Gentiana macrophylla exhibits a potential therapeutic effect on osteoarthritis (OA) via modulating disease-related proteins

Type

Research paper

Keywords

osteoarthritis, COX-2, Chondrocyte, PGE2, MMPs, Gentiana macrophylla

Abstract

Introduction
Prostaglandin E2 (PGE2) has been reported to cause cartilage degradation in the pathogenesis of osteoarthritis (OA). Matrix metallopeptidases (MMPs) play important roles in the pathogenesis of OA, while p-AKT and p-P39 signaling pathways were reported to be activated in the pathogenesis of OA. In this study, we aimed to investigate the effect of Gentiana macrophylla (GM) on the treatment of OA.

Material and methods
Primary rat chondrocytes were treated with PBS, IL-1β, and IL-1β+GM respectively to established in vitro models, and in vivo models were set up as a SHAM group, a monoiodoacetic acid (MIA) group, a MIA+GM (low dose) group and a MIA+GM (high dose) group.

Results
In primary rat chondrocytes, the IL-1β treatment elevated the expression of PGE2 and COX2 mRNA. However, the GM treatment reduced the expression of PGE2 mRNA and COX2 mRNA. Also, the GM treatment reduced the expression of above MMPs in primary rat chondrocytes treated with IL-1β. Moreover, unlike P38 and AKT, GM treatment could reduce the expression of p-P38 and p-AKT in primary rat chondrocytes treated with IL-1β. Also, GM treatment reduced the up-regulated expression of COX2, MMPs including MMP-1, MMP-3 and MMP-13, and p-P38 and p-AKT in OA rat models, thus exhibiting a therapeutic effect on OA pathology.

Conclusions
Our study demonstrated the inhibitory effect of GM on the up-regulated expression of PGE2, Cyclooxygenase-2 (COX-2), MMPs including MMP-1, MMP-3 and MMP-13, AKT and P38 in OA models, thus verifying the therapeutic effect of GM on the treatment of OA.

Gentiana macrophylla exhibits a potential therapeutic effect on osteoarthritis (OA) via modulating disease-related proteins

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Abstract

Background: Prostaglandin E2 (PGE2) has been reported to cause cartilage degradation in the pathogenesis of osteoarthritis (OA). Matrix metallopeptidases (MMPs) play important roles in the pathogenesis of OA, while p-AKT and p-P39 signaling pathways were reported to be activated in the pathogenesis of OA. In this study, we aimed to investigate the effect of *Gentiana macrophylla* (GM) on the treatment of OA. **Method**: Primary rat chondrocytes were treated with PBS, IL-1β, and IL-1β+GM respectively to established in vitro models, and in vivo models were set up as a SHAM group, a monoiodoacetic acid (MIA) group, a MIA+GM (low dose) group and a MIA+GM (high dose) group. **Results**: In primary rat chondrocytes, the IL-1β treatment elevated the expression of PGE2 and COX2 mRNA. However, the GM treatment reduced the expression of PGE2 mRNA and COX2 mRNA. Also, the GM treatment reduced the expression of above MMPs in primary rat chondrocytes treated with IL-1β. Moreover, unlike P38 and AKT, GM treatment could reduce the expression of p-P38 and p-AKT in primary rat chondrocytes treated with IL-1β. Also, GM treatment reduced the up-regulated expression of COX2, MMPs including MMP-1, MMP-3 and MMP-13, and p-P38 and p-AKT in OA rat models, thus exhibiting a therapeutic effect on OA pathology. **Conclusion**: Our study demonstrated the inhibitory effect of GM on the up-

regulated expression of PGE2, Cyclooxygenase-2 (COX-2), MMPs including MMP-1, MMP-3 and MMP-13, AKT and P38 in OA models, thus verifying the therapeutic effect of GM on the treatment of OA.

Running title: *Gentiana macrophylla* inhibits osteoarthritis by modulating the expression of disease-related proteins

Keywords: *Gentiana macrophylla*, Osteoarthritis, PGE2, COX-2, MMPs, COX-2, chondrocyte

Abbreviation

OA: osteoarthritis

GM: *Gentiana macrophylla*

MIA: monoiodoacetic acid

Introduction

As a type of degenerative illness of the articular cartilage, osteoarthritis (OA), which is usually caused by the wear and tear of cartilage, impacts the normal life of roughly 1/3 of the worldwide population [1]. Characterized by articular cartilage degradation and hypertrophic joint changes, the incidence of OA increases in older people. In China alone, more than half of the people with an age of > 65 years old struggle with OA, which drastically influences their life quality [2]. OA is caused by numerous reasons, such as the aging of the worldwide population, joint stress, injury, as well as overweight, each one of which can lead to damages to the articular cartilage, triggering articular as well as subchondral bone hyperplasia. Thereby, OA causes symptoms including chronic joint inflammation, pain, as well as joint deformity [3-5].

GM is mainly used in China as well as Siberia. The root of GM contains some materials which are commonly utilized in Chinese medicine for the treatment of many health conditions, including diabetes, paralysis, apoplexy, as well as rheumatism [6]. GM belongs to the Gentianaceae family. The dried root of GM has been widely used as a Chinese medicinal herb. The active compounds of GM, including gentiopicroside, loganic acid, swertiamarin and sweroside, are enriched in the plant and exert extensive pharmacological effects, including choleretic, stomachic, anti-

inflammatory, anti-hepatotoxic, as well as also antihistamine effects [7, 8]. Therefore, GM is often made use of to handle rheumatoid arthritis [7]. Moreover, GM has been reported to be useful in the management of apoplexy, diabetes, liver injuries, and rheumatism [9-11]. Especially, GM has been demonstrated to be a useful anti-inflammatory agent in the control of inflammation of rheumatoid arthritis [11].

MMPs belong to the family of proteolytic enzymes dependent on zinc and play a role in protein degradation [12]. MMPs may degrade any kind of extracellular matrix component, such as vitronectin, proteoglycans, laminin, fibronectin, as well as collagens. From a standpoint of disease progression, MMPs are very important during tumor growth as well as metastasis [13, 14]. For that reason, the levels of MMP-1, MMP-9 as well as MMP-2 proteins in OA patients were increased to promote the progress of tumors and illnesses such as OA. Among MMPs, attention has been drawn to MMP-13 considering that it is substantially overexpressed in the articular cartilage as well as joint tissues in OA patients but is lowly expressed in normal tissues. In a mouse model of OA, the level of MMP-13 was associated with the chondrocytes collected from OA rats during the onset of OA [15]. The over-expression of MMP-13 can trigger the onset of OA via excessive degradation of the extracellular matrix (ECM) [16, 17].

The family of p38 mitogen-activated protein kinases (MAPK) is made up of 4 proteins: p38α, p38β, p38γ, as well as p38δ. Among these kinases which are expressed in a tissue-specific manner, p38α is ubiquitously expressed at significant levels in most cell types [18]. Besides, p38 MAPKs act as substrates of 3 MAP2K enzymes, i.e., MKK6, MKK4 as well as MKK3). The treatment with inhibitors of p38 MAPK prevents inflammation as well as joint degradation in mice, showing the potential of using p38 MAPK inhibitors in the treatment of OA [19]. Past studies also revealed that the cAMP response element-binding protein (CREB) activation led to boosted MMP13 expression in chondrocytes [20, 21]. In addition, the activation of CREB induced by IL-1β is mostly depending on the action of p38 kinases [22, 23]. Thus, the expression of p-P38, as well as p-CREB, was elevated in osteoarthritic cartilages [24].

It has been shown that IL-18 may induce the production of PGE2, which led to cartilage degradation in the pathogenesis of OA [25]. Meanwhile, celecoxib could diminish the levels of

COX-2 and promote the degeneration of cartilage in the progression of OA and bone healing [26]. Moreover, MMPs including MMP-1, MMP-3 and MMP-13 were reported to play important roles in the pathogenesis of OA [15-17]. It was also reported that the down-regulated p-AKT in the cartilage tissue could promote chondrocyte apoptosis and autophagy in the pathogenesis of OA [27]. And the p-P38 signaling pathway up-regulated the expression of iNOS and COX-2, which induced apoptosis in the pathogenesis of OA [28]. In this study, we hypothesized that GM might exhibit its therapeutic effect upon OA via modulating the expression of the above-mentioned genes and proteins. To validate our hypothesis, we established cellular and animal models and administrated GM to observe the effect of GM during the treatment of OA via observing its effect on the expression of PGE2, COX-2, AKT, P38 and MMPs including MMP-1, MMP-3 and MMP-13.

Materials and Methods

Extract preparation

The extract preparation was accomplished following instructions given by a previous publication [9]. Briefly, we utilized Gentiana macrophylla roots which were harvested in autumn and dried naturally under the sun (Gansu Provincial Medical Company, Gansu, China). 500 g of the filtered fine powder (by 40 mesh filter) of the roots of GM (harvested from field) were treated with 70% ethanol for 1 h under reflux, and the extraction was repeated twice and the extract from both extractions was pooled. In the next step, the extract was concentrated via running through a gel column packed with molecular adsorption silica (200-400 mesh) and then eluted by using chloroform. Then, the mixture of the extract of GM and chloroform was dried and then ground to produce a brownish powder. During use, the powder was reconstituted using distilled water to produce solutions at desired concentrations.

Animal model and treatment

A total of 28 Sprague-Dawley Male Rats (with an average age of 3 months old and an average bodyweight of 250 g) were utilized in this study. All SD rats were placed in animal cages with each cage holding five rats. In addition, the conditions of the animal room complied with the requirements of the SPF grade. During the study, all rats were given unlimited access to water and food, and the environment in the animal room was set to a humidity level of 50% and a

constant temperature of 22°C. The animal model was established according to a previous publication [29]. Briefly, during the experiments, the rats were divided randomly into 4 groups with 4 rats in each group: 1. SHAM group (SD rats treated with sterile saline); 2. MIA group (SD rats treated with MIA to induce OA; 3. MIA + GM group-low dose (SD rats treated with MIA to induce OA and at the same time given a 100 mg/kg body weight of GM); 4. MIA + GM group-high dose (SD rats treated with MIA to induce OA and at the same time given a 200 mg/kg body weight of GM). All rats in the MIA groups were given MIA through intra-articular injection via the infra-patella ligament in the right knee, and the dose of MIA was 50 μl of sterile saline containing 1 mg of MIA for each rat. During the injection of MIA, all rats were anesthetized intraperitoneally with ketamine (75 mg/kg) + dexmedetomidine (0.5 mg/kg). The rats in the SHAM group were given the intra-articular injection of sterile saline in the same volume. In terms of administration of GM, the rats in the two MIA + GM groups were given the extract of GM, which was obtained via the method described above, via oral administration at a fixed dose of one hundred milligrams per one kilogram of body weight. The cartilage tissues of the rat hip joints were collected for subsequent analysis. At the end of the study, the rats were sacrificed by a lethal intravenous dose of sodium pentobarbital (100 mg/kg) in strict compliance with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH). All experimental operations involving animal treatment were carried out upon approval from the Animal Ethics Committee of our university.

Isolation of primary rat chondrocytes

After SD rats were adapted to the conditions of the SPF environment, primary chondrocytes were collected from the hip joints of the rats. In brief, the cartilage tissues of the rats were collected from their hip joints and then minced into 1 mm3 cubes under a sterile environment. After that, the cartilage tissues were trypsinized for 1 h at room temperature with 0.25% trypsin before they were incubated for 6 h at 37 ° C and under 5% carbon dioxide in DMEM-F12 (Gibco, Thermo Fisher Scientific, Waltham, MA) containing 0.2% collagenase II. Afterward, the suspension of chondrocytes was centrifuged for 5 minutes at 241.5 xg and then cultured in DMEM-F12 containing 10% FBS as well as 1% suitable antibiotics.

Cell culture and treatment

Chondrocytes were harvested from SD rats and then maintained in a special growth medium of chondrocytes in an environment of 5% CO_2 and 37 °C under saturated humidity. Then, cellular models were established according to a previous publication [30]. Briefly, the cells were divided into 3 groups, i.e., 1. PBS group (rat chondrocytes treated with PBS only); 2. IL-1β group (rat chondrocytes treated with IL-1β); 3. IL-1β + GM group (rat chondrocytes treated with IL-1β and GM). In the IL-1β groups, the growth medium was replaced by a medium containing no serum for overnight culture before the cells were treated by 10 ng/mL of IL-1β for 2 h. In the IL-1β + GM group, the cells were treated with 10 ng/mL of IL-1β along with 5 μM of GM for 2 h. Subsequently, the cells were harvested for real-time PCR and Western blot to measure the expression of target genes.

RNA isolation and real-time PCR

The isolation of total RNA from tissue and cell samples was carried out by using a Trizol reagent (Invitrogen, Carlsbad, CA) based on the instruction manual provided by the assay kit manufacturer. To determine the expression of COX2 mRNA, MMP-1 mRNA, MMP-3 mRNA, and MMP-13 mRNA in the samples, the extracted total RNA in each sample was first reverse transcribed into cDNA by using an iScript cDNA Synthesis assay kit (Bio-Rad Laboratories, Hercules, CA) based on the instruction manual provided by the assay kit manufacturer. In the next step, real-time PCR was done by utilizing an iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) based on the instruction manual provided by the assay kit manufacturer, on a 7900HT real-time PCR machine (Applied Biosystems, Foster City, CA). Finally, the relative expression of COX2 mRNA, MMP-1 mRNA, MMP-3 mRNA, and MMP-13 mRNA was calculated using the 2 delta delta Ct method. GAPDH was used as the internal reference.

Western blot analysis

To determine the protein expression of COX2, MMP-1, MMP-3, MMP-13, P38, p-P38, AKT, and p-AKT in each sample, the tissue and cell samples were first lysed in a RIPA buffer (Invitrogen, Carlsbad, CA) based on the instruction manual provided by the reagent manufacturer. In the next step, the isolated protein from each sample (50 μg) was resolved by using 10% SDS-PAGE and

blotted onto PVDF membranes, which were then blocked for 2 h at room temperature with 5% non-fat milk and then treated with primary antibodies against COX2 (Dilution 1:1000; ab179800; Abcam, Cambridge, MA), MMP-1 (Dilution 1:1000; ab134184; Abcam, Cambridge, MA), MMP-3 (Dilution 1:1000; ab52915; Abcam, Cambridge, MA), MMP-13 (Dilution 1:1000; ab51072; Abcam, Cambridge, MA), P38 (Dilution 1:1000; # 9212S; Cell Signaling Technology, Danvers, MA), p-P38 (Dilution 1:1000; #9211S; Cell Signaling Technology, Danvers, MA), AKT (Dilution 1:500; # 9272S; Cell Signaling Technology, Danvers, MA), and p-AKT (Dilution 1:1000; # 9271S; Cell Signaling Technology, Danvers, MA) based on the suggested conditions of antibody incubation provided by the antibody manufacturer. Subsequently, the PVDF membranes were washed and further incubated with HRP-labeled secondary antibodies for 1 h at room temperature (Beyotime, Shanghai, China). Finally, the protein bands were developed by using an enhanced chemiluminescence (ECL) assay kit (Millipore, Billerica, MA) based on the instruction manual provided by the assay kit manufacturer and then visualized to determine the relative expression of COX2, MMP-1, MMP-3, MMP-13, P38, p-P38, AKT, and p-AKT proteins in each sample. The protein quantification was performed by optic density analysis of each protein band by Image J software (Version no. 1.44e, National Institutes of Health). β-actin was used as the control.

ELISA

The expression of PGE2 in collected samples was determined by using standard commercial enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, Waltham, MA) based on the instruction manual provided by the assay kit manufacturer.

IHC assay

To determine the protein expression of p-P38 and p-AKYT in the rat cartilage tissue samples collected from the hip joints, the samples were first fixed in 4% paraformaldehyde (Sigma Aldrich, St Louis, MO), embedded in paraffin, sliced into 5 μm sections, stained with anti-p-P38 and anti-p-AKYT primary and biotinylated secondary antibodies (Abcam, Cambridge, MA), and counter-stained with hematoxylin before the positive expression of p-P38 and p-AKYT proteins was analyzed under an Olympus light microscope (magnification, x200; Olympus Corporation) by semi-quantitative analysis.

Statistical analysis

All statistical analyses were carried out by using SPSS 21.0 (IBM, Chicago, IL). The differences between different groups were compared by using one-way ANOVA. A P value of < 0.05 was deemed statistically significant. Each experiment was repeated in triplicate.

Results

PGE2 production and COX-2 mRNA expression were elevated by IL-1β treatment and inhibited by GM treatment

Primary rat chondrocytes were treated with PBS, IL-1β, and IL-1β+GM, respectively. ELISA assay was performed to compare the production of PGE2 among different cell groups. As shown in Fig.1A, the PGE2 production was evidently increased in the IL-1β treatment group while GM treatment inhibited the expression of PGE2. Also, the relative gene expression of COX-2 (Fig.1B) was increased by the IL-1β treatment and was reduced by the co-treatment of GM. Therefore, it was validated that IL-1β treatment elevated the expression of PGE2 mRNA and COX2 mRNA in primary rat chondrocytes, while the GM treatment reduced the gene expression of PGE2 mRNA and COX2 mRNA to a certain degree.

MMP expression was elevated by IL-1β and inhibited by GM treatment

Moreover, real time qPCR results showed that the IL-1β treatment caused a significant elevation in the relative gene expression of MMPs, including MMP-1 (Fig.2A), MMP-3 (Fig.2B) and MMP-13 (Fig.2C), in primary rat chondrocytes, and the gene expression of MMPs was suppressed by the co-treatment with GM. Moreover, the protein expression of MMPs, including MMP-1 (Fig.3A), MMP-3 (Fig.3B) and MMP-13 (Fig.3C), was also increased in the IL-1β group, and GM treatment reduced the protein expression of these MMPs. Therefore, it was validated that IL-1β treatment elevated the expression of MMPs, including MMP-1, MMP-3 and MMP-13 in primary rat chondrocytes, while the GM treatment reduced the expression of these MMPs to a certain degree.

P-P38 and p-AKT production was elevated by IL-1β treatment and inhibited by GM treatment

The production of P38, p-P38, AKT and p-AKT was measured by Western blot assays. Accordingly, the levels of P38 (Fig.4A) and AKT (Fig.4C) remained similar among different groups, while the levels of p-P38 (Fig.4C) and p-AKT (Fig.4D) were both increased by the treatment of IL-1β. Moreover, the up-regulated production of p-P38 and p-AKT in the IL-1β group was both down-regulated by GM treatment. Therefore, it was validated that the GM treatment partly down-regulated the expression of p-P38 and p-AKT elevated in the primary rat chondrocytes treated with IL-1β.

GM treatment inhibited the up-regulated COX2 and MMPs in OA rats

Four groups of rats were established in our study: a SHAM group, an MIA group, a MIA+GM (low dose) group and a MIA+GM (high dose) group. Keen articular cartilage tissues were collected and subjected to PCR and Western blot assays to measure the expression of COX2 mRNA and MMPs. As shown in Fig.5, the relative expression levels of COX2 mRNA (Fig.5A), MMP-1 mRNA (Fig.5B), MMP-3 mRNA (Fig.5C) and MMP-13 mRNA (Fig.5D) were all significantly increased in OA rats (MIA group), and GM treatment suppressed the up-regulated gene expression of COX2 and MMPs in a dose-dependent manner. Moreover, the relative protein expression of COX2 (Fig.6A), MMP-1 (Fig.6B), MMP-3 (Fig.6C) and MMP-13 (Fig.6D) was all markedly up-regulated in OA rats. A low dose of GM treatment alleviated the up-regulated COX2 and MMPs in the MIA group, while a high dose of GM treatment exhibited a more significant inhibitory effect on COX2 and MMPs in the MIA group. Therefore, it was concluded that GM treatment alleviated the up-regulated COX2, MMP-1, MMP-3 and MMP-13 in OA rats, thus exhibiting a therapeutic effect on OA pathology.

GM treatment inhibited the up-regulated p-P38 and p-AKT in OA rats

Furthermore, the production of P38, p-P38, AKT and p-AKT was observed in rats via Western blot and IHC assays. As shown in Fig.7, the Western blot assay exhibited no evident difference of P38 production (Fig.7A) or AKT expression (Fig.7C) among the rat groups, while the production of p-P38 (Fig.7B) and p-AKT (Fig.7D) was significantly increased in OA rats, but GM treatment reduced the p-P38 and p-AKT production in OA rats. Similarly, the results from IHC assays also indicated the inhibitory effect of GM on the p-P38 (Fig.8A) and p-AKT (Fig.8B) production evidently up-regulated in OA rats.

Discussion

In this study, we found that GM treatment of OA rats alleviated the up-regulated expression of COX2, MMP-1, MMP-3 and MMP-13, thus exhibiting a therapeutic effect on OA pathology. In addition, GM treatment inhibited the up-regulated p-P38 and p-AKT expression in OA rats. The results of this present study are consistent with previous studies about the therapeutic role of GM in the treatment of various human medical conditions. For instance, it was found that iridoid glycosides from G. macrophylla (GMI) could remarkably reduce the levels of COX-2 as well as iNOS. It was speculated that COX-2 as well as iNOS signaling is involved in the GM activity in RA treatment [31]. Another research showed that the treatment of OA with COX-2 inhibitors as well as metformin could lower the rates of joint replacement surgery [32]. The different therapeutic effect of GM on different diseases may be attributed to its effects on different disease-related genes or signaling pathways and its effect on OA involves COX2, MMP-1, MMP-3 and MMP-13.

IL-1β activates COX-2 expression and PGE2 production in chondrocytes [33]. In addition, the expression of COX-2 proteins is often delayed after the activation of chondrocytes by IL-1β in OA [34]. It was shown that the level of PGE2 was more effectively decreased by GM than by prednisone. When an extract of GM rats was administered into rats, the level of PGE2 was obviously decreased to reduce chondrocyte damage [9]. Since the increase in PGE2 can result in inflammatory responses in RA, including edema as well as pain, the significantly decreased PGE2 level in rat models of RA may be a result from COX2 inhibition by GM [35]. It was revealed that exogenous administration of PGE2 in chondrocytes of rats induced the incorporation of [3H] thymidine as well as the synthesis of aggrecan which was reported to be involved in the pathogenesis of OA [36]. In human cartilages affected by OA, exogenous administration of PGE2 up-regulated the levels of glucocorticoid receptors, suggesting a possible negative feedback loop of PGE2 [37, 38]. All those above previous reports indicate a pivotal role of COX2/PEG2 in the development of OA and the inhibitory effect of GM on this signaling pathway might be the major reason for its therapeutic effect of GM in OA.

It was shown that MMP-13 was expressed abnormally in human cartilages in different stages of OA [39]. Due to the fact that MMP-13 plays a core role in the degradation of cartilage, the

inhibition of expression of MMP-13 has been thought to be an effective way to limit the development of OA and the regulation of MMP-13 expression could be moderated at different levels, such as post-transcriptional regulation, epigenetic regulation, as well as transcriptional regulation [40-42]. The result of this study indicated that GM treatment down-regulated expression of MMP-13 but how GM regulated the expression of MMP-13 is still under investigation. In addition, MMP-13 is suggested to contribute to the turnover of cartilage materials via cleaving ECM molecules including type II collagen [43, 44]. MMP-13 is constitutively generated and endocytosed by chondrocytes [41]. During the progression of OA, the induction of collagenase as well as MMP-13 activity was noticed in cartilages [45-47]. In this study, we found that IL-1β treatment elevated the expression of PGE2 and COX2 mRNAs in primary rat chondrocytes. However, the GM treatment reduced the gene expression of PGE2 mRNA and COX2 mRNA. In addition, we also found that IL-1β treatment elevated the expression of MMPs, including MMP-1, MMP-3 and MMP-13, in primary rat chondrocytes, but the GM treatment reduced the expression of these MMPs elevated by IL-1β. Meanwhile, we also found that GM treatment reduced the expression of p-P38 and p-AKT elevated by IL-1β in primary rat chondrocytes.

MAPK signaling, such as JNK as well as p38 MAPK pathways, were suggested to be widely associated with the progression of OA [48, 49]. Thereby, the p38 MAPK activation is associated with chondrocyte apoptosis, while the p38 MAPK inhibition is associated with reduced cartilage damage in OA [50]. A past study actually illustrated that AKT controls the proliferation of chondrocytes during skeletal development [51, 52]. Thus, the inhibition of the PI3K/ AKT/mTOR pathway might play a role in preventing chondrocyte expansion.

Conclusion

Our study demonstrated the inhibitory effect of GM on the expression of PGE2, COX-2, MMPs including MMP-1, MMP-3 and MMP-13, AKT and P38 up-regulated in IL-1β cell group or MIA rat group, thus verifying the therapeutic effect of GM on the treatment of OA.

Conflict of interest

None

Acknowledgement

This work was supported by Shanghai Sailing Program (19YF1449700).

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

YXZ and HTX planned the study, HTX and NYG collected the literature,collected and analyzed the data, YXZ and NYG composed the manuscript, and all the other co-authors approved the final manuscript.

Figure legends

Fig.1

Expression of PGE2 and COX-2 mRNAs elevated by the IL-1β treatment was inhibited by the GM treatment (N=3; * P value < 0.05 vs. PBS group; ** P value < 0.05 vs. IL-1β group; one-way ANOVA)

A: ELISA assay indicated that PGE2 production was increased in primary rat chondrocytes treated with IL-1β, while GM treatment inhibited the increased PGE2 production in IL-1β group;

B: Real time PRC indicated that the relative expression of COX2 mRNA was increased in primary rat chondrocyte s treated with IL-1β, while GM treatment inhibited the increased PGE2 production in IL-1β group.

Fig.2

The expression of MMPs elevated by the IL-1β treatment was inhibited by the GM treatment (N-3; * P value < 0.05 vs. PBS group; ** P value < 0.05 vs. IL-1β group; one-way ANOVA)

A: Real time PRC indicated that the relative expression of MMP-1 mRNA was increased in primary rat chondrocyte s treated with IL-1β, while GM treatment inhibited the increased MMP-1 mRNA level in IL-1β group;

B: Real time PRC indicated that the relative expression of MMP-3 mRNA was increased in primary rat chondrocyte s treated with IL-1β, while GM treatment inhibited the increased MMP-3 mRNA level in IL-1β group;

C: Real time PRC indicated that the relative expression of MMP-13 mRNA was increased in primary rat chondrocyte s treated with IL-1β, while GM treatment inhibited the increased MMP-13 mRNA level in IL-1β group.

Fig.3

Protein expression of MMPs elevated by the IL-1β treatment was inhibited by the GM treatment (N=3; * P value < 0.05 vs. PBS group; ** P value < 0.05 vs. IL-1β group; one-way ANOVA)

A: Western blot assay indicated that the protein level of MMP-1 was up-regulated in primary rat chondrocyte s treated with IL-1β, while GM treatment suppressed the up-regulation of MMP-1 protein in IL-1β group;

B: Western blot assay indicated that the protein level of MMP-3 was up-regulated in primary rat chondrocyte s treated with IL-1β, while GM treatment suppressed the up-regulation of MMP-3 protein in IL-1β group;

C: Western blot assay indicated that the protein level of MMP-13 was up-regulated in primary rat chondrocyte s treated with IL-1β, while GM treatment suppressed the up-regulation of MMP-13 protein in IL-1β group.

Fig.4

The protein production of p-P38 and p-AKT elevated by the IL-1β treatment was inhibited by the GM treatment (N=3; * P value < 0.05 vs. PBS group; ** P value < 0.05 vs. IL-1β group; one-way ANOVA)

A: Western blot assay indicated that the protein levels of P38 remained stable among different primary rat chondrocyte groups;

B: Western blot assay indicated that the up-regulated protein level of p-P38 in primary rat chondrocytes treated with IL-1β was evidently reduced by the administration of GM;

C: Western blot assay indicated that the protein levels of AKT remained stable among different primary rat chondrocyte groups;

D: Western blot assay indicated that the up-regulated protein level of p-AKT in primary rat chondrocytes treated with IL-1β was evidently reduced by the administration of GM.

Fig.5

GM treatment inhibited the gene expression of COX2 and MMPs up-regulated in OA rats (N=3; * P value < 0.05 vs. sham group; ** P value < 0.05 vs. MIA group; one-way ANOVA)

A: Real time PCR indicated that the relative gene expression of COX2 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

B: Real time PCR indicated that the relative gene expression of MMP-1 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

C: Real time PCR indicated that the relative gene expression of MMP-3 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

D: Real time PCR indicated that the relative gene expression of MMP-13 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner.

Fig.6

GM treatment inhibited the protein production of COX2 and MMPs up-regulated in OA rats (N=3; * P value < 0.05 vs. sham group; ** P value < 0.05 vs. MIA group; one-way ANOVA)

A: Western blot assay indicated that the relative protein expression of COX2 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

B: Western blot assay indicated that the relative protein expression of MMP-1 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

C: Western blot assay indicated that the relative protein expression of MMP-3 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

D: Western blot assay indicated that the relative protein expression of MMP-13 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner.

Fig.7

The Western blot assay showed that the GM treatment inhibited the p-P38 and p-AKT production which were up-regulated in OA rats (N=3; * P value < 0.05 vs. sham group; ** P value < 0.05 vs. MIA group; one-way ANOVA)

A: Western blot assay indicated that the relative protein expression of P38 remained stable among different rat groups;

B: Western blot assay indicated that the relative protein expression of p-P38 was evidently promoted in MIA group, and GM treatment partly restored the p-P38 up-regulation in MIA+GM group in a dose-dependent manner.

C: Western blot assay indicated that the relative protein expression of AKT remained stable among different rat groups;

D: Western blot assay indicated that the relative protein expression of p-AKT was evidently promoted in MIA group, and GM treatment partly restored the p-AKT up-regulation in MIA+GM group in a dose-dependent manner.

Fig.8

IHC assay showed that GM treatment inhibited the p-P38 and p-AKT production which were up-regulated in OA rats (Scale bar: 50 μm)

A: IHC assay demonstrated inhibitory effect of GM upon the up-regulated expression of p-P38 in the MIA rats treated with different doses of GM;

B: IHC assay demonstrated inhibitory effect of GM upon the up-regulated expression of p-AKT in the MIA rats treated with different doses of GM.

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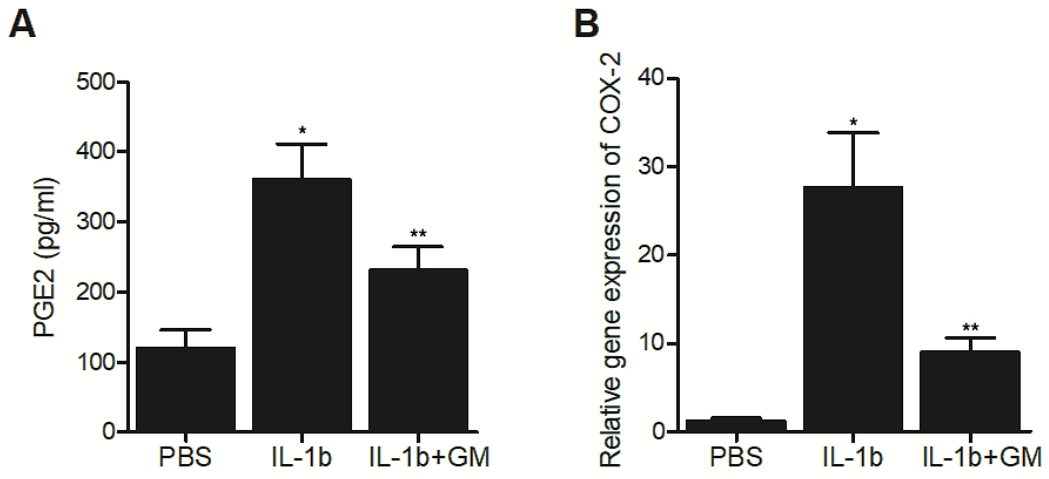

Fig.1
Expression of PGE2 and COX-2 mRNAs elevated by the IL-1β treatment was inhibited by the GM treatment (N=3; * P value < 0.05 vs. PBS group; ** P value < 0.05 vs. IL-1β group; one-way ANOVA)
A: ELISA assay indicated that PGE2 production was increased in primary rat chondrocytes treated with IL-1β, while GM treatment inhibited the increased PGE2 production in IL-1β group;
B: Real time PRC indicated that the relative expression of COX2 mRNA was increased in primary rat chondrocyte s treated with IL-1β, while GM treatment inhibited the increased PGE2 production in IL-1β group.

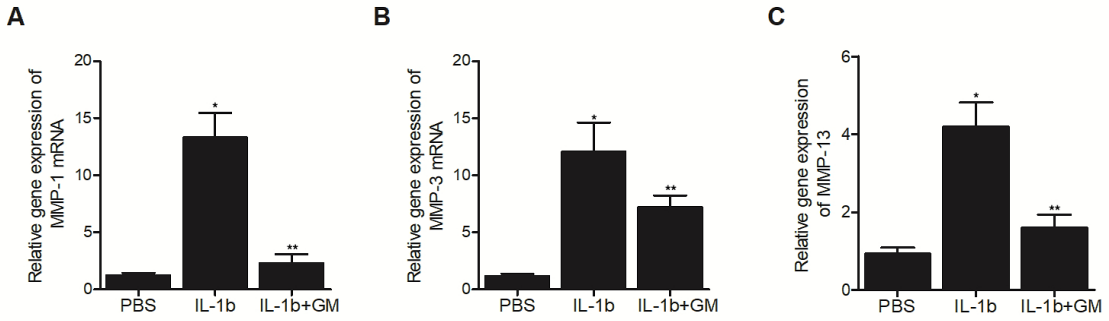

Fig.2
The expression of MMPs elevated by the IL-1β treatment was inhibited by the GM treatment (N-3; * P value < 0.05 vs. PBS group; ** P value < 0.05 vs. IL-1β group; one-way ANOVA)
A: Real time PRC indicated that the relative expression of MMP-1 mRNA was increased in primary rat chondrocyte s treated with IL-1β, while GM treatment inhibited the increased MMP-1 mRNA level in IL-1β group;
B: Real time PRC indicated that the relative expression of MMP-3 mRNA was increased in primary rat chondrocyte s treated with IL-1β, while GM treatment inhibited the increased MMP-3 mRNA level in IL-1β group;
C: Real time PRC indicated that the relative expression of MMP-13 mRNA was increased in primary rat chondrocyte s treated with IL-1β, while GM treatment inhibited the increased MMP-13 mRNA level in IL-1β group.

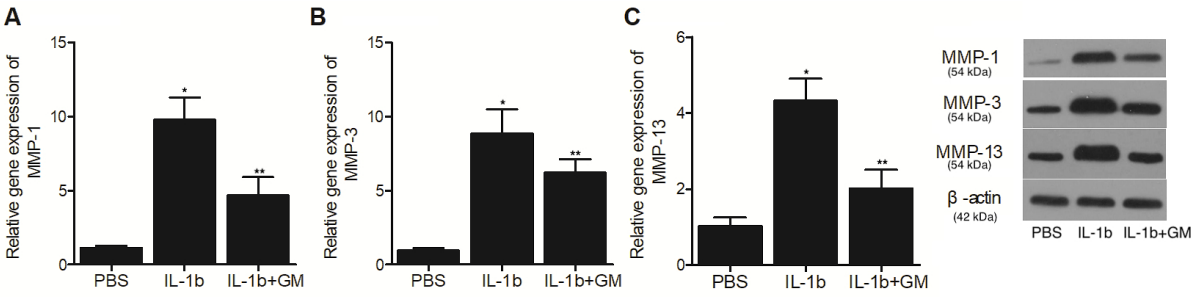

Fig.3

Protein expression of MMPs elevated by the IL-1β treatment was inhibited by the GM treatment (N=3; * P value < 0.05 vs. PBS group; ** P value < 0.05 vs. IL-1β group; one-way ANOVA)

A: Western blot assay indicated that the protein level of MMP-1 was up-regulated in primary rat chondrocyte s treated with IL-1β, while GM treatment suppressed the up-regulation of MMP-1 protein in IL-1β group;

B: Western blot assay indicated that the protein level of MMP-3 was up-regulated in primary rat chondrocyte s treated with IL-1β, while GM treatment suppressed the up-regulation of MMP-3 protein in IL-1β group;

C: Western blot assay indicated that the protein level of MMP-13 was up-regulated in primary rat chondrocyte s treated with IL-1β, while GM treatment suppressed the up-regulation of MMP-13 protein in IL-1β group.

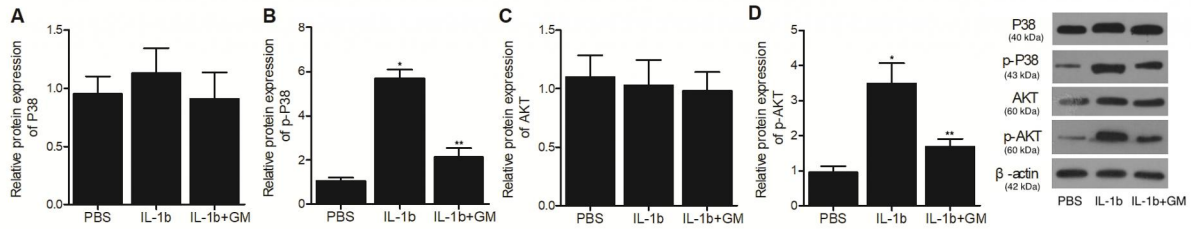

Fig.4

The protein production of p-P38 and p-AKT elevated by the IL-1β treatment was inhibited by the GM treatment (N=3; * P value < 0.05 vs. PBS group; ** P value < 0.05 vs. IL-1β group; one-way ANOVA)

A: Western blot assay indicated that the protein levels of P38 remained stable among different primary rat chondrocyte groups;

B: Western blot assay indicated that the up-regulated protein level of p-P38 in primary rat chondrocytes treated with IL-1β was evidently reduced by the administration of GM;

C: Western blot assay indicated that the protein levels of AKT remained stable among different primary rat chondrocyte groups;

D: Western blot assay indicated that the up-regulated protein level of p-AKT in primary rat chondrocytes treated with IL-1β was evidently reduced by the administration of GM.

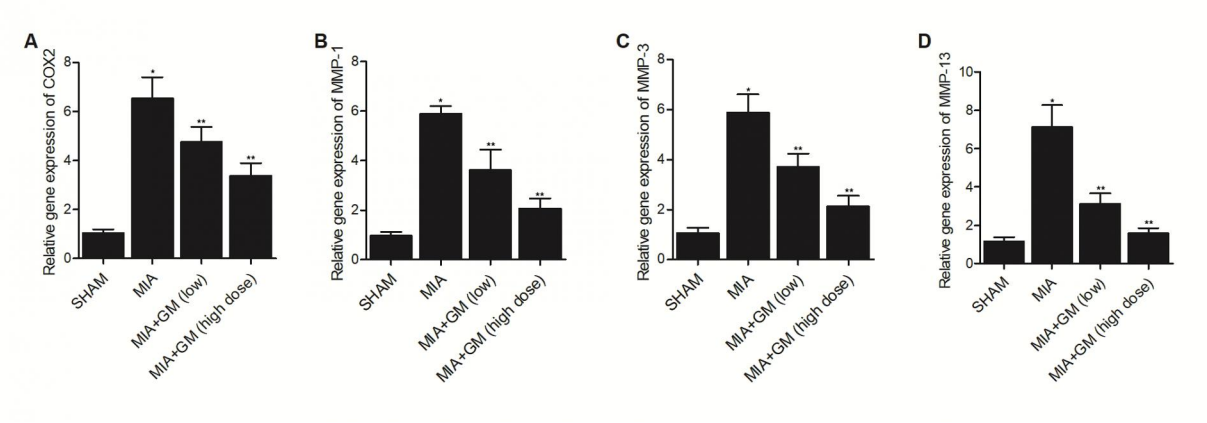

Fig.5
GM treatment inhibited the gene expression of COX2 and MMPs up-regulated in OA rats (N=3; * P value < 0.05 vs. sham group; ** P value < 0.05 vs. MIA group; one-way ANOVA)
A: Real time PCR indicated that the relative gene expression of COX2 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;
B: Real time PCR indicated that the relative gene expression of MMP-1 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;
C: Real time PCR indicated that the relative gene expression of MMP-3 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;
D: Real time PCR indicated that the relative gene expression of MMP-13 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner.

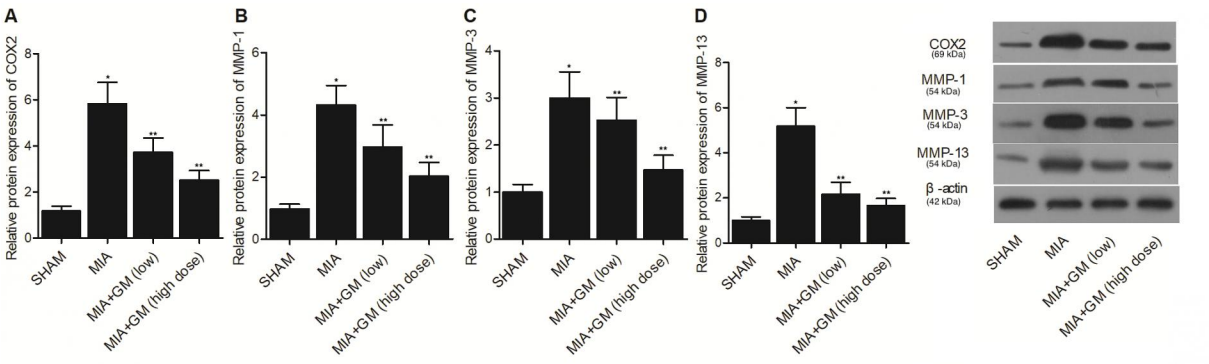

Fig.6

GM treatment inhibited the protein production of COX2 and MMPs up-regulated in OA rats (N=3; * P value < 0.05 vs. sham group; ** P value < 0.05 vs. MIA group; one-way ANOVA)

A: Western blot assay indicated that the relative protein expression of COX2 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

B: Western blot assay indicated that the relative protein expression of MMP-1 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

C: Western blot assay indicated that the relative protein expression of MMP-3 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner;

D: Western blot assay indicated that the relative protein expression of MMP-13 was significantly increased in MIA group, while being partly restored in MIA+GM group in a dose-dependent manner.

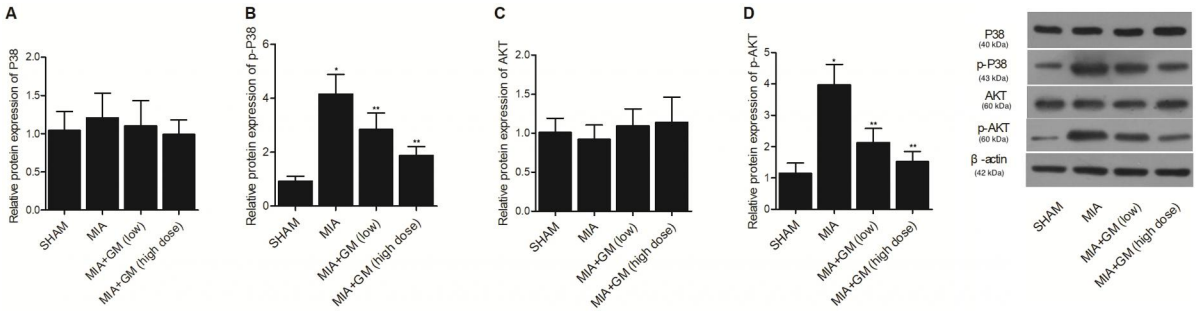

Fig.7

The Western blot assay showed that the GM treatment inhibited the p-P38 and p-AKT production which were up-regulated in OA rats (N=3; * P value < 0.05 vs. sham group; ** P value < 0.05 vs. MIA group; one-way ANOVA)

A: Western blot assay indicated that the relative protein expression of P38 remained stable among different rat groups;

B: Western blot assay indicated that the relative protein expression of p-P38 was evidently promoted in MIA group, and GM treatment partly restored the p-P38 up-regulation in MIA+GM group in a dose-dependent manner .

C: Western blot assay indicated that the relative protein expression of AKT remained stable among different rat groups;

D: Western blot assay indicated that the relative protein expression of p-AKT was evidently promoted in MIA group, and GM treatment partly restored the p-AKT up-regulation in MIA+GM group in a dose-dependent manner.

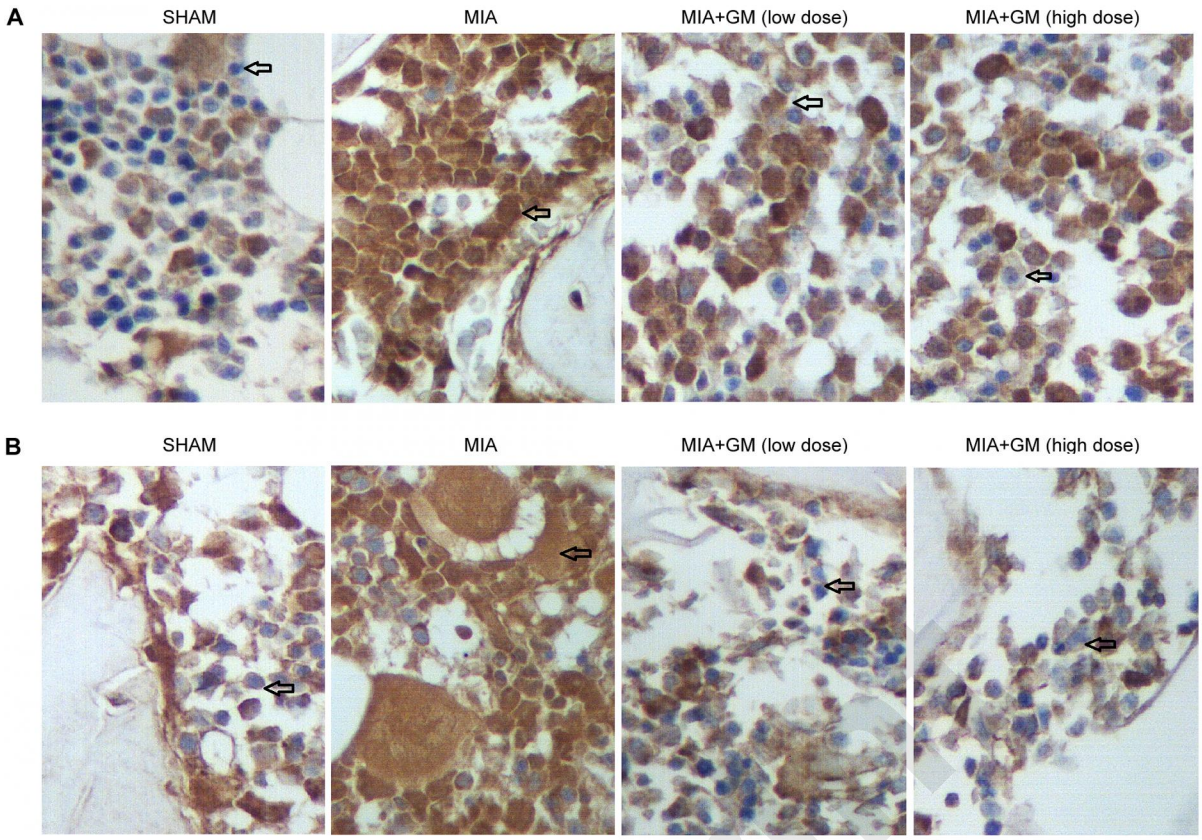

Fig.8 IHC assay showed that GM treatment inhibited the p-P38 and p-AKT production which were up-regulated in OA rats (Scale bar: 50 μm) A: IHC assay demonstrated inhibitory effect of GM upon the up-regulated expression of p-P38 in the MIA rats treated with different doses of GM; B: IHC assay demonstrated inhibitory effect of GM upon the up-regulated expression of p-AKT in the MIA rats treated with different doses of GM.