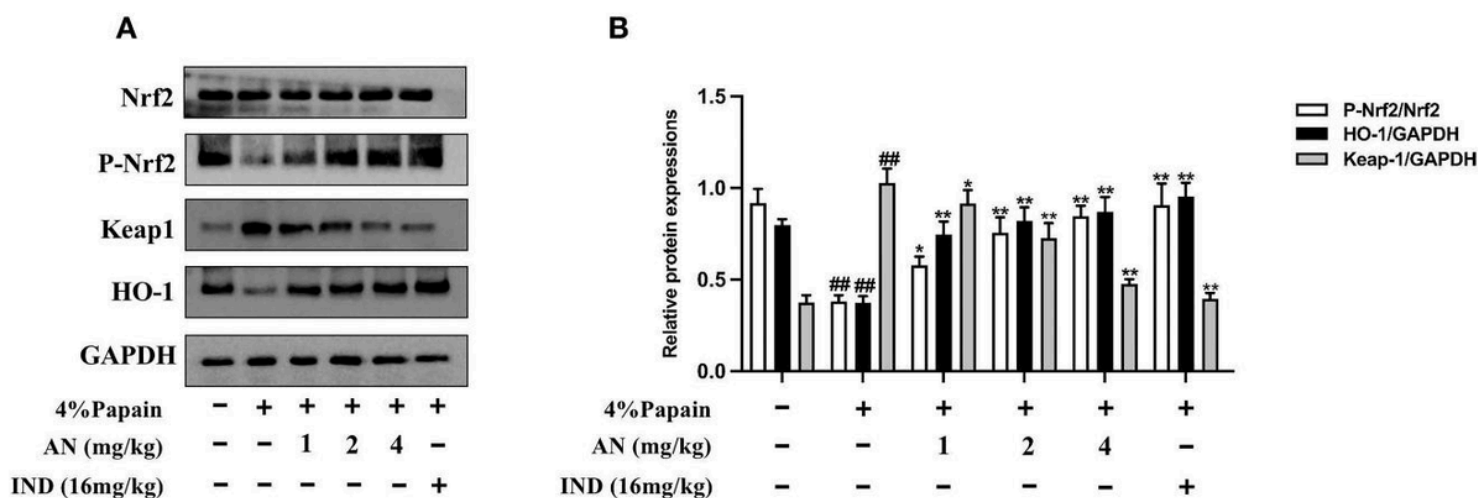


Figure 6

Effects of AN on the expression levels of Nrf2/HO-1 signaling pathway-related proteins in the knee joint cartilage tissue of rats (Mean $\pm$ SD, n = 3). A. Electrophoresis images of Nrf2, p-Nrf2, Keap1 and HO-1 proteins (Western Blot); B. Analysis of P-Nrf2/Nrf2, Keap1/GAPDH and HO-1/GAPDH grayscale. Compared with CON group, $^{\#}P < 0.05$, $^{##}P < 0.01$; Compared with MOD group, $^{*}P < 0.05$, $^{**}P < 0.01$.

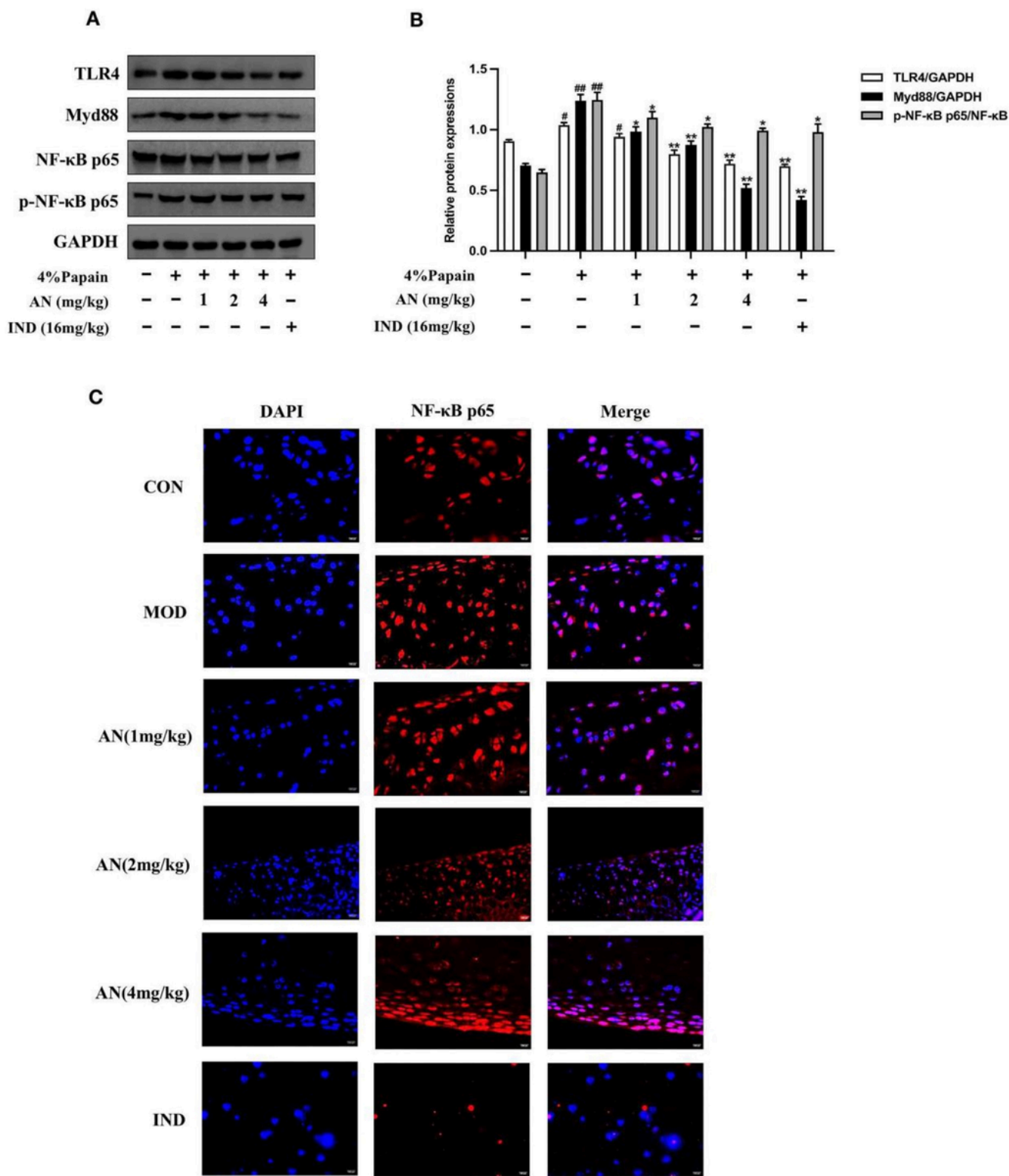


Figure 7

Effects of AN on the expression levels of NF-κB signaling pathway-related proteins in the knee joint cartilage tissue of rats (Mean ± SD, n = 3). A. Electrophoresis images of TLR4, Myd88, NF-κB p65 and p-NF-κB p65 proteins (Western Blot); B. Analysis TLR4/GAPDH, Myd88/GAPDH, and p-NF-κB p65/NF-κB p65 grayscales. C. Effects of AN on the nuclear translocation of NF-κB p65 by immunofluorescence analysis. Compared with CON group, [#]*P* < 0.05, ^{##}*P* < 0.01; Compared with MOD group, ^{*}*P* < 0.05, ^{**}*P* < 0.01.

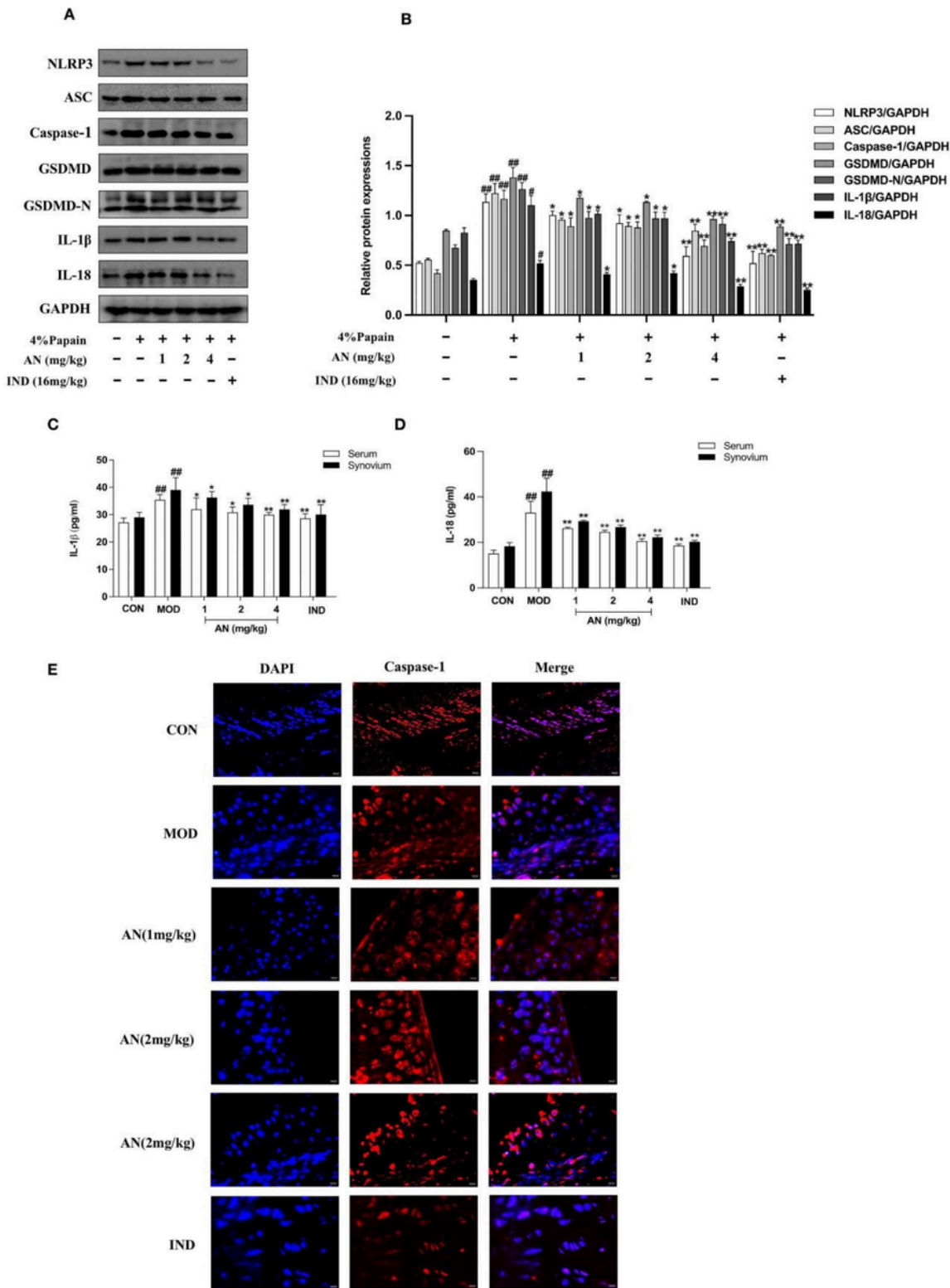


Figure 8

Effects of AN on expression levels of pyroptosis signaling pathway-related proteins, IL-1 β and IL-18 in knee joint cartilage tissue cells treated with of rats (Mean $\pm$ SD, n = 3). A. Electrophoresis images of NLRP3, ASC, caspase-1, GSDMD, GSDMD-N, 1L-1 β and IL-18 proteins (Western Blot); B. Grayscale analysis of NLRP3/GAPDH, ASC/GAPDH, Caspase-1/GAPDH, GSDMD/GAPDH, GSDMD-N/GAPDH, 1L-1 β /GAPDH and IL-18/GAPDH. Compared with CON group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with MOD group, $^*P < 0.05$, $^{**}P < 0.01$. C. Effects of AN on inflammatory factors 1L-1 β levels in the serum and knee synovial tissue (Mean $\pm$ SD, n = 8). Compared with CON group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with MOD group, $^*P < 0.05$, $^{**}P < 0.01$. D. Effects of AN on inflammatory factors 1L-18 levels in the serum and knee synovial tissue (Mean $\pm$ SD, n = 8). Compared with CON group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with MOD group, $^*P < 0.05$, $^{**}P < 0.01$. E. Analysis on the expression of Caspase-1 detected by Immunofluorescence.

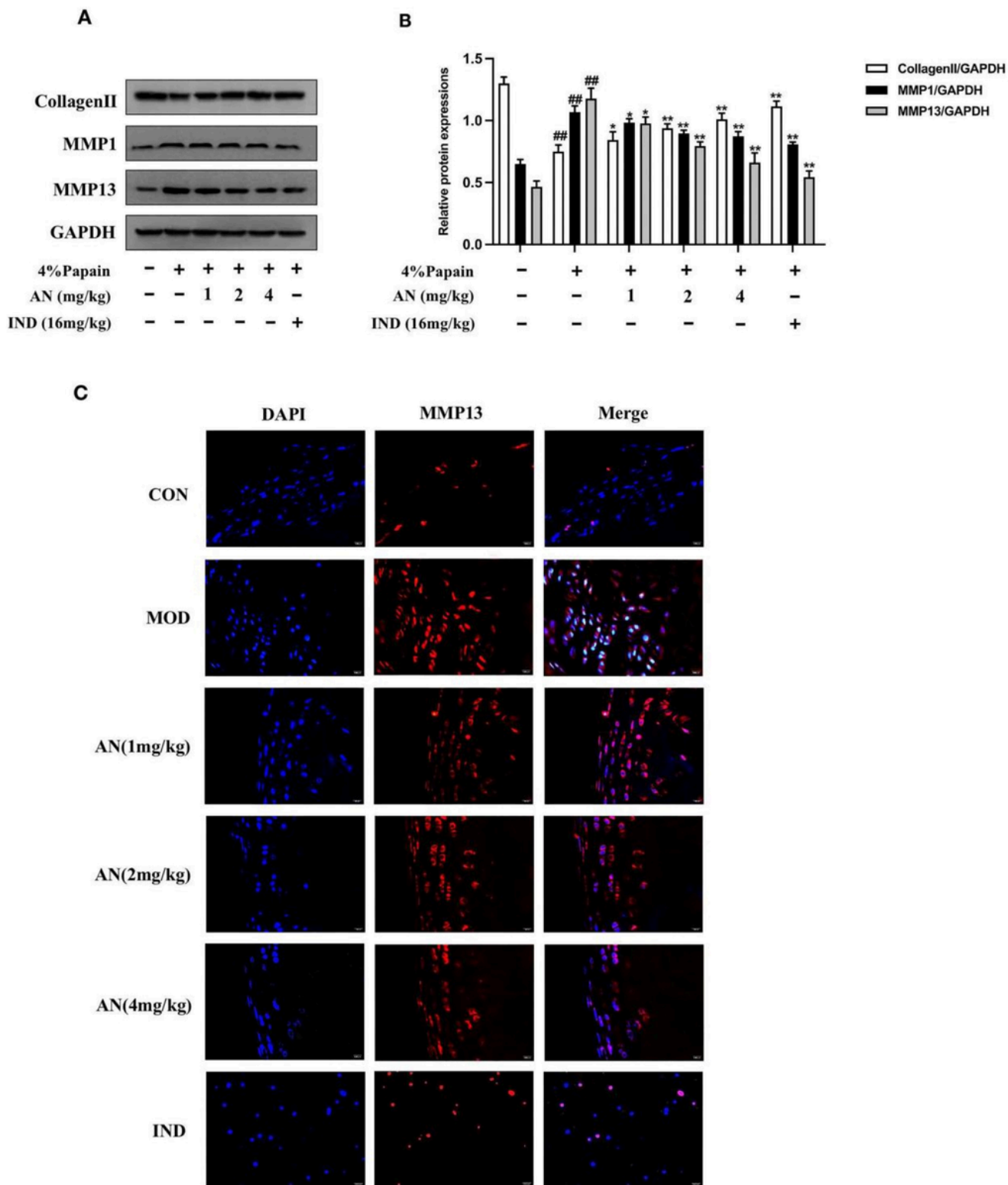


Figure 9

Effects of AN on the expression of extracellular matrix related proteins in the knee joint cartilage tissue of rats (Mean $\pm$ SD, n = 3). A. Electrophoresis images of CollagenII, MMP1 and MMP13 proteins (Western Blot); B. Analysis of CollagenII/GAPDH, MMP1/GAPDH, MMP13/GAPDH grayscales. Compared with CON group, [#] $P < 0.05$, ^{##} $P < 0.01$; Compared with MOD group, ^{*} $P < 0.05$, ^{**} $P < 0.01$. C. Analysis on the expression of MMP13 detected by immunofluorescence.

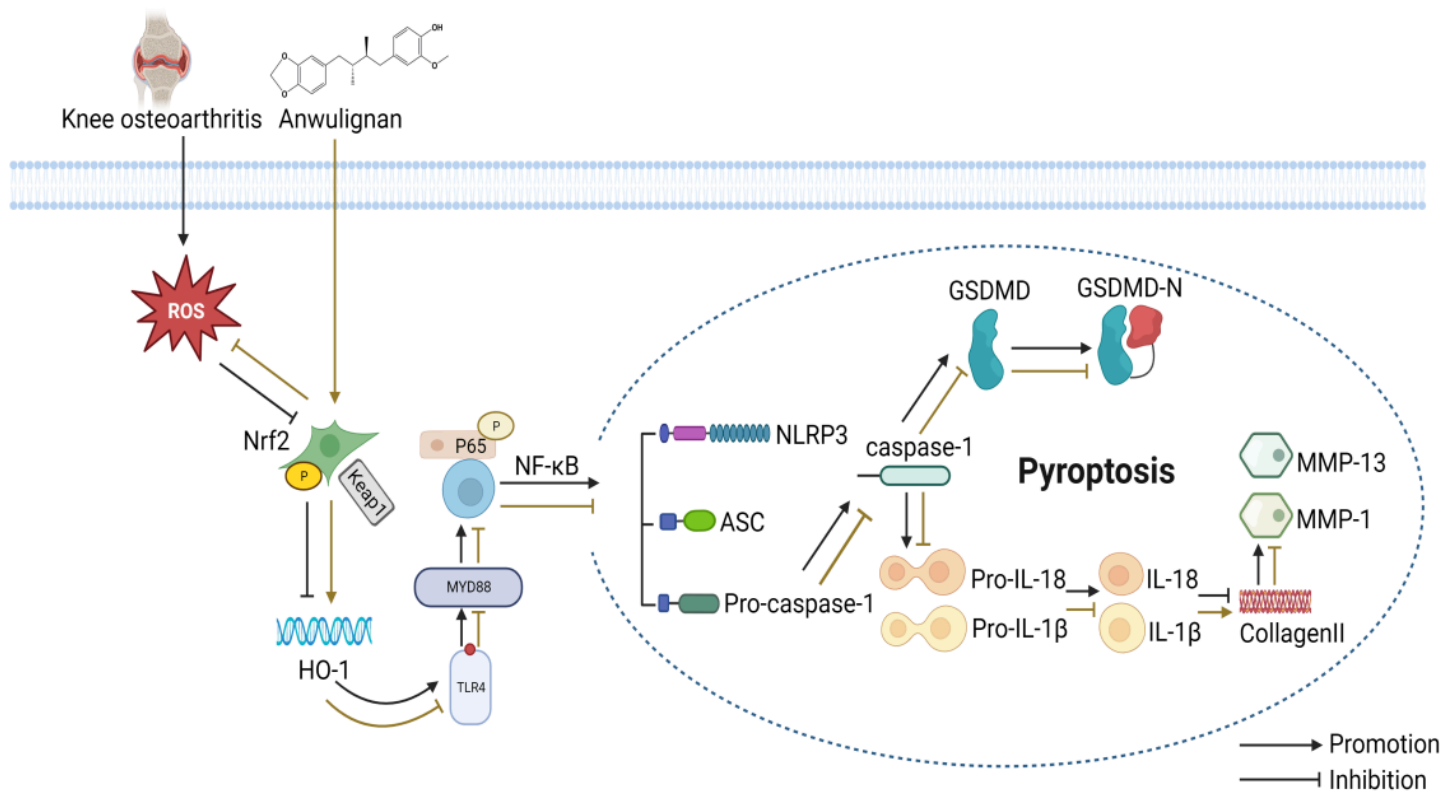


Figure 10

Mechanism of the protective effect of AN against KOA in rats

Supplementary Files

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