Matrine from *Sophora flavescens* attenuates on collagen-induced osteoarthritis by modulating the activity of miR-29b-3p/PGRN axis

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Running title: matrine attenuates arthritis by inhibiting miR-29b-3p

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Abstract

Matrine is an active ingredient in traditional Chinese medicine that has been shown to be effective in treating bone disorders. The anti-osteoarthritis (OA) effects of matrine were assessed using both in *in vitro* and *in vivo* systems, and the mechanisms underlying the effects were investigated by focusing on the activity of miR-29b-3p/PGRN axis. The miR was chosen as potential target for matrine after chondrocytes were treated with both IL-1β and matrine. Changes in cell viability, cell apoptosis, inflammation, and miR-29b-3p/PGRN axis were detected. *In vitro* assays results were validated using collagen-induced arthritis (CIA) rat models. Incubation with IL-1β reduced cell viability, induced cell apoptosis, and inhibited production of cytokines in chondrocytes, which was associated with the up-regulation of miR-29b-3p and down-regulation of PGRN. In CIA rats, matrine reduced bone destruction and weight loss in a dose-dependent manner. Matrine also reduced the systemic levels of cytokines. At the molecular level, matrine inhibited the expression of miR-29b-3p while increasing the expression of PGRN. The findings outlined in the current study showed that matrine exerted its anti-OA effects by modulating the miR-29b-3p/PGRN axis.

Keywords: inflammation; matrine; miR-29b-3p; osteoarthritis; PGRN

Introduction

Osteoarthritis (OA) is a common chronic and degenerative disorder of joints such as knee and hip joint, and has become one of the major contributors to the increased number of disabilities among the middle-aged and the elderly [1]. The disorder is commonly characterized by articular cartilage loss, synovium inflammation, and other joint tissue complications, and patients with OA are always impaired by clinical manifestation such as joint pain, joint swelling, limited motion, and have a significant burden in their daily lives. Except for joint replacement, the pathogenesis of OA is currently unraveled, resulting in a lack of effective treatment strategies to prevent or even reverse the disorder's progression. However, the treatment will cause secondary joint tissue damage [2]. Thus, the development of novel treatment strategies for OA has become an important task for the disorder's clinical management.

Previous research has shown that chondrocyte degeneration and apoptosis play an important role in the development of OA. Thus, a thorough understanding of the molecular mechanism modulating chondrocyte viability will aid in the development of novel anti-OA therapies. MicroRNA (miRs), a class of molecules involved in various biological processes, are also linked to the development of OA and regulate the apoptotic process of chondrocytes. For instance, miR-34a can effectively reduce the production of IL-1β and attenuate apoptosis in chondrocytes [3]. MiR-146a can induce chondrocyte apoptosis and promote OA pathogenesis by increasing VEGF expression and inhibiting Smad4 [4]. When compared to normal cartilage tissue, the level of miR-29b-3p is increased in the cartilaginous tissue of patients with end-stage OA [5, 6]. Other miRs, including miR-142-3p, miR-21, and miR-448, have been linked to the pathogenesis of OA [7-10]. As a result, specific modulation of specific miRs may be a promising strategy for the treatment of OA.

OA is considered as a type of "arthromyodynia" in traditional Chinese medicine, and numerous formulas have shown significant treatment effects on the disorder [11]. *Sophora flavescens* is one of the main ingredients of Qing-Luo-Yin (QLY), a representative formula of Ji-Ren Li, a well-known contemporary traditional Chinese medicine (TCM) master. For over 40 years, the formula has been used successfully to treat OA and rheumatoid arthritis (RA) [12]. Matrine is a quinolizidine alkaloid derived from *Sophora flavescens* that has been shown

in previous studies to have the potential to treat OA. Pu et al. demonstrated that matrine has a strong anti-arthritic effect on type II collagen-induced arthritis in rats [13]. Yang et al. found that matrine causes apoptosis in fibroblast-like synoviocytes from arthritis rats by inhibiting the activity of JAK/STAT signaling pathway [14]. Furthermore, by suppressing NF-κB pathway, matrine can regulate Th1/Th2 cytokine responses in RA [15]. Thus, matrine is a proven anti-OA compound as reported by various studies. However, whether miRs are involved matrine's anti-OA function has not been investigated. Given the multiple roles of miRs in the development of OA and chondrocyte apoptosis, as well as matrine's promising treatment effects on OA, it is reasonable to identify potential miRs that respond to matrine treatment on OA.

Therefore, the current study first exposed chondrocytes to matrine treatment before detecting changes in miR expression levels. MiR-29b-3p was chosen as the potential target of matrine in the treatment of OA. The miR is a well-characterized pro-OA factor that has been supported by numerous studies [5, 6, 16, 17]. According to Le et al, miR-29b-3p expression is up-regulated in mouse knee joints following DMM surgery and is involved in the onset or early stages of OA [5]. The study by Chen et al. shows that miR-29b-3p directly inhibits the anti-OA factor PGRN, further demonstrating the role of the miR in the development of OA [16]. Thus, by concentrating on the role of miR-29b-3p/PGRN axis, the effects and associated mechanisms of matrine on complications associated OA were investigated.

Materials and methods

Isolation and treatments of chondrocytes

Chondrocytes of rat were isolated from femoral heads of healthy Wistar rats following previous studies [18] with minor modification: all rats were euthanized by an overdose of pentobarbital sodium (120 mg/kg, intraperitoneal administration), after which the femoral head was exposed. The cartilage tissue surrounding the femoral head was chopped into pieces of ~1.0 mm^2, and digested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 30 min. The precipitate was collected and centrifuged at 300 g for 10 min at room temperature before being further digested at 37 °C for 3 h with 0.2% type II collagenase from Beijing Solarbio Science & Technology Co., Ltd. The precipitate containing the chondrocytes was collected after a second centrifugation at 300 g for 10 min at room temperature. Later, once the cells had reached confluency, the isolated chondrocytes were cultured with Dulbecco's modified Eagle's medium (DMEM; HyClone, Cytiva) supplemented with 15% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.). To evaluate the effects of matrine, chondrocytes were initially treated with IL-1 (10 ng/ml) for 2 hours [19] before matrine (0.75 mg/ml for 24 hours) was applied [14] to identify the probable miR target.

RT-qPCR detection

Total RNA was extracted from samples with TRIzol reagent (Sangon Biotech Co., Ltd., Shanghai, China) and was then reversely transcribed into cDNA templates with AMV First Strand cDNA Synthesis kit (Sangon Biotech Co., Ltd.). The qPCR reaction system contained 10 μl of 2×Power Taq PCR MasterMix (PR1702, BioTeke, Beijing, China), 0.5 μl of each primer (Table S1), 1 μl of the cDNA template, and 8 μl of RNase free H_2O. The amplifications were performed with a StepOne Plus™ Real-Time PCR system (Applied Biosystems, Grand Island, NY, USA) following routine conditions. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of miRs.

CCK-8 assay detection of cell viability

The CCK-8 experiment was used to assess the impact of matrine on the viability of IL-1-treated chondrocytes: briefly, cells with various treatments were cultured with 10 μl of CCK-

8 for two hours. Afterwards, the absorbance of 450 nm was recorded and served as the representative of cell viability.

Flow cytometry detection of cell apoptosis

Apoptosis rates of chondrocytes were assessed with Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology): briefly, 2 x 10^5 cells were collected from each group and re-suspended. The cell suspension was incubated in 5 μl of Annexin V-FITC and 10 μl PI for 20 min at room temperature in the dark. Apoptotic cells were analyzed using a flow cytometer (Accuri C6, BD, USA).

Enzyme-linked immune sorbent assay (ELISA)

The levels of IL-6, IL-1β, and TNF-α in serum samples were detected by ELISA using specific kits (Jiancheng Biological Science and Technology, Nanjing, China) according to the manufacturer's instructions: briefly, 1 g of fresh tissue was ground into tissue homogenate and centrifuged at 3,000 x g for 10 min. The supernatant was then retained, and cytokine concentrations were measured using corresponding kits.

Western blotting

To extract total protein, various sample were lysed in ice-cold RIPA lysis buffer (Beyotime, Jiangsu, China), and the concentration of protein was determined using a BCA protein assay kit (Applygen, Beijing, China). Following that, equivalent amounts of all proteins were separated by SDS-PAGE on 10% gels and then transferred to PVDF membranes (Thermo Fisher Scientific Waltham, MA, USA). After blocking for 30 min with solution containing 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibody against PGRN (1:500) (ab191211, Abcam, China). Thereafter, the membranes were incubated with an appropriate HRP-conjugated secondary antibody for an additional hour at room temperature. The proteins bands were visualized using ECL kits (Amersham), and the optical density of the protein bands was measured using the ImageJ software to determine the relative expression level.

Animals and establishment of collagen-induced arthritis (CIA) model

All animal experiments were performed following the ethical standards in the 1964 Declaration of Helsinki and its later amendments, were approved by the Ethics Committee of Ganzhou people's Hospital and were conducted in accordance with the ARRIVE guidelines for animal studies. Healthy six-week-old female Wistar rats (weighting between 160 and 180 g) were bought from the Changsheng BioTech Co. Ltd. (Liaoning, China) and housed with free access to food and water at a temperature of 20°C and a humidity level of 50% to 55%. To induce arthritis, 30 rats were randomly divided into five groups (six for each group). 30 rats were randomly assigned into five groups (six for each group). Rats in Control group received intraperitoneal injections of 0.9% normal saline starting on day 22 and continuing for nine weeks. Rats in the CIA group received intraperitoneal injections of 0.9% normal saline once daily beginning on day 22 after receiving two injections of type II collagen (4 ml each in 4 ml of Freund's complete adjuvant) spaced three weeks apart over the six-week study period [20]. CIA rats in Low group received daily intraperitoneal injections of 50 mg/kg matrine (purity > 98%, no.1808232, Shanxi Zhendong Pharmaceutical, China) since the 22nd day once daily. CIA rats in Hight group received daily intraperitoneal injections of 100 mg/kg matrine since the 22nd day once daily. CIA rats in MTX group received an intraperitoneal injection of 2 mg/kg methotrexate (MTX) (Sigma-Aladdin Reagent, Shanghai, China) at the 22nd day [20]. All of the rats were kept in housing for an additional three weeks. after the second immunization.

Evaluation of rat weight and arthritis index

Every two weeks during the nine-week period, changes in each rat's body weight were recorded. The arthritis index was determined following a prior work [20] as follows. 0: no swelling or erythema; 1: light swelling or erythema of one toe; 2: low-to-moderate edema; 3: severe redness and swelling of lower limbs； and 4: excessive edema with joint rigidity. All of the rats were euthanized once the arthritis index test was finished, and blood and limb samples were taken and stored for the subsequent tests.

Hematoxylin-Eosin Staining (HE Staining)

Rat limb joints underwent histological examination with HE staining after being fixed in 4% paraformaldehyde for 24 hours, paraffin-embedded, and cut into 7-mm slices. The samples were then deparaffinized, hydrated in alcohol, and incubated with a haematoxylin solution for 20 minutes and an eosin solution for another 10 minutes (Sigma-Aldrich, St. Louis, MO). The image-Pro image analysis program (Media Cybernetics, Rockville, MD) was used to determine the extent of bone damage in the joints under 20x magnification.

Statistical analysis

All the data are expressed as the mean ± standard deviation (SD) values. GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com) was used to perform one-way ANOVA with post hoc multiple comparisons testing (Tukey's test) and repeated measures ANOVA with a significance level of 0.05 (two-tailed *P* value).

Results

Effects of matrine on the expression levels of miRs in IL-1β-treated chondrocytes

The possible miR target of matrine was determined by subjecting chondrocytes to IL-1β and matrine treatments. As shown in Figure 1, the treatment with matrine increased the expression levels of miR-34a, miR-142-3p, and miR-17-5p while suppressing the expression levels of miR-146a, miR-21, and miR-29b-3p. The treatment with matrine had no appreciable impact on the levels of miR-488 and miR-26b-5p expression. Based on the fold-change in relative expression level and the p value in comparison to control chondrocytes, the miR with the highest sensitivity to the treatment with matrine was chosen. Based on the investigation, miR-29b-3p was chosen since it had the lowest p value and was known to have a pro-OA function.

Matrine increased viability while suppressing apoptosis in IL-1β-treated chondrocytes

CCK-8 and flow cytometry assays were used to confirm the possible anti-OA properties of matrine. After IL-1 incubation, a decrease in cell viability (Figure 2A) and an increased in cell apoptosis (Figure 2B) were observed, and the differences between Control groups and IL-1β groups were statistically significant ($P < 0.05$). The viability and apoptosis of chondrocytes co-treated with IL-1 and matrine were recovered, demonstrating that matrine might shield chondrocytes from IL-1-induced deficits (Figure 2).

Matrine decreased the production of pro-inflammatory cytokines and increased the expression of PGRN in IL-1β-treated chondrocytes

Pro-inflammatory cytokine production changes were also detected. When compared to Control groups, the treatment with IL-1β raised the production of IL-6, IL-1β, and TNF-α (Figure 3A-3C), which was inhibited by matrine, further demonstrating the substance's anti-inflammatory properties.Furthermore, the use of matrine suppressed expression of miR-29b-3p (Figure 3D) while increasing the expression of PGRN, which had previously been inhibited by IL-1β (Figure 3E).

Matrine reduced arthritis index and body weight loss in CIA rats

To assess the therapeutic effects of matrine on OA, the CIA method was used. Rats in the OA group displayed substantial OA symptoms, including apparent weight loss (Figure 4A) ($P < 0.05$) and increased arthritis indices (Figure 4B) ($p < 0.05$). Both dosages of matrine considerably alleviated each of these symptoms (Figure 4), and the benefits of matrine were dose-dependent, with 100 mg/kg having the most beneficial effects. Additionally, matrine showed fewer adverse effects than the positive control drug MTX, even though MTX decreased the arthritis index more than matrine did (Figure 4).

Matrine attenuated joint tissue destruction and the reduced the production of cytokines by the inhibition of miR-29b-3p and induction of PGRN levels

In contrast to normal rats, which had undamaged articular cartilage, healthy joints, and synovial tissues, the HE staining of the CIA rat joints revealed extensive bone loss and inflammation (Figure 5A). The breakdown of joint tissues was connected to elevated levels of IL-6 (Figure 5B), IL-1β (Figure 5C), and TNF-α (Figure 5D), all of which supported the development of the OA model. The deficiencies of the joint tissues improved and the cytokine production reduced after the treatment with matrine of both doses and MTX (Figure 5). The effects of matrine, however, were noticeably less potent than those of MTX and did not appear to be dose-dependent. At the molecular level, matrine and MTX treatment restored both miR-29b-3p PGRN levels after CIA administration (Figure 6), supporting the finding from *in vitro* experiments that matrine's anti-OA actions were mediated through regulation of the miR-29b-3p/PRGN axis.

Discussion

The effects of matrine on OA symptoms were investigated in the current study. In IL-1-treated chondrocytes, matrine enhanced cell viability and reduced apoptosis and inflammation. The anti-OA benefits of matrine were also confirmed in CIA rats, and the results demonstrated that matraine might attenuate collagen-II-induced systemic inflammation and bone loss. The up-regulation of PGRN and the inhibition of miR-29b-3p were both responsible for all of these effects. The results presented here give more evidence for the preventive effects of matrine against bone diseases, and the modification of the miR-29b-3p/PGRN axis adds to our understanding of the processes behind matrine's action.

An growing number of studies demonstrate that compounds originating from natural plants may be exploited as novel candidate treatments against various bone disorders like OA and RA [21, 22], and matrine is one of the compounds with promising anti-OA actions. The substance is a key ingredient in the traditional Chinese medicine Kushen, which is made from the dry root of Sophora flavescens. In China, the medication has traditionally been used to treat pruritus, eczema, and dysentery [23]. Recent clinical trials also show that Kushen injection can be utilized as an adjuvant treatment for several malignancies [24-26]. It has been demonstrated that single-component medications containing matrine have certain advantages over Kushen injections in terms of quality control because it is the principal bioactive chemical in Kushen and contributes significantly to the biological functions of the drug [23]. As new research on the bioactivity of matrine is conducted, the possible protective properties of matrine against bone illnesses like OA are being reported more frequently. For instance, the Qing-Luo-Yin formula, which contains matrine as a key ingredient, has demonstrated significant therapeutic efficacy against OA and RA for more than 40 years [12]. Both studies by Pu et al. and Yang et al. demonstrate that matrine has protective effects on chondrocytes or bone tissues [13, 14]. The results of the present study confirmed those of earlier investigations and established a link for the first time between the role of miR-29b-3p and the preventive effects of matrine against bone diseases.

Several investigations conducted over the past ten years have revealed a direct link between miRs and the onset and development of bone diseases. In osteoarthritic cartilage compared to normal cartilage, levels of miRs such miR-483, miR-22, miR-377, miR-103, etc.

was up-regulated, while miR-29a, miR-140, miR-25, and miR-373 levels were down-regulated. [16]. Thus, it is reasonable to investigate the role of miRs in the anti-OA function of matrine given the close involvement of miRs in the pathogenesis of OA. As a result, when matrine was administered to chondrocytes treated with IL-1, we noticed alterations in previously described OA-related miRs. Due to its high sensitivity and pro-OA potential, miR-29b-3p was chosen as the possible target of matrine. According to Chen et al. research, miR-29b-3p inhibits PGRN, which increases the growth of OA and causes chondrocyte death [16]. Another work by Zhi et al. found that miR-29b-3p is induced and PGRN is inhibited when lncRNA OIP5-AS1 is inhibited by IL-1 [17]. As a result, alterations in the miR-29b-3p/PRGN axis were also found in the current investigation when matrine was being administered. In IL-1-treated chondrocytes as well as in CIA rats, it was demonstrated that matrine reduced the expression of miR-29b-3p while inducing the expression of PGRN. Progranulin (PGRN), an autocrine growth factor that is highly expressed in many tissues, can slow the course of OA by altering the TNF-/-catenin signaling pathway [27-31]. The modifications to miR-29b-3p/PRGN by matrine add to our understanding of the compound's bone-protective properties and will support the therapeutic use of matraine as a therapy for various bone diseases.

The findings of this study show that matrine has strong anti-apoptotic and pro-proliferative effects on chondrocytes, which will attenuate OA in rat models. Interestingly, the effects of matrine treatment were linked to modifications in miR-29b-3p/PRGN, which suggests a brand-new therapeutic mechanism for the compound's anti-OA actions.

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Not applicable

Conflict of interests

The authors disclose no conflict of interests

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Figure legends

Figure 1 Verification of miR expression levels in chondrocytes. Chondrocytes were subjected to the co-treatment of IL-1β and matrine, and the levels of miRs involved in the progression of OA were detected with RT-qPCR detection.

Figure 2 Matrine increased viability and suppressed apoptosis in IL-1β-treated chondrocytes. A, analysis results of CCK-8 detection of cell viability. **B**, representative images and analysis results of flow cytometry detection of cell apoptosis. "*" $P < 0.05$ vs. Control group. "#" $P < 0.05$ vs. IL-1β group.

Figure 3 Matrine suppressed the production of IL-6, IL-1β, and TNF-α, and inhibited the expression of miR-29b-3p, while increased the expression of PGRN in IL-1β-treated chondrocytes. A, analysis results of ELISA detection of IL-6. **B**, analysis results of ELISA detection of IL-1β. **C**, analysis results of ELISA detection of TNF-α. **D**, analysis results of RT-qPCR detection of miR-29-3p. **E**, representative images and analysis results of western blotting detection of PGRN. "*" $P < 0.05$ vs. Control group. "#" $P < 0.05$ vs. IL-1β group.

Figure 4 Matrine inhibited the weight loss and reduced arthritis score in CIA rats. Rats were subjected to CIA and matrine administrations in a six-week period. A, analysis result of changes in body weight. **B**, analysis results of changes in arthritis score. "*" $P < 0.05$ vs. Control group. "#" $P < 0.05$ vs. CIA group.

Figure 5 Matrine attenuated bone destruction and suppressed the production of IL-6, IL-1β, and TNF-α in CIA rats. A, analysis result and representative images of H&E staining of joint tissues. **B**, analysis results of ELISA detection of IL-6. **C**, analysis results of ELISA detection of IL-1β. **D**, analysis results of ELISA detection of TNF-α. "*" $P < 0.05$ vs. Control group. "#" $P < 0.05$ vs. CIA group.

Figure 6 Matrine down-regulated the expression of miR-29b-3p and up-regulated the expression of PGRN in CIA rats. A, analysis results of RT-qPCR detection of miR-29b-3p. **C**, representative images and analysis results of western blotting detection of PGRN. "*" $P < 0.05$ vs. Control group. "#" $P < 0.05$ vs. CIA group.

Figure 1

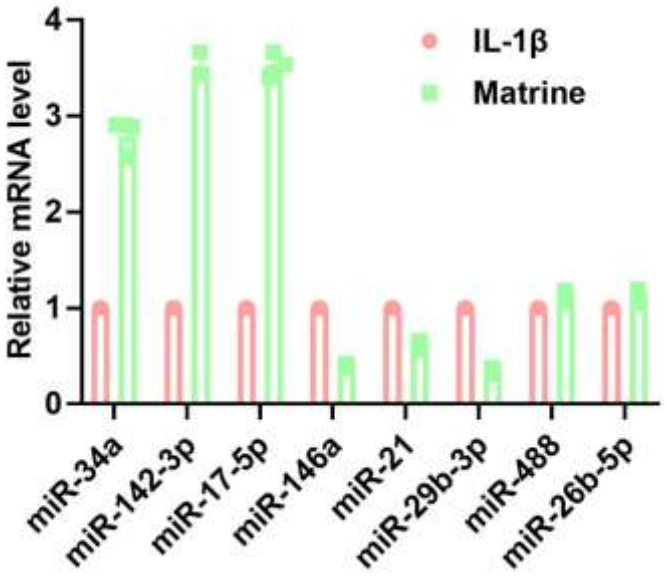
Relative mRNA level
4
3
2
1
0
IL-1β
Matrine
miR-34a
miR-142-3p
miR-17-5p
miR-146a
miR-21
miR-29b-3p
miR-488
miR-26b-5p

Figure 2

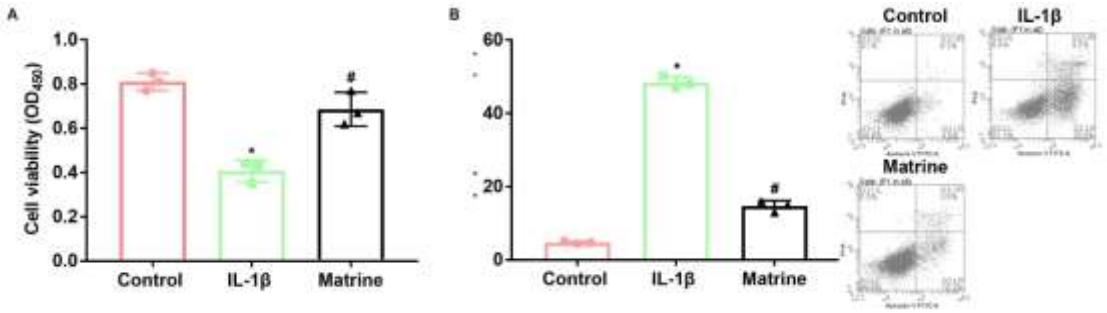
A
Cell viability (OD450)
1.0
0.8
0.6
0.4
0.2
0.0
Control
IL-1β
Matrine
B
60
40
20
0
Control
IL-1β
Matrine
Control
IL-1β
Matrine

Figure 3

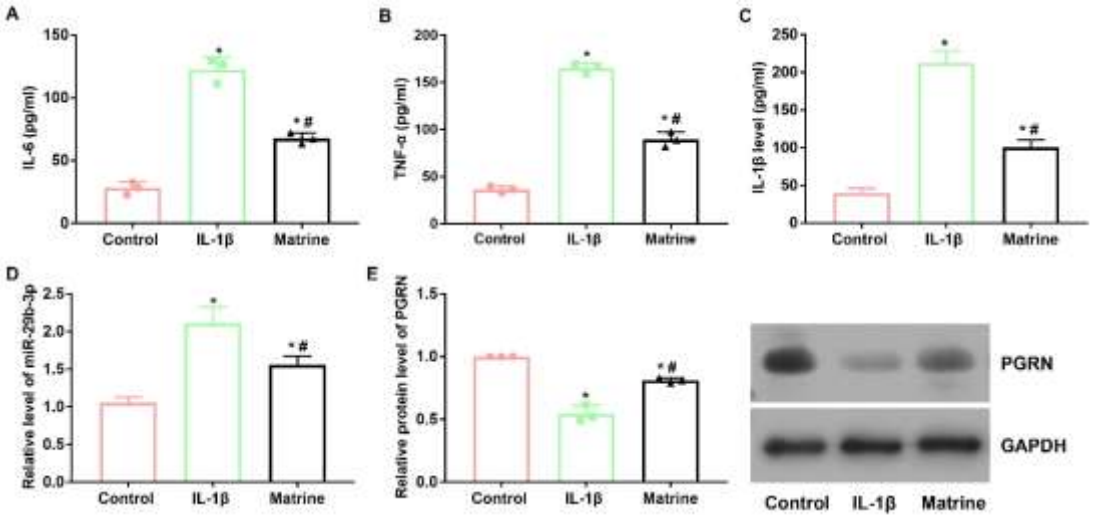
A
IL-6 (pg/ml)
150
100
50
0
Control
IL-1β
Matrine
B
TNF-α (pg/ml)
200
150
100
50
0
C
IL-1β level (pg/ml)
250
200
150
100
50
0
D
Relative level of miR-29b-3p
2.5
2.0
1.5
1.0
0.5
0.0
E
Relative protein level of PGRN
1.5
1.0
0.5
0.0
PGRN
GAPDH

Figure 4

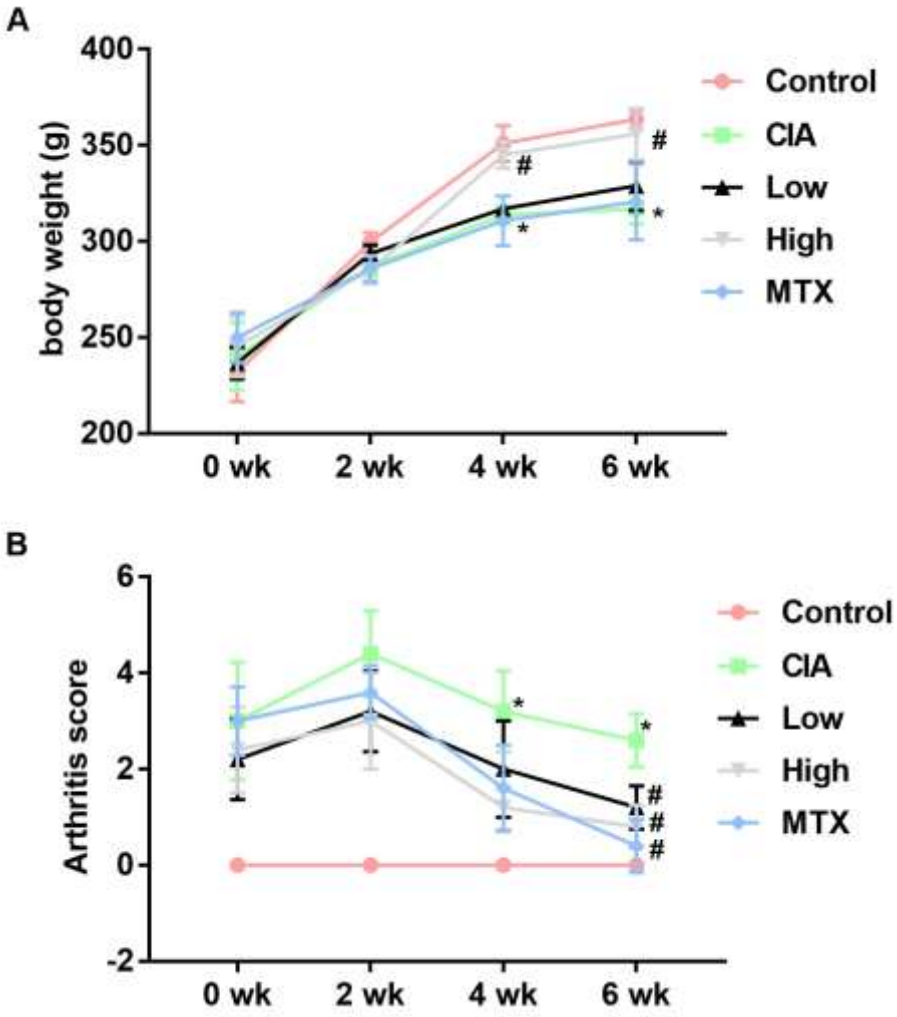
A
body weight (g)
400
350
300
250
200
0 wk
2 wk
4 wk
6 wk
Control
CIA
Low
High
MTX
#
#
*
*
B
Arthritis score
6
4
2
0
-2
0 wk
2 wk
4 wk
6 wk
Control
CIA
Low
High
MTX
*
*
#
#
#
#

Figure 5

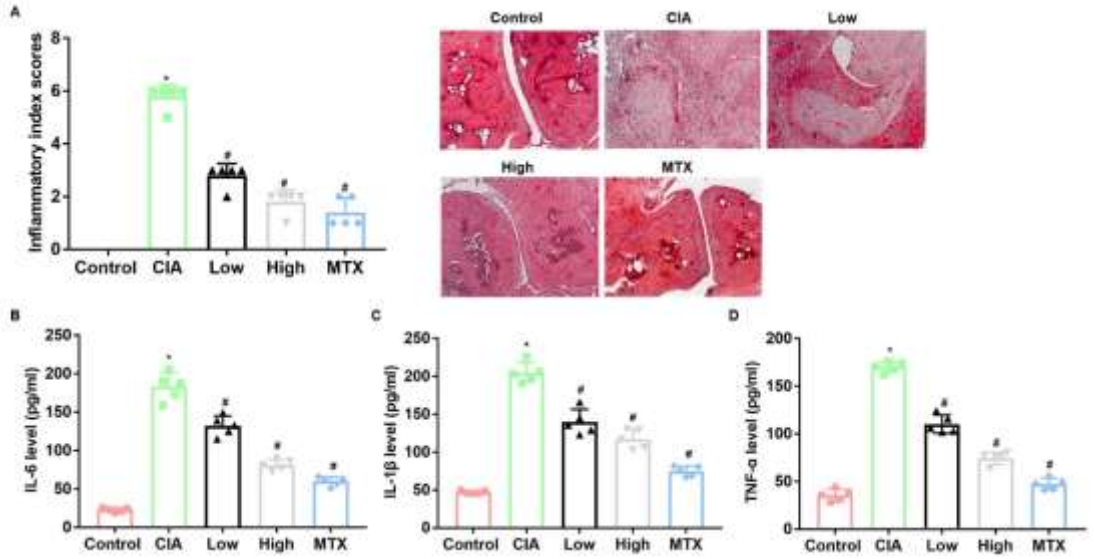
A
Inflammatory index scores
Control
CIA
Low
High
MTX
B
IL-6 level (pg/ml)
C
IL-1β level (pg/ml)
D
TNF-α level (pg/ml)

Figure 6

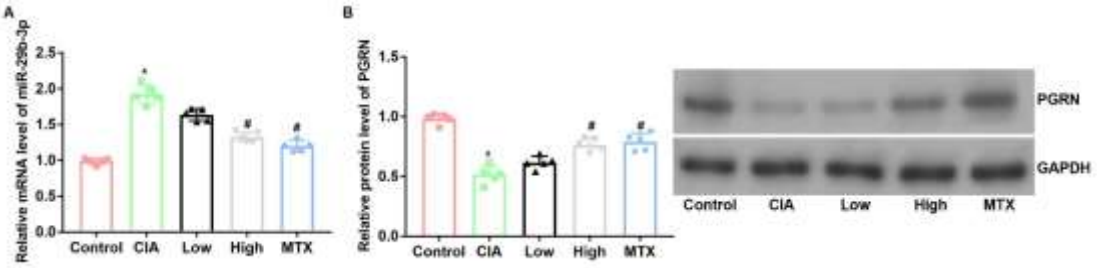
A
Relative mRNA level of miR-296-3p
2.5
2.0
1.5
1.0
0.5
0.0
Control
CIA
Low
High
MTX
B
Relative protein level of PGRN
1.5
1.0
0.5
0.0
Control
CIA
Low
High
MTX
PGRN
GAPDH
Control
CIA
Low
High
MTX

Table S1 Primer information

Gene	Direction	Sequence (5’-3’)
miR-34a	Forward	GAATCTACGTCACCCGAAAAG
	Reverse	CAAACACGTGGGACAGCCAAG
miR-142-3p	Forward	CGCCGTGTAGTGTTTCCTAC
	Reverse	CAGTGCAGGGTCCGAGGT
miR-17-5p	Forward	TGCGCCAAAGTGCTTACAGTGCA
	Reverse	CCAGTGCAGGGTCCGAGGTATT
miR-146a	Forward	TGAGAACTGAATTCCATGGGTT
	Reverse	ATCTACTCTCTCCAGGTCCTCA
miR-21	Forward	CAGTGCAGGGTCCGAGGT
	Reverse	GCCCGCTAGCTTATCAGACTGATG
miR-29b-3p	Forward	UAGCACCAUUUGAAAUC
	Reverse	GTGCAGGGTCCGAGGT
miR-488	Forward	GATGCTACCCAGATAATGGCACT
	Reverse	CAGTGCGTGTCGTGGAGT
miR-26b-5p	Forward	TTCAAGTAATTCAGGATAGGT
	Reverse	GTGCGTGTCGTGGAGTC
U6	Forward	CTCGCTTCGGCAGCACATATACT
	Reverse	ACGCTTCACGAATTTGCGTGTC