

Empagliflozin ameliorated chondrocytes inflammation, catabolism and senescence and osteoarthritis via suppressing the nuclear factor kappa-B signal pathway.

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Abstract

Background Extracellular matrix (ECM) degradation, chondrocyte inflammation, and cellular senescence contribute to the pathology of osteoarthritis (OA). Empagliflozin, a selective inhibitor of sodium-glucose cotransporter-2 (SGLT2), has been reported to show the anti-inflammatory properties in several conditions. However, whether empagliflozin can be used to improve OA is still unknown. **Methods** The protective effects and underlying mechanism of empagliflozin in OA were investigated in vitro and in vivo. Cell viability, catabolic markers, inflammatory mediators, cellular senescence level, cartilage degeneration were evaluated by CCK-8 assay, safranin O staining, ELISA, real-time PCR, western blot, β -galactosidase Staining, and histological analysis. **Results** We found that empagliflozin significantly downregulated the expression of catabolic enzymes (MMP9 and MMP13), and decreased the expression of inflammatory mediators (NO, PGE2, IL-6, COX2, and INOS), and reduced the cellular senescence level in IL-1 β -treated chondrocytes by inhibiting the nuclear factor kappa-B (NF- κ B) signaling pathway. What's more, empagliflozin prevented cartilage degeneration in DMM-induced OA mice model with significant lower OARSI grade. **Conclusion** Our findings reveal that empagliflozin inhibited chondrocytes ECM degradation, inflammation and cellular senescence in vitro and prevented cartilage degeneration in vivo by suppressing NF- κ B signal pathway, indicating a therapeutic potential in OA treatment.

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Abstract

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Methods

The protective effects and underlying mechanism of empagliflozin in OA were investigated in vitro and in vivo. Cell viability, catabolic markers, inflammatory mediators, cellular senescence level, cartilage degeneration were evaluated by CCK-8 assay, safranin O staining, ELISA, real-time PCR, western blot, β -galactosidase Staining, and histological analysis.

Results

We found that empagliflozin significantly downregulated the expression of catabolic enzymes (MMP9 and MMP13), and decreased the expression of inflammatory mediators (NO, PGE2, IL-6, COX2, and INOS), and reduced the cellular senescence level in IL-1 β -treated chondrocytes by inhibiting the nuclear factor kappa-B (NF- κ B) signaling pathway. What's more, empagliflozin prevented cartilage degeneration in DMM-induced OA mice model with significant lower OARSI grade.

Conclusion

Our findings reveal that empagliflozin inhibited chondrocytes ECM degradation, inflammation and cellular senescence in vitro and prevented cartilage degeneration in vivo by suppressing NF- κ B signal pathway, indicating a therapeutic potential in OA treatment.

Keywords: empagliflozin; chondrocyte; inflammation; senescence; NF- κ B.

1 Introduction

Osteoarthritis (OA) is a common joint disease with high rate of incidence and wide coverage in the elderly population, which causes a severe economic burden on families and society^{1,2}. Current research has shown that OA is characterized by cartilage degradation, underlying bone remodeling, and synovial inflammation³. Several risk factors, such as age, gender, obesity and genetics, may affect the development of OA^{2,4}. Nevertheless, the underlying molecular mechanism among OA remains obscure.

Increasing evidence suggests that low-grade inflammation is a key mediator of the pathogenesis of OA⁵. Chondrocytes, as the only cellular component of cartilage, play a central role in the synthesis of cartilage matrix. OA is defined as the accumulation of degenerative factors leading to the decrease of chondrocytes and the degradation of extracellular matrix (ECM). During OA, pro-inflammatory factors, such as IL-1 β , TNF- α , trigger a series of pathogenic reactions of chondrocytes, promoting inflammation, senescence, and apoptosis⁶. Current treatment is aimed at relieving the symptoms of OA but cannot slow down disease progression. Therefore, it is necessary to further facilitate identification of emerging pharmaceutical therapies.

Empagliflozin (Empa), a selective inhibitor of sodium-glucose cotransporter-2 (SGLT2), lowers blood sugar by reducing glucose reabsorption in the renal tubules. Various studies showed favorable effects of Empa

on glycaemic improvement, body weight, blood pressure, arterial stiffness, and endothelial function⁷⁻⁹. In recent years, Empa has been reported to regulate macrophage polarity and exert anti-inflammatory effects¹⁰. Also, Empa has hepatic protective effect against cholestatic liver injury via its anti-oxidant and anti-inflammatory properties¹¹. However, the effect of Empa on inflammation and senescence in OA has not been reported yet

In our study, we hypothesized that Empa might inhibit inflammation in chondrocytes during the process of OA. We concentrated on the anti-inflammatory effect of Empa on chondrocytes and the underlying mechanism. We hope that our study will provide a new therapeutic approach for the treatment of OA in the future.

2 Materials and methods

Reagents

Recombinant mouse IL-1 β was purchased from R&D Systems, UK. Empagliflozin was purchased from Med-ChemExpress, China. Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), and 0.5% trypsin were obtained from Gibco BRL, Grand Island, NY, USA. Collagenase type II was purchased from Sigma-Aldrich, St, Louis, MO, USA.

Chondrocyte isolation and culture

Primary chondrocytes were obtained from 5-day-old C57BL/6 mice as previously described¹². Briefly, the articular cartilage from the knee joint of mice was cut into 1 mm³ particles and then was digested with DMEM containing 1% penicillin/streptomycin, 0.5% trypsin, and 1% Collagenase type II for 2 h. After filtered through a 40 μ m cell strainer, the digestive suspensions were placed in a centrifuge at 1500 rpm. The centrifuged cell deposits were resuspended and grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO₂. The cells were passaged to passage 3 and then used for the following experiments.

Cell viability assay

5 x 10³ cells were seeded in 96-well plates and treated with different concentrations (0, 0.5, 1, and 5 μ M) of empagliflozin with or without IL-1 β (10 ng/ml) for 24 h. The equal volume of vehicle was used in the control group. To analyze cytotoxicity of empagliflozin on chondrocytes, CCK-8 assays (MCE, HY-K0301) were conducted according to the manufacturer's instruction. The optical density (OD) value was read at a wavelength of 450 nm.

Safranin O staining

Chondrocytes were incubated with different concentrations (0, 1, and 5 μ M) of empagliflozin with or without IL-1 β (10 ng/ml) for 24 h. Then the cells were fixed with 4% paraformaldehyde (PFA) solution for 20 min. After washing with PBS, 0.1% Safranin O dye solution were added to stain the cells for 5 min. After washing with PBS twice, cells were imaged by gross camera.

Western blot

After treatment, equal amounts of cells were lysed with RIPA for 60min. The lysates were quantified by BCA kit (Beyotime, China). The samples were then separated with SDS-polyacrylamide gels and transferred into nitrocellulose membranes. After being blocked and cut into proper sections, membranes were incubated with different primary antibodies against GAPDH (Abcam, #ab181602), MMP9 (Abcam, #ab76003), MMP13 (Abcam, #ab39012), COX2 (CST, #12282), INOS (Abcam, #ab3523), P53 (Abcam, #ab90363), P21 (Abcam, #ab215971), NF- κ B P65 (CST, #8242), P-NF- κ B P65 (Ser536) (CST, #3033), I κ B (CST, #4812), and P-I κ B (Ser32) (CST, #2859) at 4 °C overnight. The membranes were then incubated with luminesced secondary antibodies and detected with the BioRad System. GAPDH was used as control.

Real-time PCR (RT-PCR)

Chondrocytes were pretreated with empagliflozin (0, 1, and 5 μM) for 2 h, and followed by incubating with IL-1 β (10 ng/ml) for 24 h. Afterward, RNAiso reagent (Takara, China) was used to extract total RNA from chondrocytes. The total RNA was reversely transcribed into cDNA with cDNA synthesis kit (Takara). SYBR Green PCR Master Mix (Takara) was used for RT-PCR with an ABI StepOnePlus System (Applied Biosystems, UK). The primers used for the RT-PCR were shown in Table 1.

Assessment of nitric oxide (NO)

The NO concentration in cell suspensions was measured with Griess reagent (Sigma, G4410). Chondrocytes were pretreated with empagliflozin at various concentrations (0, 1, and 5 μM) for 2 h and incubated with IL-1 β (10 ng/ml) for 24 h, the NO levels in the cell culture medium were assessed with Griess reagent according to the manufacturer. The relative levels of NO were normalized to cell count.

ELISA

The levels of MMP9, MMP13, PGE2 and IL-6 were measured using ELISA kits (AdipoGen LIFE SCIENCES, Germany). Chondrocytes were incubated with IL-1 β (10 ng/ml) for 24 h before pretreatment for 2 h with empagliflozin at various concentrations (0, 1, and 5 μM). The levels of MMP9, MMP13, PGE2 and IL-6 were detected by collecting the cell culture supernatants from each sample with ELISA kits according to the manufacturer. The relative levels were normalized to cell count.

Senescence analysis

β -galactosidase Staining assay was performed according to the manufacture's instruction. After treatment, chondrocytes were fixed for 20 min and then incubated with β -galactosidase staining buffer (Beyotime, China) at 37°C for 24h and subsequently observed under a microscope.

Animal experiments

DMM-induced OA mouse model was used in the animal experiments as previously described¹³. Thirty 12-week-old C57BL/6 mice were randomly divided into 3 groups. Mice in the OA group and the OA+Empa group were anesthetized and the medial meniscus in their knee joints was surgically removed while mice in the Sham group were anesthetized and performed a sham operation. A week after surgery, intra-articular injection of 5 μM empagliflozin was conducted once a week in the OA+Empa group, and equal volume of vehicle was conducted in the OA group and the Sham group. After 4 weeks of treatment, the mice were sacrificed and the knee samples were harvested for histological analysis. This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China.

Histological analysis

The mouse knee samples were fixed with 4% paraformaldehyde solution for 48 h and then decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA) until the tissues became soft. The samples were embedded in paraffin and cut into 5 mm sections by a microtome. The tissue sections were subsequently stained with safranin O and then observed by optical microscopy. The OARSI grading system (0–6) was used to evaluate the sections.

Statistical analysis

All data are presented as means \pm SDs. One-way ANOVA with a subsequent post hoc Tukey's test was used for comparisons. The value of $p < 0.05$ was considered to indicate significant differences.

3 Results

3.1 Εμφέρειση της εμπάγλιφλοζίνης στην ΙΛ-1β στην σεξουαλική διαίρεση των χονδροκυττάρων

The effects of empagliflozin and IL-1 β on cell viability and chondrocyte phenotype maintenance were evaluated by CCK-8 assay and the safranin O staining. The results demonstrated that empagliflozin had no cytotoxicity on mouse chondrocyte viability with concentration [?] μM at 24 h. Besides, chondrocytes

treated with 1 and 5 μM concentrations of empagliflozin could significantly improve the down-regulated cell viability caused by IL-1 β (Figure 1A). Similarly, the results of safranin O staining revealed that chondrocytes were apparently less stained after 24 h IL-1 β treatment, but the mouse chondrocytes treated with 1 and 5 μM of empagliflozin were observably reverse the IL-1 β caused safranin O stain loss (Figure 1B). Thus, 1 and 5 μM concentrations of empagliflozin were used for the following in vitro tests, and a concentration of 5 μM was used for the following in vivo tests.

3.2 Εμφεξης οφ εμπανλιφλοζιν ον ΙΑ-1β-ινδυσεδ ΕΜ δεγραδατιον ιν μουσε σιονδροςψτες

Here the expression levels of catabolic markers of ECM were detected by ELISA, RT-PCR and western blot to evaluate the effects of empagliflozin on IL-1 β -induced ECM degradation in mouse chondrocytes. As shown in Figure 2, administration of empagliflozin could counteract the increments of MMP9 and MMP13 secretion level in the cell medium caused by IL-1 β . In addition, pretreatment with empagliflozin significantly decreased IL-1 β -induced upregulation of matrix-degrading genes (MMP9, MMP13) in a dose-dependent manner at both mRNA level and protein level. Based on the results above, empagliflozin could protect mouse chondrocytes from ECM degradation by inhibiting the MMPs.

3.3 Εμφεξης οφ εμπανλιφλοζιν ον ΙΑ-1β-ινδυσεδ ινφλαμματιον ιν μουσε σιονδροςψτες

To discover the anti-inflammation effects of empagliflozin on mouse chondrocytes, the expression of several inflammatory mediators in mouse chondrocytes was assessed by ELISA, RT-PCR and western blot. As illustrated in Figure 3A, the production and secretion of proinflammatory factors (NO, PGE2, and IL-6) was increased under IL-1 β treatment, and empagliflozin pretreatment could dose-dependently offset these increments. Moreover, the results also showed that empagliflozin significantly decreased IL-1 β -induced upregulation of other inflammatory genes (COX2 and INOS) in both mRNA and protein expression levels (Figure 3B-C). These data demonstrated that empagliflozin could significantly inhibited the production of IL-1 β -induced inflammatory mediators in mouse chondrocytes.

3.4 Εμφεξης οφ εμπανλιφλοζιν ον ΙΑ-1β-ινδυσεδ σενεσενε ιν μουσε σιονδροςψτες

Senescence analysis was conducted to evaluate the effects of empagliflozin on the IL-1 β -induced senescence in mouse chondrocytes by western blot and β -galactosidase Staining Assay. It was found that the levels of senescence markers (P21 and P53) were upregulated by IL-1 β treatment, but this effect was largely weakened by empagliflozin (Figure 4A). Besides, β -galactosidase Staining results showed that the proportion of SA- β -Gal-positive cells obviously increased after IL-1 β treatment but decreased in the presence of empagliflozin (Figure 4B). These data collectively suggested that empagliflozin exerted the protective role in IL-1 β -induced cellular senescence of mouse chondrocytes.

3.5 Εμφεξης οφ εμπανλιφλοζιν ον ΙΑ-1β-ινδυσεδ ΝΦ-κΒ πατηωαψ αστιατιον ιν μουσε σιονδροςψτες

To explore the underlying mechanism, the effects of empagliflozin on NF- κ B pathway activation were investigated. As illustrated in Figure 5, empagliflozin activated the NF- κ B pathway in mouse chondrocytes represented as higher levels of the activated status of P65 (phosphor-P65, p-P65) and I κ B (phosphor-I κ B, p-I κ B). The above IL-1 β -induced NF- κ B pathway activation was inhibited by empagliflozin pretreatment. The results indicated that empagliflozin could inhibit IL-1 β -induced NF- κ B pathway activation in mouse chondrocytes.

3.6 Effects of empagliflozin on cartilage degeneration in mouse OA model

To investigate the anti-osteoarthritic effects of empagliflozin in vivo, DMM-induced OA mouse model was used. The articular cartilage tissue samples from mice in the OA group exhibited typical OA features with reduced abundance of chondrocytes and loss of ECM. However, 4-week intra-articular injection of 5 μM empagliflozin could improve these changes (Figure 6A). The OARSI grade of the OA+Empa group was lower than the OA group with significant difference. The data demonstrated that empagliflozin showed anti-osteoarthritic effect in mouse model.

4 Discussion

OA is a common clinical degenerative joint disease in elderly population, which is one of the main causes of chronic pain and joint disability. OA can severely reduce the quality of life of affected individuals. Current therapeutic goal of OA in clinical practice is to relieve the symptoms but cannot slow down the disease progression. Non-steroidal anti-inflammatory drugs (NSAIDs) are generally considered to be the first-line pharmacological treatments for OA. However, NSAIDs cannot achieve the long-term pain relief for OA patients, and they are associated with significant side effects such as gastric ulceration, renal impairment, and cardiovascular accidents¹⁴. At the end stage of OA, a replacement surgery remains to be the only treatment option. Thus, identification of emerging pharmaceutical therapies is urgently needed in the treatment of OA. Empagliflozin, a selective inhibitor of sodium-glucose cotransporter-2 (SGLT2), has been reported to show the anti-inflammatory properties in several conditions such as cardiovascular diseases, Alzheimer's disease, liver injury, and chronic kidney disease¹⁵⁻¹⁹. In this study, we discovered the protective effect of empagliflozin on cartilage degeneration via inhibition of NF- κ B pathway.

Previously thought to simply be damaged from "wear and tear", OA is now understood as a complex cell-mediated process. Current research has shown that OA is characterized by progressive cartilage degradation, but also underlying bone remodeling, osteophyte formation, and synovial inflammation^{3,20}. A series of pathological factors such as ECM degradation, chondrocyte inflammation, oxidative stress, mitochondrial dysfunction, abnormal mechanical load, and senescent molecules contribute to the degeneration of chondrocytes, ultimately causing cartilage damage and OA development^{21,22}. Chondrocytes, as the only cellular component of cartilage, play a central role in the balance of ECM metabolism through the synthesis of cartilage matrix. However, the inflammatory factors like IL-1 β can disrupt the balance of ECM metabolism by increasing the expression of catabolic enzymes including MMPs, causing the gradual loss of ECM. MMPs inhibitors could be promising agents for the treatment of OA²³. Our data revealed that IL-1 β upregulated the expression of matrix-degrading enzyme (MMP9 and MMP13) and activated the inflammatory cascade reaction by the secretion of inflammatory cytokines (NO, PGE2, IL-6, COX2, and INOS) in mouse chondrocytes, whereas these effects could be offset by the administration of empagliflozin. These findings indicated that empagliflozin could protect mouse chondrocytes from IL-1 β -induced ECM degradation and inflammatory reaction. What's more, chondrocyte senescence is also involved in the pathological process of OA. Jeon et al found that senescent cells accumulated in the articular cartilage and synovium after anterior cruciate ligament transection, and local clearance of these senescent cells attenuated the development of post-traumatic OA²⁴. Therefore, the senescence analysis of chondrocytes was conducted in this study. Cellular senescence is generally regarded as a cell state characterized by an irreversible cell-cycle arrest²⁵. P21 and P53 are the key factors for promoting senescence. We found that empagliflozin could counteract the elevated expression of senescence markers (P21 and P53) induced by IL-1 β . Empagliflozin could also decrease the SA- β -Gal-positive cells. Thus, empagliflozin could alleviate IL-1 β -induced senescence in mouse chondrocytes in vitro. All these results displayed the antidegradation role of empagliflozin on chondrocytes in vitro. The effect of empagliflozin on cartilage damage in vivo was also observed in DMM-induced OA mice model. We found that empagliflozin could also protect mouse knee cartilage from wear and matrix degeneration.

Multiple signaling pathways are involved in OA such as NF- κ B, Wnt/ β -catenin, HIFs, TGF β /BMP and so on²⁶. NF- κ B signaling participates in many OA-associated events, including chondrocyte catabolism, survival, and inflammation²⁷. NF- κ B transcription factor has been considered as a disease-contributing factor of OA for a long time²⁷. When NF- κ B exists in the cytoplasm, it is in an inactive state and cannot enter the nucleus to play its function because it binds to the inhibitory protein I κ B. Once cells are stimulated by certain inductive factors, I κ B is phosphorylated and the molecular conformation of I κ B changes, resulting in the activation and nuclear translocation of NF- κ B^{28,29}. Activation of NF- κ B, on the one hand, can directly bind to the promoters of MMPs genes to promote the expression of matrix-degrading enzyme³⁰. On the other hand, activation of NF- κ B regulates the transcription of many nuclear genes related to inflammation, including cyclooxygenase (COX2) and inducible nitric oxide synthase (iNOS) genes^{31,32}. COX2 and iNOS can promote the production and secretion of induce prostaglandin (PGE2) and nitric oxide (NO). Increased PGE2 and NO levels can upregulate the expression of MMPs, ultimately causing cartilage degeneration.

NF- κ B signaling pathway was focused in this study. Our results showed that empagliflozin inhibited the phosphorylation of I κ B and P65, displaying the suppressive role on NF- κ B signaling. Thus, the underlying mechanism of empagliflozin in mouse cartilage protective roles is related to the inhibition of NF- κ B pathway.

However, there are still some limitations in our study. First at all, we confirmed the protective effect of empagliflozin on preventing cartilage degeneration via inhibiting the activation of NF- κ B pathway. But whether NF- κ B is the direct target of empagliflozin needs to be further identified. Secondly, we have only demonstrated the effects of empagliflozin on cellular inflammation and senescence in chondrocytes. The effects of empagliflozin on other aspects involving the onset and progression of OA deserve further investigation.

5 Conclusions

We demonstrated that empagliflozin inhibited chondrocytes ECM degradation, inflammation and cellular senescence in vitro and prevented cartilage degeneration in vivo by suppressing the NF- κ B pathway activation. Our findings indicate a therapeutic potential of empagliflozin in OA treatment.

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Tables

Table 1 Primers used for RT-PCR in this study

Gene	Forward	Reverse
MMP9	CTTCACCGGCTAAACCACCT	TGTCCCTAACGCCAGTAGA
MMP13	TGATGGACCTTCTGGTCTGGC	CATCCACATGGTTGGGAAGTTCTG
COX2	GGTCTGGTGCCTGGTCTGATGAT	GTCCTTTCAAGGAGAATGGTGC
INOS	GCAAACCCAAGGTCTACGTTCA	GAGCACGCTGAGTACCTCATTG
GAPDH	TCACTGCCACCCAGAC	TGTAGGCCATGAGGTCCAC

Figure legends

Figure 1. Effects of empagliflozin and IL-1 β on cell viability and chondrocyte phenotype maintenance of mouse chondrocytes. (A) CCK-8 analysis, * $p < 0.05$ versus the IL-1 β group. (C) Gross view of safranin O stained mouse chondrocytes that treated with empagliflozin at various concentrations (0, 1, and 5 μ M) with or without IL-1 β (10 ng/ml) for 24 h.

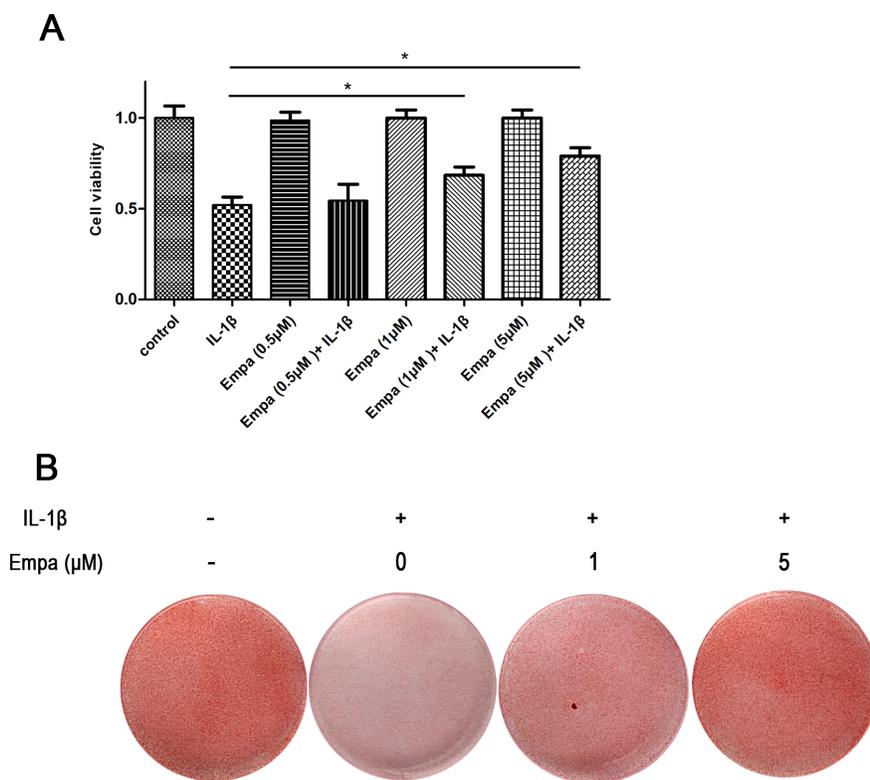
Figure 2. Effects of empagliflozin on IL-1 β -induced ECM degradation in mouse chondrocytes. Chondrocytes were pretreated with empagliflozin at various concentrations (0, 1, and 5 μ M) for 2 h and then incubated with IL-1 β (10 ng/ml) for 24 h. (A) ELISA analysis of MMP9 and MMP13 in treated cells. (B) The mRNA expression levels of MMP9 and MMP13 in treated cells. (C) The protein expression levels of MMP9 and MMP13 in treated cells. * $p < 0.05$ versus control group, # $p < 0.05$ versus model group.

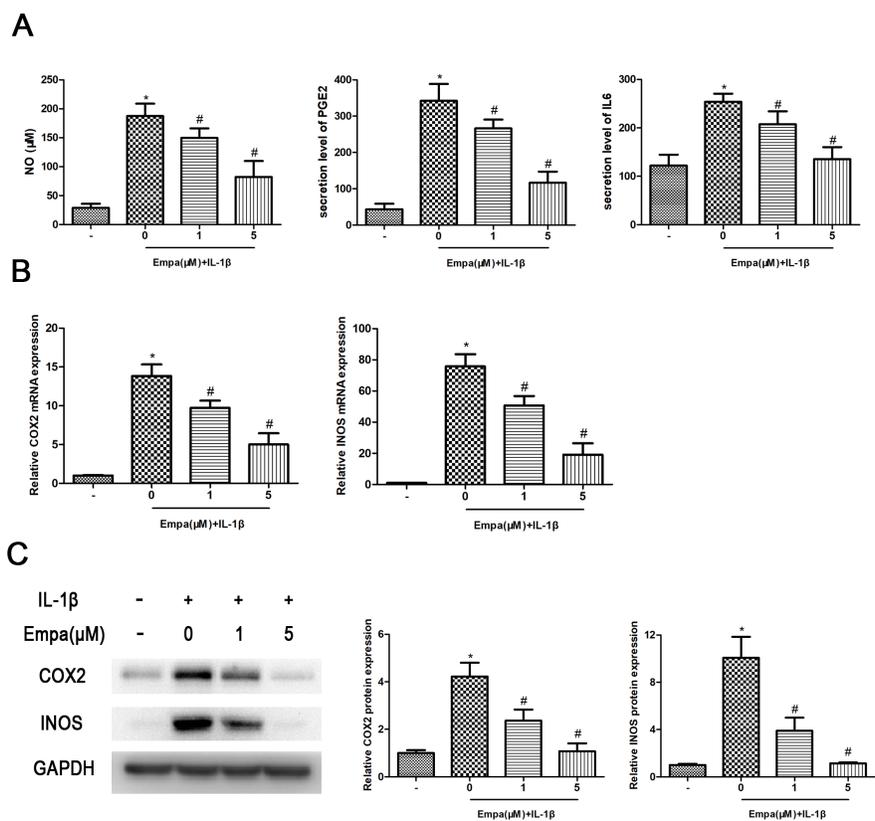
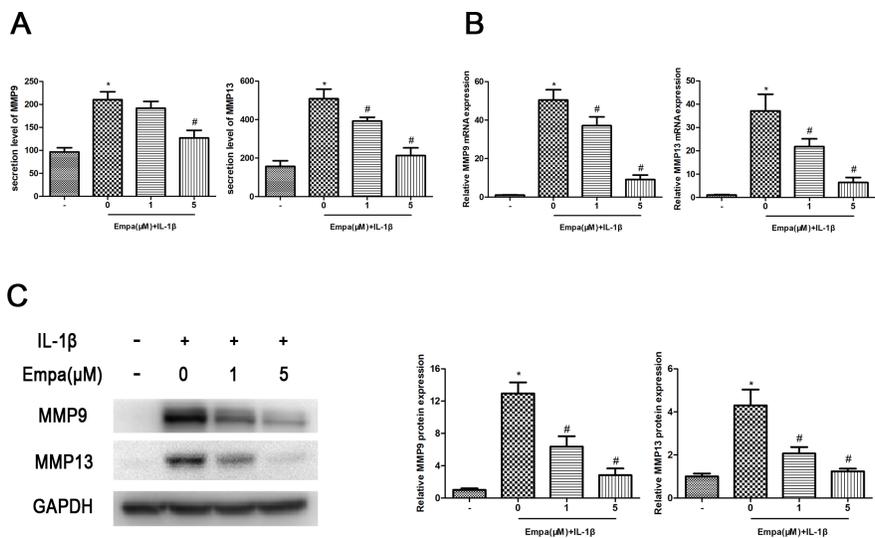
Figure 3. Effects of empagliflozin on IL-1 β -induced inflammation in mouse chondrocytes. Chondrocytes were pretreated with empagliflozin at various concentrations (0, 1, and 5 μ M) for 2 h and then incubated with IL-1 β (10 ng/ml) for 24 h. (A) Nitrite production, PGE2 secretion, and IL-6 secretion in treated cells. (B) The mRNA expression levels of COX2 and INOS in treated cells. (C) The protein expression levels of COX2 and INOS in treated cells. GAPDH as an internal control. * $p < 0.05$ versus control group, # $p < 0.05$ versus model group.

Figure 4. Effects of empagliflozin on IL-1 β -induced senescence in mouse chondrocytes. Chondrocytes were pretreated with empagliflozin at various concentrations (0, 1, and 5 μ M) for 2 h and then incubated with IL-1 β (10 ng/ml) for 24 h. (A) The protein expression levels of P21 and P53 in treated cells. GAPDH as an internal control. (B) β -galactosidase staining results of treated cells. * $p < 0.05$ versus control group, # $p < 0.05$ versus model group. Bar = 50 μ m

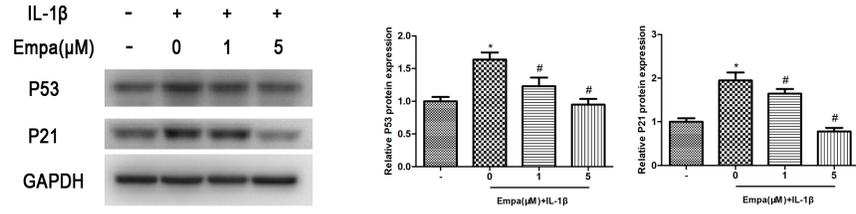
Figure 5. Effects of empagliflozin on the IL-1 β -induced NF- κ B pathway activation in mouse chondrocytes. Chondrocytes were pretreated with empagliflozin at various concentrations (0, 1, and 5 μ M) for 2 h and then incubated with IL-1 β (10 ng/ml) for 15 min. The protein expression levels of P-P65, P65, p-I κ B, and I κ B in treated cells. The ratio of p-P65 to P65 as well as the ratio of p-I κ B to I κ B was calculated. GAPDH as an internal control. * $p < 0.05$ versus control group, # $p < 0.05$ versus model group.

Figure 6. Effects of empagliflozin on cartilage degeneration in mouse OA model. (A) Microscopic images of safranin O stained mouse knee joint sections. Bar = 500 μ m. (B) The OARSI grades. * $p < 0.05$ versus control group, # $p < 0.05$ versus model group.

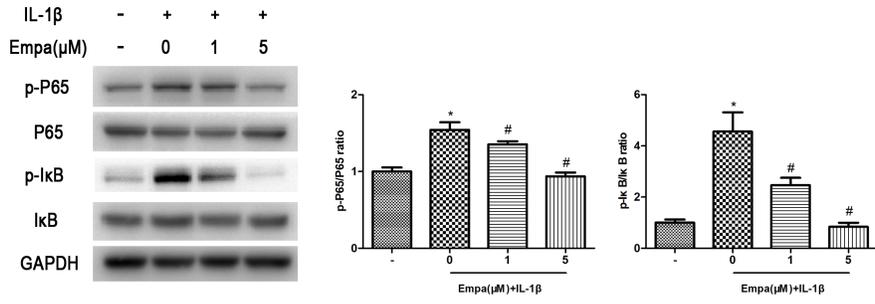
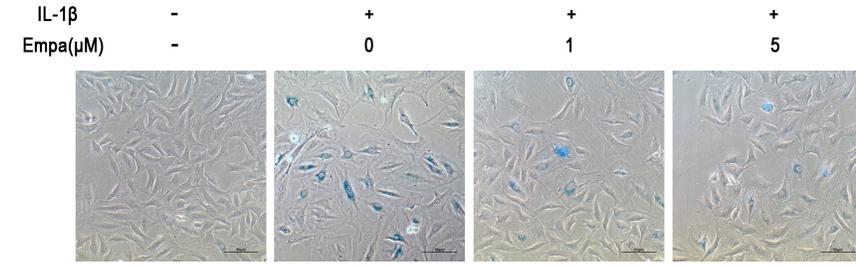




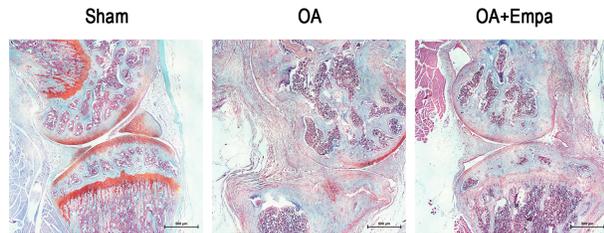
A



B



A



B

