The protective effect of mangiferin on osteoa

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Summary

Mangiferin is a kind of polyphenol chemical compound separated from these herbal medicines of Mangifera indica L., Anemarrhena asphodeloides Bge. and Belamcanda chinensis (L.) DC., which has anti-inflammatory, anti-virus, and other physiological activities without toxic effects. Osteoarthritis (OA) is a chronic disease, that is also a kind of arthritis disease in which articular cartilage or bones under the joint is damaged. In addition, artificial replacements are required in severe cases. At present, there are not too much researches on the potential biological activities of mangiferin that plays a protective role in the treatment of OA. In this study, we evaluated the protective effect of mangiferin on osteoarthritis (OA) *in vitro* and *in vivo*. First, the effect of different concentrations of mangiferin on rat chondrocytes was determined by MTT assay. Second, the effects of mangiferin on the expression levels of matrix metalloproteinase (MMP)-13, TNF-α, Collagen II, Caspase-3, and Cystatin-C in interleukin-1β (IL-1β)-induced rat chondrocytes were examined by the real-time polymerase chain reaction *in vitro*, meanwhile the effects of mangiferin on the nuclear factor kappa-B (NF-κB) signaling pathway were also investigated by Western Blot. Finally, the anti-osteoarthritic protective effect of mangiferin was evaluated in the rat model by anterior cruciate ligament transection (ACLT) combined with bilateral ovariectomy-induced OA *in vivo*. The results showed that the mangiferin was found to inhibit the expression of MMP-13, TNF-α, and Caspase-3 which also increased the expression of Collagen II and Cystain-C in IL-1β-induced rat chondrocytes. In addition,

IL-1β-induced activation of nuclear factor kappa-B (NF-κB) and the degradation of inhibitor of κB (IκB)-α were suppressed by Mangiferin. For the *in vivo* study in a rat model of OA, 100 μL of mangiferin was administered by intra-articular injections for rats, the results showed that the cartilage degradation was suppressed by mangiferin through Micro CT and Histological Examination. According to both *in vitro* and *in vivo* results, mangiferin has a protective effect in the treatment of OA which may be a promising therapeutic agent for OA.

Key words: Mangiferin, Osteoarthritis (OA), Cytokines, Gene expression, *In vitro*, *I*n vivo

Introduction

Nowadays, osteoarthritis (OA) is one of the most frequent chronic diseases which is complex and multifactorial epidemiology, meanwhile, the most important factor initiating and amplifying this disease is the inflammatory response [1]. Interleukin-1 (IL-1) was first described as a monocyte/macrophage product in articular tissue. As a lymphokine, interleukin-1 (IL-1) also could induce the production of collagenase and prostaglandin in synovial fibroblast cultures [2]. Further, Mengshol et al. reported that IL-1 significantly down-regulated the expression of matrix metalloproteinases (MMPs) [3] and caused the degradation of extra-cellular matrix (ECM).

The exact mechanism of OA has not been elucidated [4], which is not yet discovered in the early phase with an effective drug for the treatment of OA. In the clinical, non-steroidal anti-inflammatory drugs (NSAIDs), hyaluronan and corticosteroids have been used in the treatment of OA [5]. However, these drugs could not reverse the cartilage damage, and this disease continues to progress significantly to the stage in which prosthetic replacement is need required. Therefore, there is an urgent need for better therapeutics that can impede cartilage damage so that the later stages of OA can be better treated. In recent years, more researchers are interested in natural herbal compounds, which are regarded as promising remedial agents in immunological disorders that could halt the progression of the disease without any toxicity [6].

Mangiferin is a polyphenol that has been used as a non-prescription drug [7]. However, the anti-inflammatory properties of mangiferin in OA chondrocytes remain unclear [7,8].

However, some studies have revealed that mangiferin dampens the inflammatory response in tumor necrosis factor- alpha (TNF-α)-induced RAW264.7 cells *in vitro* by inhibiting the activation of the nuclear factor kappa-B (NF-κB) pathway [9]. Therefore, the mangiferin was speculated that could be effective in protecting against OA because of its anti-inflammatory effects. In this study, the mangiferin was proposed that had a protective effect against OA due to it was initiated by the inflammatory response in the early phase.

Methods

Primary rat chondrocytes culture

Rat chondrocytes were prepared by combine digestion with collagenase-neutral protease which was isolated from the Procell laboratory, with a total cell volume of approximately 5×10^5cells/bottle (Wuhan Procell Life Technology Co. Ltd, China). The cells were grown and passaged in Dulbecco's modified Eagle medium which was supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) (37°C, 5% CO2). Cells from the third generation were used in this study.

Assay of chondrocytes proliferation

Each well was inoculated with 8000 (cells/well) rat chondrocytes in a 96-well plate containing a serum-free medium. The concentrations of 10, 20, 40, 60, 80, and 100 μmol/L mangiferin were added to a 96-well plate and incubated for 24h (cells were

grown to confluence in Dulbecco's modified Eagle's medium supplemented with, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37℃ with 5% CO_2). After that, 20uL of MTT (Sigma Chemical Co, St. Louis, MO, USA) solution (5 mg/mL in serum-free medium for 24 h) were added into the wells and incubated for another 4 h. Next, the culture medium was removed and 150 uL of dimethyl sulfoxide (DMSO) was added into the wells. Finally, the absorbance was measured by a microplate reader at 570 nm [10]. Furthermore, this absorbance determination needed repeat three times. The results were expressed as chondrocytes proliferative index (CPI), which was calculated as the ratio of optical density (OD) of the treatment group to control cells.

$$CPI = OD_{\text{treatment groups}}/OD_{\text{control group}}$$

Assay for chondrocytes inducement by IL-1β

Subconfluent cells were serum-starved overnight before the experiments were performed. The final concentrations of 10, 20, and 40 μmol/L of mangiferin were added into the wells after seeding in six-well plates (1×10^5 cells/well). Then, they were incubated at 37℃ with 5% CO_2 for 1 h. The final concentration of 10 ng/mL of IL-1β was added into each well and continued to culture for 24 hours. The cells were harvested and the optimum mangiferin concentration was assessed for subsequent experiments, such as western blot analysis [11,12] .

Gene expression analysis (rat chondrocytes inducement by IL-1β)

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (600 μg), 1 μL dNTPs (10 mM), DEPC-treated water (15μL), and primer mixture were mixed into a 200 μL RNase-free centrifuge tube. Then the tube was incubated at 70℃ for 5min after it was incubated on ice. Next, 5× first-strand buffer, 0.1 M dithiothreitol, 25 units of RNase inhibitor, and 200 units of Superscript II reverse transcriptase (Invitrogen) were added into the centrifuge tube. The RNA was reverse-transcribed into cDNA. A quantitative real-time polymerase chain reaction conducted by iCycler system (BioRad, Hercules, CA, USA) and iQ SYBR Green Supermix PCR kit (BioRad) based on sequence information (Table 1) which had been described in our previous study [10]. The relative levels of targeted gene expressions were calculated following the formula:

$$2^{(\Delta ct\ 18s\ rRNA-\Delta ct\ target\ gene)}$$

Western blot analysis

Cytoplasmic protein and nuclear protein from the above samples (normal group, IL-1β-induced group, and mangiferin-treated group) were prepared by nuclear/cytoplasmic Protein Extraction Kit (Signosis, Santa Clara, CA, USA). Membranes were incubated with antibodies(IκB-α, p- IκB-α, NF-κB p65, p-NF-κB p65, β-actin) at 4°C for overnight after blocking in Tris-buffered saline-Tween. Then, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were developed using an

enhanced chemiluminescence kit (GE Healthcare, Shanghai, China) which was exposed by X-ray films (Kodak, Hangzhou, China) for detecting the proteins [10].

Mangiferin treatment in the induction of OA rats (in vivo)

Twenty-four eight-week-old female Sprague Dawley rats (SPF Biotechnology Co, LTD, Beijing, China) were chosen, which were weighed at 300–340 g. As a result of OA was induced in the left knee joint [13] so that the left knee joint of rats was used as the modeling experiment. The patella and patellar tendon were exposed after the rats were anesthetized by sodium pentobarbital (40 mg/kg), in which the patella was dislocated, and the ACL was cut with sharp scissors. Then, the patella was reset. Six rats were also used as sham-operated controls. All rats were allowed to move freely in the feeding conditions (23 ± 2 ℃, the humidity of 40–60%, 12 h light/dark cycles with food and water).

The animals were removed from the experiment, if the rat's knee joint was associated with infection or whether the animal died. In this study, all 24 rats met the inclusion criteria. Rats had been divided into group 1 (control group treated with solvent alone, n = 6,); group 2 (20 μmol/L mangiferin group; n = 6); group 3 (40 μmol/L mangiferin group; n = 6); and group 4 (sham-operated group, n = 6).

In group 2 and 3, rats were injected with 100 μL of mangiferin (20 μmol/L) and intra-articular injections of 100 μL of mangiferin (40 μmol/L) respectively in the left knee once a week for four weeks. Group 1 and 4 were injected with 100 μL of solvent

alone in the left knee once per week for six weeks. Rats were sacrificed seven days after the last injection. All rats were sacrificed after they have surged for nine weeks.

Micro-computed tomography (CT) and gross morphology imaging

After surgery for nine weeks, the knee joints of the rats were scanned and imaged by micro-computed tomography (CT) scanner (SkyScan 1174, Bruker, Kontich, Belgium). The femur condyles of the rats from the four groups were harvested after the CT scanning. The gross morphological changes of the femur condyles were assessed in a blind manner: grade 1: intact surface; grade 2: minimal surface fibrillation; grade 3: overt surface fibrillation; grade 4: erosion [14].

Histological examination

Knee joints specimens were fixed in 10% neutral buffer formalin and then decalcified in EDTA for seven days, after that the knee joints specimens were cut into sections (5 μm) for safranin O-fast green staining and H&E staining. The damage was graded according to the Mankin score system by a blind investigator [15-17]. The definition of different damage grades was as follows the Table 2.

Statistical analysis

SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for data statistical analysis. Data were expressed as the mean ± standard deviation (SD). The data of MTT assay,

194 histological, gross morphological changes, western blot, and gene expression were
195 statistically analyzed by paired t-test, in which *p*-values less than 0.05 were considered
196 statistically significant.
197
198 **Results**
199 *Effect of mangiferin on the viability proliferation*
200 Rat chondrocytes were initially plated in each well of a 96-well plate and added in
201 different concentrations of mangiferin within 0, 10, 20, 40, 60, 80, 100 μmol/L. The
202 number of viable cells was measured by MTT assay after incubated for 24 hours. The
203 results (Fig. 1) showed that the rat chondrocytes were proliferated in three different
204 concentrations of mangiferin (10, 20, 40 μmol/L) groups, among which the
205 concentrations of 10 μmol/L and 40 μmol/L mangiferin presented significant
206 promotions as compared with the control group (0 μmol/L) ($p<0.05$). Meanwhile, the rat
207 chondrocytes proliferation indexes (60, 80, 100 μmol/L groups) were seriously damaged
208 when the concentration was more than 40 μmol/L, which that means it had toxic effects
209 on rat chondrocytes. ($p<0.05$). Therefore, the concentrations of mangiferin used in the
210 follow-up experiments were 10, 20, and 40 μmol/L.
211
212 *Gene expression of MMP-13, TNF-α, Col II, caspase-3, and cystatin C*
213 The expression levels of MMP-13, TNF-α, Col II, caspase-3, and cystatin C were
214 measured in rat chondrocytes. The expression of MMP-13, TNF-α, and caspase-3 (Fig.

2c, d and e) was upregulated by stimulation with IL-1β and the expression of Col II and cystatin C (Fig. 2 a, b) was downregulated. As predicted, IL-1β-induced an upregulation of MMP-13, TNF-α, and caspase-3, meanwhile the downregulation of Col II and cystatin C gene expression in rat chondrocytes were dramatically inhibited by mangiferin (Fig. 2). As this result show, the highest concentration of mangiferin (40 μmol/L) was used for the western blot experiments.

Analysis mangiferin blocking IL-1β-mediated induction of NF-κB signaling pathway

The results showed that IκB-α and the phosphorylation of IκB-α were reduced significantly by IL-1β in the cytoplasm of chondrocytes (Fig. 3 d, e and f), meanwhile, this reduction was significantly blocked by mangiferin. Moreover, phosphorylation of NF-κB p65 was dramatically inhibited by mangiferin (Fig. 3 a,c). However, the content of nuclear NF-κB p65 was not significantly affected in the IL-1β group but it was significantly decreased in the mangiferin-treated group (Fig. 3 a, b).

Analysis of cartilage histomorphology

In the control group within solvent only, general characteristics of OA were shown in Fig. 4 a. In group 2, less bone wear was observed which was compared to the control group, as it was determined by gross appearance (Fig. 4 b). In group 3, the macroscopic examination indicated that the cartilage on the femoral condyles was nearly normal, which was shown in Fig. 4 c. Furthermore, the score in group 3 was lower than the control group (Fig. 4 d).

Meanwhile, the same pattern was observed by micro-CT(Fig. 5). In the control group, the knee joints had a rough and irregular surface at the medial and lateral femur areas (Fig. 5 a). In group 2, there was only slight damage to the cartilage surface(Fig. 5 b). But in group 3 and sham group, no obvious macroscopic changes were found (Fig. 5 c, d).

Histopathological changes in articular cartilage

In the control group, the characteristics of OA were obvious, such as chondrocyte degeneration, depletion, and irregular cartilage surface (Fig. 6 a). But well-formed cartilaginous tissues containing cytoplasm and nuclei, and a smooth and regular cartilage surface, which were observed in group 3 (Fig. 6 c). However, some erosions were exhibited at the cartilage surface which rats in group 2 (Fig. 6 b). There was a normal cartilage matrix in the sham group (Fig. 6 h) and the treatment group of the concentration with 40 μmol/L (Fig. 6 h) (the cartilage surface was uniform red matrix which in the picture), furthermore, the cartilage matrix was distributed uniformly, the chondrocyte nuclei were arranged neatly, the tide line was neat. Meanwhile, the control group (Fig. 6 e) cartilage has degenerated, the cartilage was showed irregularly, there were a large number of cracks, red color was lost staining, cell nucleus was arranged disorderly and showed clustered, the number of nuclei was significantly reduced, and the tide line was disordered. The expression mediation in the mangiferin-treated group (20 μmol/L) was introduced between the control group and the mangiferin-treated group

of 40 μmol/L. Moreover, ACLT led to histopathological changes such as the surface depletion and the reduction of Safranin O-fast green-staining in the cartilage (Fig. 6 e), and the cartilage degradation was inhibited by the treatment group of mangiferin, which was developed in the progression of OA (Fig. 6 f, g). Consistent with these findings, the modified Mankin score was reduced in the mangiferin-treated group as compared with the control group (Fig. 7).

Discussion

In this study, the effects of mangiferin were progressed on OA which were evaluated *in vitro* and *in vivo*. The breakdown of cartilage macromolecules could cause by many biochemical factors such as proteolytic enzymes, MMPs, and cytokines [18,19] . IL-1β was played a critical role in cartilage degradation through the induction of MMPs, especially MMP-13, which was secreted by chondrocytes. Thus, IL-1β had been widely used in *in vitro* studies to generate a micro environment that mimics that of OA [11,12]. Moreover, MMP-13, a predominant proteinase, had the distinctive ability to cleave Col II, a major component of the ECM in OA. Our study found that the IL-1β-mediated induction of MMP-13 in rat articular chondrocytes which was inhibited by mangiferin (Fig. 2 c), this result was consistent with previous studies [20,21].

Previously, Lotz (2001) found that TNF-α could inhibit chondrocyte compensatory biosynthesis pathways. In this study, the TNF-α expression was observed that decreased in the mangiferin-treated groups, especially the group in which was pretreated with the

concentration of 40 μmol/L of mangiferin (Fig. 2 d). Moreover, as IL-1 was contributed to cartilage degradation through upregulating some cytokines, the inhibition of IL-1 was proposed that in chondrocytes could treat OA. An IL-1 receptor antagonist was shown to inhibit the cleavage of Col II and the release of glycosaminoglycan in the cartilage of OA [13]. Our findings were demonstrated that the mangiferin was reversed the IL-1β-induced decrease in the Col II expression in chondrocytes, which may partly be due to the anti-inflammatory effects of mangiferin (Fig. 2 a). These findings suggest that TNF-α, Col II, and MMP influenced and restricted each other at the gene expression level. Thus, the joint cartilage was might be protected by mangiferin with influencing the presence of Col II and maintaining the integrity of cartilage by promoting Col II expression [22-25].

The chondrocyte oxidative stress-induced apoptosis was found that its caused by the development of OA, and caspase-3 was a key enzyme in the mechanism of apoptosis [26-28]. Gao [29] demonstrated a dramatically enhanced caspase-3 gene expression in H2O2-induced injury of chondrocytes. In this study, the caspase-3 gene expression was decreased by following treatment with mangiferin under IL-1β induction (Fig. 2 e**)**, which was suggested that mangiferin could inhibit the progression of OA by caspase-3.

Surprisingly, the cystatin C gene expression was noted that was upregulated in the mangiferin-treated groups (Fig. 2 b). In the previous report, cystatin C could block cathepsin activity by forming a reversible enzyme–inhibitor complex to counteract preexisting OA [30]. A low gene expression of cystatin C would likely contribute to OA

pathology. Thus, mangiferin might be used in the treatment of OA in the early stage, which still should be further researched.

NF-κB plays a critical role in inducing proinflammatory cytokines [31,32]. Many proinflammatory response genes of the expression are controlled by the transcription factor NF-κB. Our study indicated that mangiferin was inhibited the NF-κB activation in chondrocytes via the inhibition of IκB-α degradation. Both IκB-α and the phosphorylation of IκB-α were reduced by IL-1β in the cytoplasm of chondrocytes which were blocked by treatment with mangiferin (Fig. 3 d, e, and f). The IL-1β-induced increase in the NF-κB phosphorylation in chondrocyte nuclei was inhibited by the mangiferin (Fig. 3 a, b, and c). That means, NF-κB was retained in the inactive cytoplasm, but NF-κB was activated by IL-1β and led to the translocation of NF-κB p65 from the cytoplasm to the nucleus. This effect was significantly inhibited by mangiferin. Overall, these results showed that mangiferin could inhibit IL-1β-induced inflammation.

The rat model of OA has been widely used [17,33]. Furthermore, cartilage degradation was induced by ACLT. Our study showed that ACLT in rats caused cartilage degradation due to its mechanical instability. The cartilage degradation (Fig. 4), micro-CT (Fig. 5), and histological evaluation (Fig. 6, 7) were inhibited by delivering mangiferin to the joint. These outcomes were similar to the in-vitro study, which confirmed the protective effect for OA both *in vitro* and *in vivo*.

Conclusion

Mangiferin possesses chondroprotective effects *in vitro* and *in vivo*. The CPI of different concentrations of mangiferin was different in the MTT analysis, in which the CPI was significantly increased at the concentration of 10, 20, and 40 umol/L of mangiferin. The CPIIn IL-1β-induced rat chondrocytes, mangiferin not only inhibited the expression of MMP-13, TNF-α, and caspase-3 but also increased the expression of Col II and cystatin C at the mRNA levels by NF-κB pathway. Through micro-CT and histological examination after *in vivo* injection for OA model rats, it was found that mangiferin could inhibit the degradation of cartilage. The results had indicated that mangiferin would be a promising agent for the treatment of OA.

Abbreviations:

OA: Osteoarthritis; MG: Mangiferin; ACLT: Anterior Cruciate Ligament Transection; MMPs: Matrix Metalloproteinases; IL-1β: Interleukin-1β; ECM: Extracellular Matrix; NSAIDs: Non-steroidal Anti-inflammatory Drugs; TNF-α: Tumor Necrosis Factor Alpha; NF-κB: Nuclear Factor kappa-B; IκB-α: Inhibitor of κB-α; Col II: Type II Collagen; CT: Computed Tomography.

Declarations

Ethics approval and consent to participate

This study had been approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

Consent for publication

Not applicable

Conflict of interest

No conflict of interest.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Figure Captions:

Fig. 1 The effects of mangiferin on chondrocyte proliferation index (CPI) as determined by the MTT assay (n=5). (*p<0.05 compared with the control group)

Fig. 2 Effects of mangiferin on gene expression of MMP-13, TNF-α, Caspase-3, Collagen II, and Cystain-C in rat chondrocytes induced by IL-1β (n=3) (* p<0.05 compared with cells stimulated with IL-1β alone).

Fig. 3 Effects of mangiferin on cytoplasmic protein levels of IκB-α and phosphorylation of IκB-α,and nucleoprotein levels of nuclear factor kappa-B p65 (NF-κB p65) and phosphorylation of NF-κB p65 in chondrocytes induced by IL-1β. (n=3) (* p<0.05 compared with cells stimulated with IL-1β alone).

Fig. 4 The picture and data of effects of the gross morphological changes index with different concentrations of mangiferin group (20umol/L and 40 umol/L) and sham group (n=6) (e, * p<0.05 compared with control group).

Fig. 5 The images of micro-computed tomography (micro-CT) of the knee joints from the rat models (original magnification is ×100) (control group (a and e), treatment group by 20μmol/L Mangiferin (b and f), treatment group by 40μmol/L Mangiferin (c and g), sham group (d and h)).

Fig. 6 The representative pictures with each staining which the effects of different concentrations of mangiferin on the cartilage in vivo (original magnification is ×100), control group (a and e), treatment group by 20μmol/L Mangiferin (b and f), treatment group by 40μmol/L Mangiferin (c and g), sham group (d and h).

Fig. 7 The data of Mankin scores (n=6) (* $p<0.01$ compared with control group， * # $p<0.05$ treatment groups comparison between 20 umol/L and 40 umol/L).

Fig.1

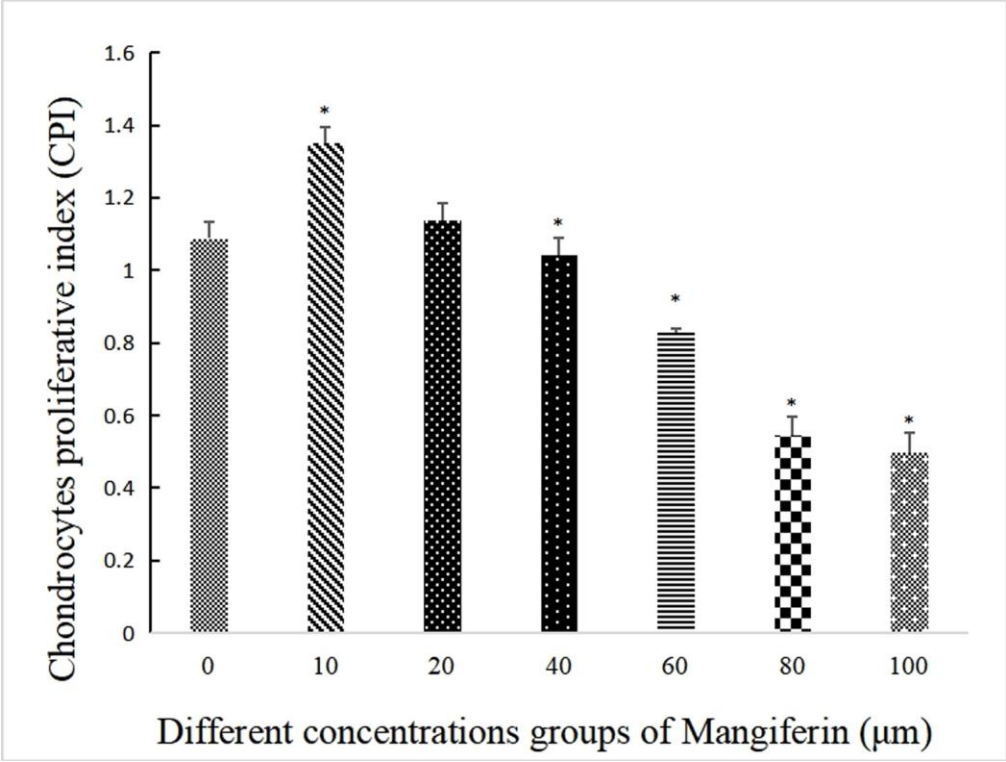


Fig. 2

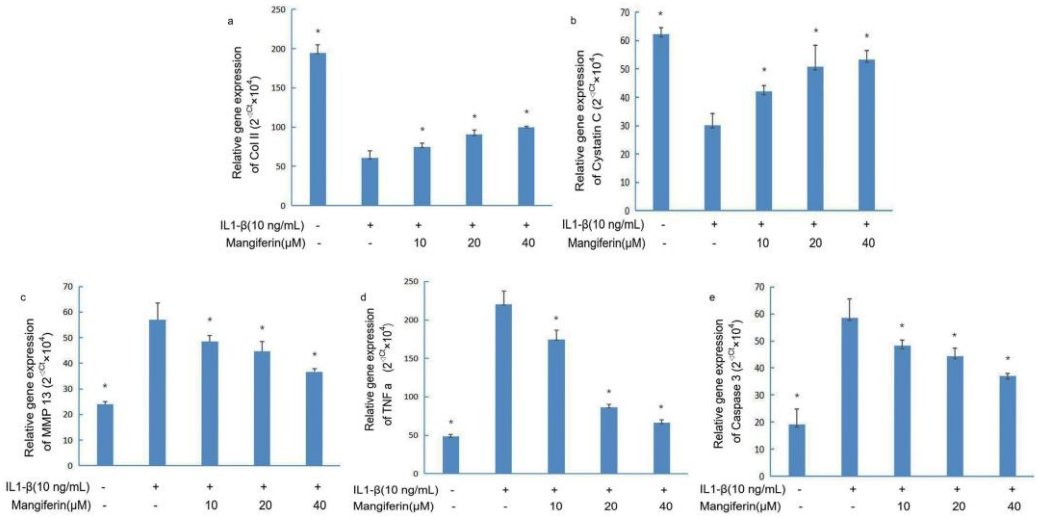

a
Relative gene expression of Col II (2^-CT×10^4)
b
Relative gene expression of Cystatin C (2^-CT×10^4)
c
Relative gene expression of MMP 13 (2^-CT×10^4)
d
Relative gene expression of TNF a (2^-CT×10^4)
e
Relative gene expression of Caspase 3 (2^-CT×10^4)
IL1-β(10 ng/mL)
Mangiferin(μM)
10
20
40

Fig. 3

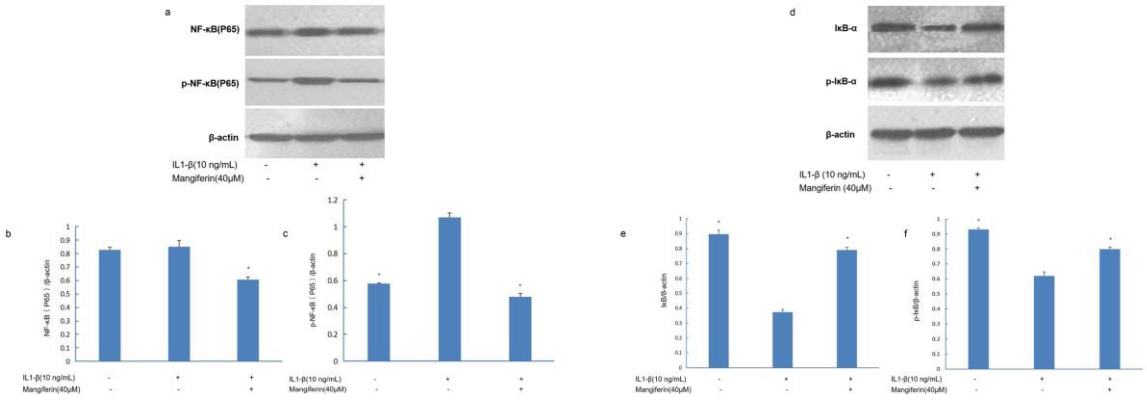

a
NF-κB(P65)
p-NF-κB(P65)
β-actin
IL1-β(10 ng/mL)
Mangiferin(40μM)
b
c
d
IκB-α
p-IκB-α
β-actin
IL1-β (10 ng/mL)
Mangiferin (40μM)
e
f

Fig. 4

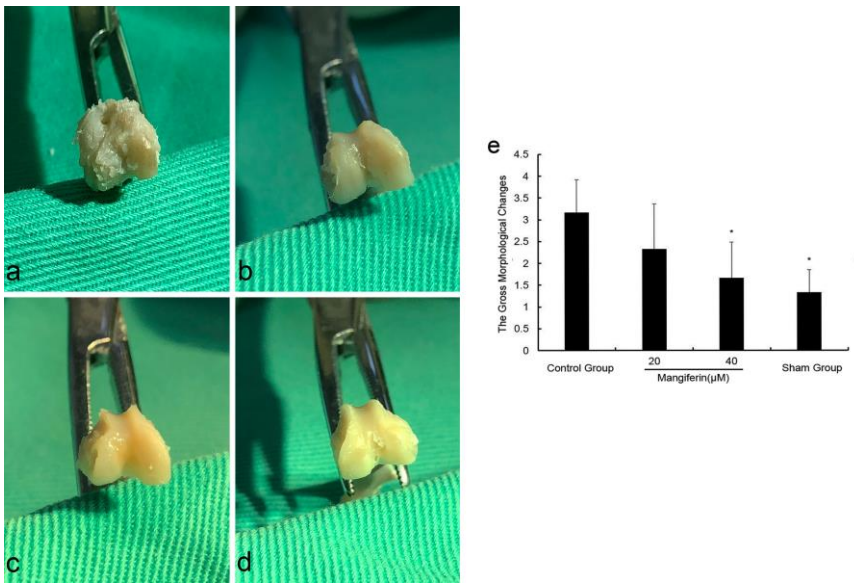

a
b
c
d
e
The Gross Morphological Changes
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control Group
20
40
Mangiferin(μM)
Sham Group

Fig. 5

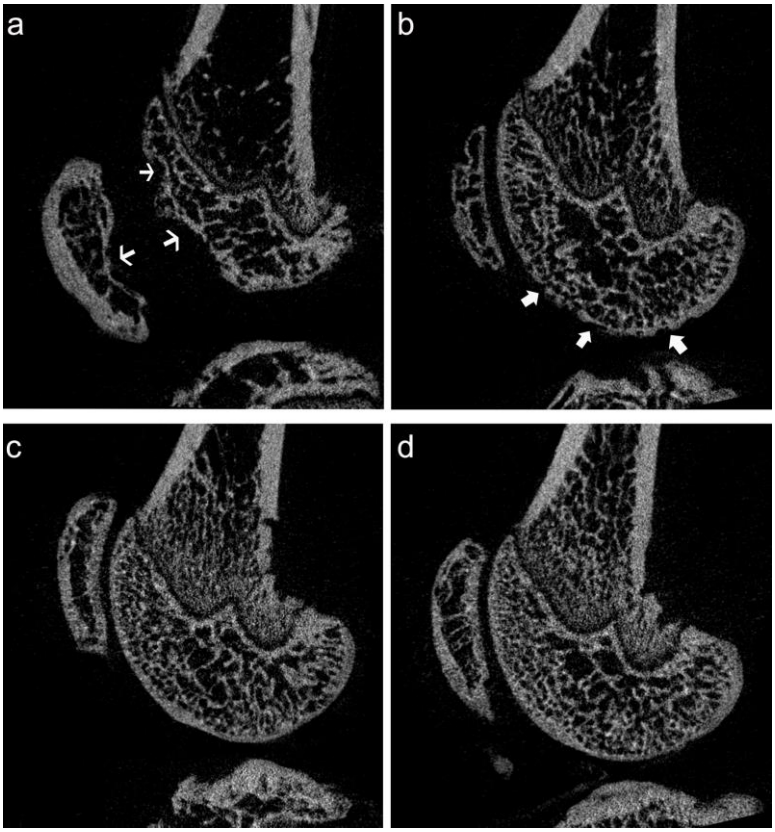

a
b
c
d

Fig. 6

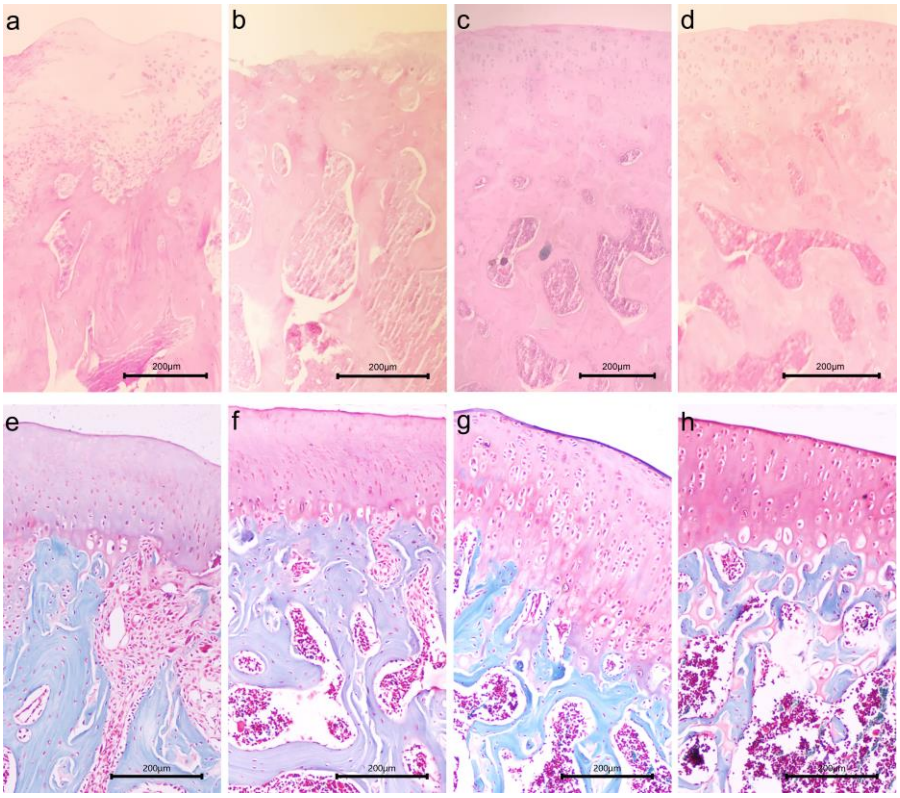
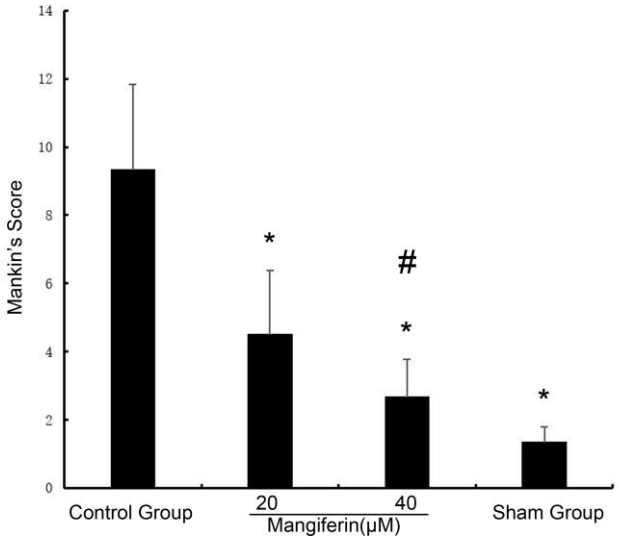

a
b
c
d
e
f
g
h
200μm

Fig. 7


Mankin's Score
0
2
4
6
8
10
12
14
*
#
*
*
Control Group
20
40
Mangiferin(μM)
Sham Group

527 **Table 1.** Real-Time PCR Primers and Conditions

Gene	GenBank Accession	Primer Sequences	Size（bp）	Annealing (℃)
Rat-18S	M11188	5’GAATTCCCAGTAAGTGCGGG TCATA 3’ 5’CGAGGGCCTCACTAAACCAT C3’	105	62
Rat MMP-13	NM_133530	5' CAACCCTGTTTACCTACCCACT TAT 3' 5' CTATGTCTGCCTTAGCTCCTGT C 3'	85	62
Rat-TNF-α	NM_012675	5’ GGTCCCAACAAGGAGGAGAAG TTC3’ 5’CCGCTTGGTGGTTTGCTACGA C3’	136	64
Rat col II	L48440	5' CTGGTGGAGCAGCAAGAGC 3' 5' GTGGACAGTAGACGGAGGAAA G 3'	144	64
Rat Caspase3	NM_012922	5’ AGAGTTGGAGCACTGTAGCAC ACA3’	187	64

		5’TCATGTCCACCACTGAAGGA TGGT-3’		
Rat Cystatin C	NM_01283 7.1	5’ ACTTCGCCGTAAGCGAGTACA ACA3’ 5’ TCGGCCCATCTCCACATCCAAA TA3’	128	64

528

529

530 **Table 2.** The definition of different damage grades

Grades	**Modified Mankin score system**	**Cellular abnormalities**	**Matrix staining**
0	Normal	Normal	Normal
1	Surface irregularities	Diffuse hypercellularity	Slight reduction
2	Pannus and surface	Cloning	Moderate reduction
3	irregularities	Hypocellularity	Severe reduction
4	Clefts to transitional zone	/	No dye note
5	Clefts to radial zone	/	/
6	Clefts to calcified zone	/	/
	Complete disorganization		

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