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Intra-articular injection of magnesium chloride attenuates osteoarthritis progression in rats

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SUMMARY

Objective: To explore the effects of Mg^{2+} on the expression of osteoarthritic markers in human cartilage and synovium tissue explants. To investigate the therapeutic effect of intra-articular injection of Mg^{2+} in an established rat OA (Osteoarthritis) model of anterior cruciate ligament transection with partial medial meniscectomy (ACLT + PMM).

Design: Human cartilage and synovium explants were collected from total knee replacement surgeries and incubated with $MgCl_2$ (20 mmol/L) *in vitro*. A rat OA model was established by ACLT + PMM surgery in 450–500 g male Sprague Dawley (SD) rats. To select the optimal dose, intra-articular injections of $MgCl_2$ (0.05, 0.5, 5 mol/L) were performed at 4 weeks after the surgery every 3 days for 2 weeks. The effect of optimized $MgCl_2$ was further determined by histology, immunohistochemistry, and quantitative real-time polymerase chain reaction.

Results: The expressions of osteoarthritic markers in human cartilage and synovium explants were inhibited by Mg^{2+} *in vitro*. Immunohistochemical analysis further suggested the inhibitory effects of Mg^{2+} on the expression of MMP-13 and IL-6 in the human tissue explants. Cartilage degeneration and synovitis in ACLT + PMM rats were significantly improved by intra-articular injections of Mg^{2+} (0.5 mol/L). Immunohistochemical analysis also showed the regulatory effects of Mg^{2+} on osteoarthritic markers in both cartilage and synovium in rats, consistent with *in vitro* results.

Conclusion: Intra-articular injections of Mg^{2+} at 0.5 mol/L attenuate the progression of OA in the ACLT + PMM rat model. Such effect was at least in part explained by the promotion of cartilage matrix synthesis and the suppression of synovial inflammation.

Introduction

Osteoarthritis (OA) is a progressive and degenerative joint disease caused by multiple factors including age, weight, trauma, and genetic factors. It affects about 3.3% of the population worldwide and has several pathological changes known as cartilage degeneration and synovitis[1]. OA involves the whole joint that leads to pain, swelling as well as deformity at the late stage. Hyaluronic acid (HA) and corticosteroid (CS) are the most commonly used injectable drugs. However, the efficiency and recommendation of HA and CS are still controversial according to the international guidelines[2,3]. The anti-osteoarthritic mechanism of HA and CS which respectively refers to lubrication and anti-inflammation exert very limited efficacy to delay the progression of OA. Other studies have also investigated the efficacy of platelet-rich plasma (PRP) or

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mesenchymal stem cells (MSCs)[4,5]. Despite positive effects, the complicated preparation process and high cost of PRP and MSCs limit their clinical applications. A joint replacement is the final option for symptoms relief and functional improvement at late stage OA. Therefore, it is desirable to develop a novel intra-articular injectable product that is cost-effective and can effectively attenuate the progression of OA.

Cations play very important roles in numerous physiological activities in the musculoskeletal system of a human body. Strontium (Sr) was reported to cause osteophytes formation at early stage of OA[6], while zinc (Zn) was indicated to promote the synthesis and secretion of matrix metalloproteinase-3, 13 (MMP-3, 13) which aggravated the progression of OA[7]. However, a safe concentration range of cations can be beneficial. Zn (10^{-8} M) was reported to be able to inhibit the proliferation of bone marrow stem cells (BMSCs) *in vitro*[8], Magnesium ions (Mg^{2+}) do not have such limitations and may therefore be a potential candidate for treating OA. At present, Mg^{2+} has already been widely used to treat tachyarrhythmia, pre-eclampsia, and pain after orthopaedic surgery[9,10]. Strikingly, Mg-based implants show great potential in promoting the healing of bone-fracture and at the tendon-to-bone junction[11–13]. Previous clinical data suggested an inverse relationship between the serum Mg^{2+} concentration (affected by dietary Mg^{2+} intake) and radiographic OA[14–16]. Besides, previous studies showed that Mg^{2+} deficiency could induce low-grade inflammation with an elevation of C-reaction protein (CRP), tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6)[17,18]. Moreover, Mg^{2+} supplementation also exerts a promotive effect on chondrogenic differentiation[19,20], contrarily, Mg^{2+} deficiency leads to pathological changes in both the articular cartilage and growth plate[21]. These studies provided a putative efficiency of Mg^{2+} on OA treatment. Based on the evidence mentioned above, we hypothesize that intra-articular injections of magnesium chloride ($MgCl_2$) may effectively prevent the progression of OA. In the current study, we first investigated the effects of Mg^{2+} on human tissue explants. We then further evaluated the extent of cartilage degeneration and inflammation during the progression of OA in rats receiving intra-articular injections of $MgCl_2$.

Methods

Human tissue explants

Human cartilage and synovium were collected from two male and one female OA patients who underwent total knee replacement surgeries. Patients' informed consent were obtained (Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee, Ref. No. 2017.053) prior to specimen collection. Our studies conformed to the Helsinki Declaration. The mean age of the patients was 70.7 years old. Tissue explants was processed as previously described[22]. In brief, articular cartilage dissected from the lateral femoral condyle was minced and incubated with either complete Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12) medium as the control group, complete DMEM/F12 medium + 20 mmol/L $MgCl_2$ as Mg^{2+} group, complete DMEM/F12 medium + 10 ng/ml interleukin-1β (IL-1β) as IL-1β group, or complete DMEM/F12 medium + 10 ng/ml IL-1β + 20 mmol/L $MgCl_2$ as IL-1β + Mg^{2+} group for 3 or 6 days. Similarly, the processing of tissue explants and group designs were conducted on human synovium and incubated for 2 or 4 days. The cartilage and synovium tissue explants were collected for RNA extraction or histological assessments. Experiments were performed in triplicate.

Quantitative reverse transcriptional polymerase chain reaction (RT-qPCR) analysis

Human tissue explants were harvested and homogenized in liquid nitrogen with RNiso Plus Kit (AA6002-1, Takara). The messenger RNA (mRNA) was reverse-transcripted to cDNA using PrimeSript RT reagent Kit (RR047A, Takara). We measured the expression of *Col-2a1*, *Acan*, *Sox-9*, *HIF-1α*, *NFκB* in cartilage explants and *ADAMTS-4*, *ADAMTS-5*, *MMP-9*, *MMP-13*, *IL-6* and *IL-1ß* in both cartilage and synovium explants. The primer sequences were provided in the Supplementary Table 1. All samples were performed in triplicate. The relative expression was calculated using $2^{-\Delta\Delta CT}$.

Establishment of OA model

Male Sprague–Dawley rats (450 g–500 g, 6-months-old) were purchased after obtaining approval from the Animal Experimentation Ethics Committee in The Chinese University of Hong Kong (Ref. No. 17-060-GRF). Anterior cruciate ligament transection with partial medial meniscectomy (ACLT + PMM) were performed on rats as described previously[23]. Briefly, a medial parapatellar incision was made on the right knee of the rats under anaesthesia by intraperitoneal injection of Ketamine (75 mg/kg) and Xylazine (10 mg/kg). After removing the patella fat pad, the anterior horn of the medial meniscus was dissected. Thereafter, the anterior cruciate ligament was transected. A patella relocation was followed, and the wound was sutured. All rats were kept under a normal light/dark cycle, 24 °C and allowed free access to food (regular diet containing 0.18% magnesium) and water. The body weight and right knee joint of each rat were measured and checked once per week to monitor their health status.

Grouping and intra-articular injection of $MgCl_2$

A total of 96 rats were used in the current animal experiment (Supplementary Table 2). To determine the critical dose of $MgCl_2$, 72 rats were used (n = 6 per group in each time point). All rats were randomly assigned to different experimental groups, either saline (100 μl), 0.05 mol/L $MgCl_2$ (100 μl), 0.5 mol/L $MgCl_2$ (100 μl) or 5 mol/L $MgCl_2$ (100 μl), for intra-articular injections at 4 weeks after the ACLT + PMM surgery. The intra-articular injections were performed twice per week for two consecutive weeks. The knees were harvested on week 2, 4 and 8 after the last injection.

To further investigate the efficacy of $MgCl_2$ at critical dosage, 24 OA rats were further prepared (n = 6 per group at each time point). At 4 weeks after ACLT + PMM surgery, rats were divided into 2 groups. Rats in the Mg^{2+} group were injected with 100 μl $MgCl_2$ solution, while rats in the control group were injected with 100 μl saline. All rats were sacrificed on week 12 and 16 after treatment.

Histomorphometric analysis

The collected knees were fixed in buffed formalin and decalcified in 9% formic acid prior to paraffin sectioning. The histological sections were stained with either Haematoxylin and Eosin (H&E), Safranin O/Fast green, or Toluidine Blue[24]. Cartilage degeneration of medial plateau and femoral condyle was evaluated using the Osteoarthritis Research Society International (OARSI) scoring

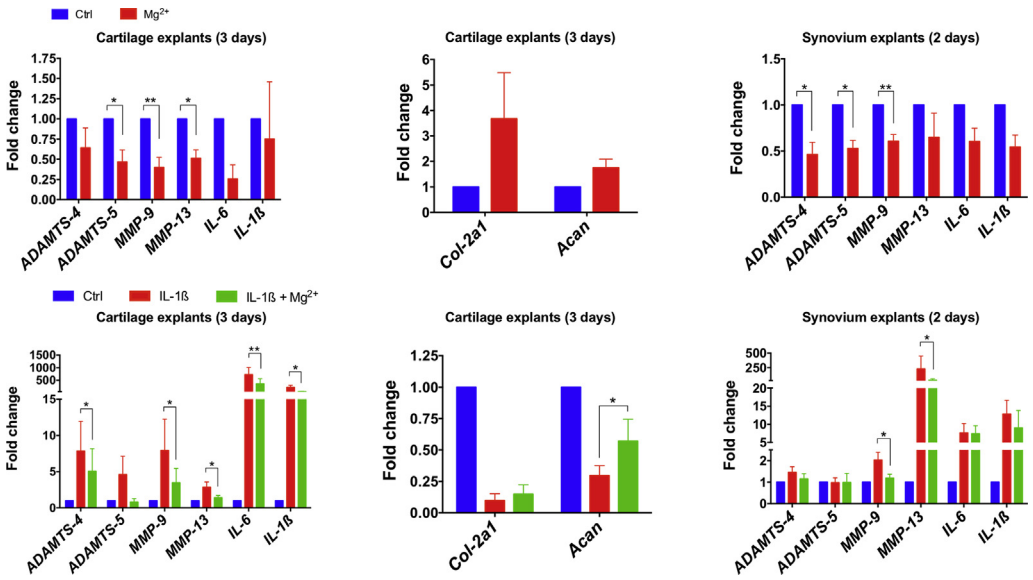

Fig. 1. The expression of osteoarthritic markers in human cartilage and synovium tissue explants, after incubating with either Mg^{2+} (20 mmol/L), IL-1β (10 ng/ml) or IL-1β (10 ng/ml) + Mg^{2+} (20 mmol/L), determined by PCR. (*$P < 0.05$, **$P < 0.01$).

system[25]. The inflammatory response in the synovium at the medial and lateral compartment of the knee was also analysed[26].

Immunohistochemical (IHC) staining

The histological sections were incubated with primary antibody against either collagen type-2a1 (Col-2a1) (1:100, M2139, Santa Cruz), transcription factor sex determining region Y-box 9 (Sox-9) (1:150, ab26414, Abcam), hypoxia-inducible factor 1-alpha (HIF-1α) (1:200, ab2185, Abcam), IL-6 (1:300, Ab9324, Abcam) or MMP-13 (1:200, Ab39012, Abcam) overnight at 4 °C. On the next day, sections were incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:300–600, Ab6789 from Abcam and 7074 from Cell Signaling Technology) for 1 h at room temperature, followed by visualization using a DAB solution kit (TA-060-QHDX, Thermo fisher).

Statistical analysis

Sample size was estimated based on our pilot study using the power calculation. Six rats per time point per group was sufficient to provide a 30% difference in the total OARSI score ($\alpha = 0.05$, power = 0.9) from 8.9 ± 1.6 (Mean ± SD) in the control group to 6.2 ± 1.1 (Mean ± SD) in the Mg^{2+} (optimal dosage: 0.5 mol/L) group. The histological scoring and semi-quantitative analysis on IHC staining were performed on 3 sections from different location of the knee samples (anterior, middle, posterior of the knee joint) using ImageJ. The maximum values were used for statistical

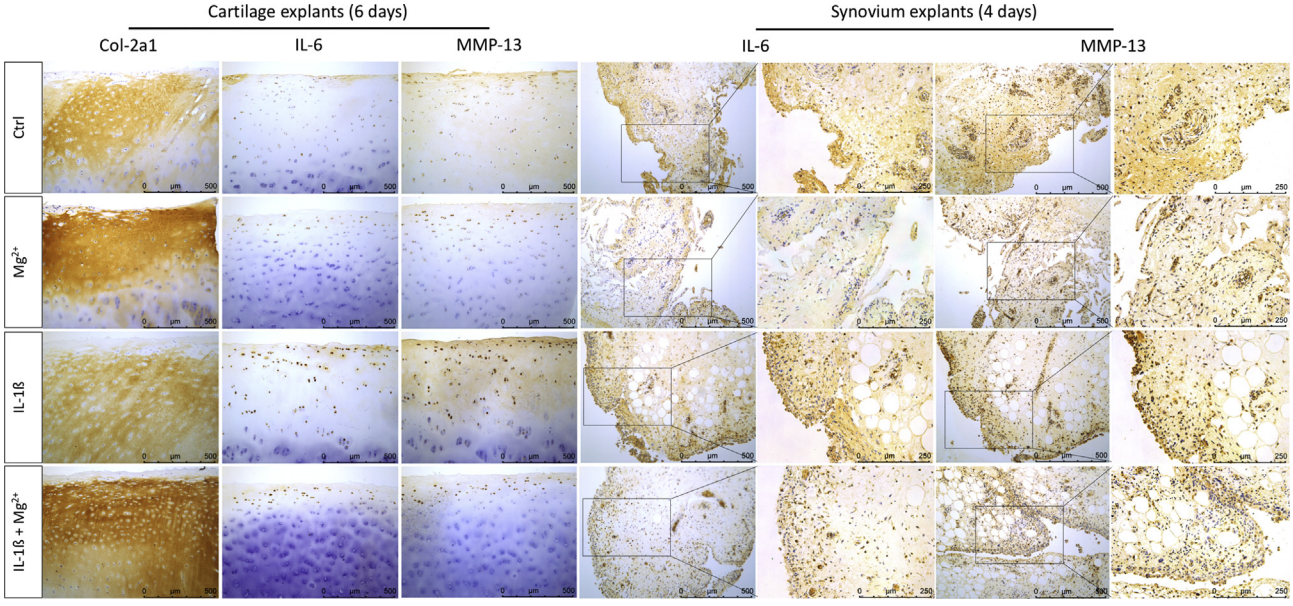

Fig. 2. Representative IHC staining further confirmed the changes of Col-2, IL-6 and MMP-13 in human cartilage and synovium explants.

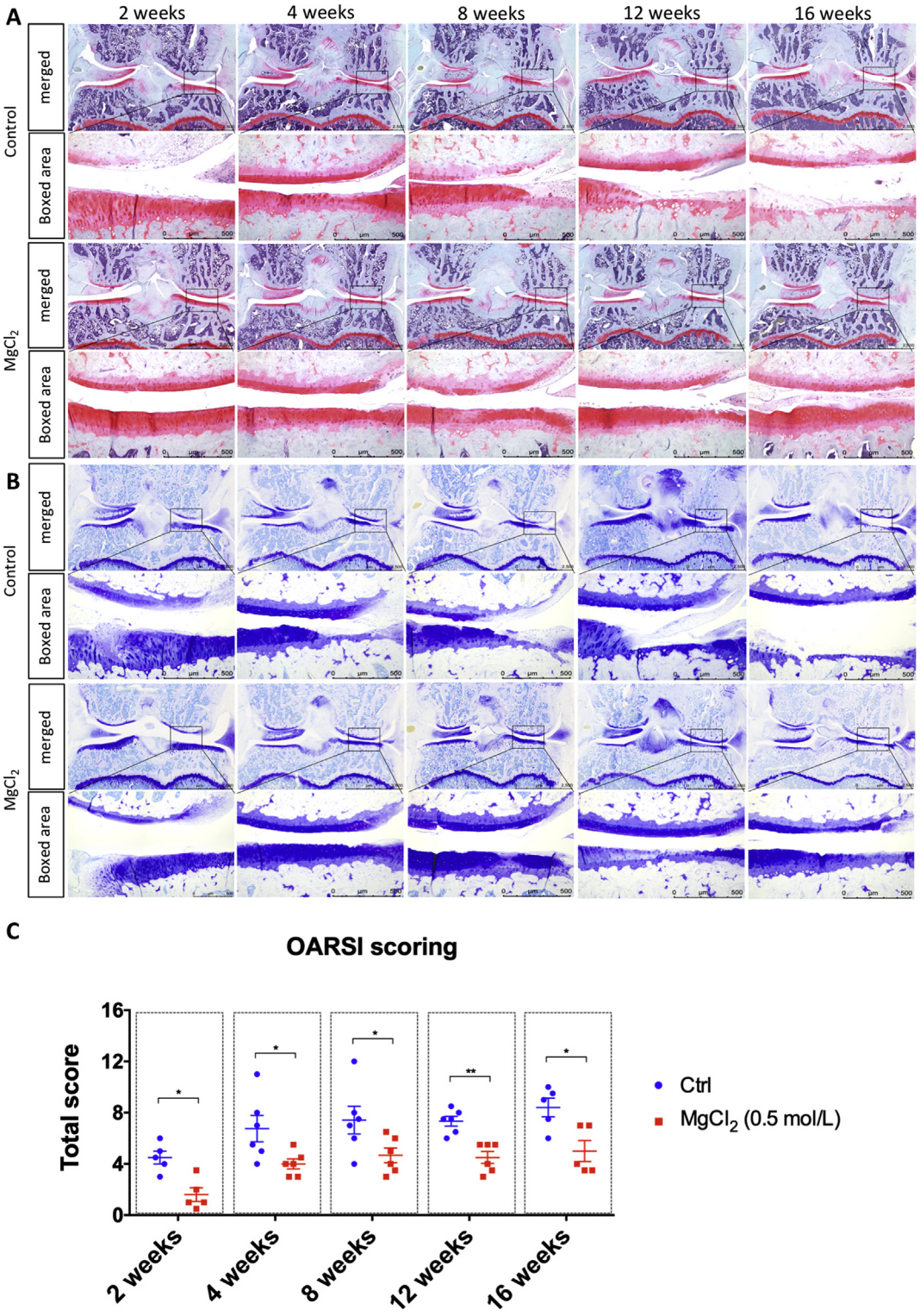

Fig. 3. The degeneration of articular cartilage was significantly attenuated by the intra-articular injections of $MgCl_2$ (0.5 mol/L). (A) Representative Safranin O/Fast green staining showed the changes on cartilage in the specified group (n = 6 per time point) from week 2–16 after treatment. (B) Representative Toluidine Blue staining further confirmed the efficacy of Mg^{2+}. (C) Cartilage degeneration in medial plateau and femoral condyle was evaluated by OARSI scoring system (*$P < 0.05$, **$P < 0.01$).

analysis. The scoring was performed blindly by two colleagues. Any disagreements were solved by seeking opinions from a third party. The data were analysed using the Wilcoxon-Signed Rank test or Mann–Whitney *U* test when comparing between two groups at each time point using the GraphPad Prism software (Version 6.01). The significance is defined at the $P < 0.05$.

Results

Effect of Mg^{2+} on human cartilage and synovium explants in vitro

Chondrogenic markers were increased whereas inflammatory markers were inhibited in human cartilage explants with Mg^{2+} supplementation

RT-qPCR results showed that the expression of *Col-2a1* ($P = 0.313$) and *Acan* ($P = 0.063$) were elevated, while the expression of *ADAMTS-4* ($P = 0.313$), *ADAMTS-5* ($P = 0.031$), *MMP-9* ($P = 0.008$), *MMP-13* ($P = 0.016$), *IL-6* ($P = 0.063$) and *IL-1ß* ($P = 0.625$) were decreased in Mg^{2+}-treated cartilage explants as compared to the control group (Fig. 1). Mg^{2+} supplementation significantly rescued the inhibitory effects of IL-1β on *Col-2a1* ($P = 0.063$) and *Acan* ($P = 0.039$), while reducing the expression of *ADAMTS-4* ($P = 0.031$), *ADAMTS-5* ($P = 0.063$), *MMP-9* ($P = 0.047$), *MMP-13* ($P = 0.031$), *IL-6* ($P = 0.008$) and *IL-1ß* ($P = 0.031$) (Fig. 1). As shown in IHC staining, the expression of Col2a1 was enhanced in the Mg^{2+} group as compared to the control group (Fig. 2). As compared to IL-1β group, the expression of Col-2a1 was robustly increased in the IL-1β + Mg^{2+} group (Fig. 2). A significant decrease in the expression of MMP-13 and IL-6 in cartilage explants was observed in the Mg^{2+} group as compared to the control group (Fig. 2). Furthermore, the IL-1β-elevated expression of MMP-13 and IL-6 were significantly suppressed by Mg^{2+} (Fig. 2).

Inflammatory markers in human synovium explants were suppressed by Mg^{2+}

In synovium explants, Mg^{2+} could suppress the expression of *ADAMTS-4* ($P = 0.031$), *ADAMTS-5* ($P = 0.016$), *MMP-9* ($P = 0.008$), *MMP-13* ($P = 0.313$), *IL-6* ($P = 0.078$) and *IL-1ß* ($P = 0.063$) when compare to the control group (Fig. 1). Mg^{2+} supplementation also significantly suppressed the elevation of *MM-9* ($P = 0.039$) and *MMP-13* ($P = 0.047$) induced by additional IL-1β (Fig. 1). Consistently, IHC stating demonstrated decreased expression levels of MMP-13 and IL-6 in Mg^{2+}-treated synovium explants as compared to either the control or IL-1β-treated ones (Fig. 2).

Therapeutic efficiency of intra-articular injection of $MgCl_2$ on OA in vivo

Six rats were eliminated from our experiments due to anaesthetic accidents or being severely bitten by other rats. In the first set of experiments, three dosage of $MgCl_2$ (0.05 mol/L, 0.5 mol/L and 5 mol/L) were used. H&E staining showed that intra-articular injections of $MgCl_2$ at 0.05 mol/L and 0.5 mol/L were not cytotoxic with no destructive effects on the morphology of chondrocytes as compared to the control group (saline). However, the unstainable nucleus of chondrocytes and destroyed cartilage structure were observed in rats injected with 5 mol/L $MgCl_2$, indicating toxicity of $MgCl_2$ at a high dose (Supplementary Fig. 1). Thus, 0.5 mol/L $MgCl_2$ was selected as the critical dosage for further experiments.

Mg^{2+} alleviated cartilage degeneration

In the control group, obvious osteoarthritic changes with abrasion in the cartilage surface and focal cartilage matrix degradation as indicated by cationic stain matrix depletion were observed in the stained histological slides at week 2 and week 4 after intra-articular injections. In contrast, the cartilage matrix degeneration was significantly alleviated in the Mg^{2+} group (Fig. 3A and B). At week 8 to week 16, the cartilage matrix was further degenerated with significant disrupted cartilage in the control group. Intriguingly, Mg^{2+} could maintain the cartilage matrix up to week 12 after intra-articular injections of $MgCl_2$. Although cartilage matrix degeneration was seen at week 16 after intra-articular injections of $MgCl_2$, the cartilage integrity remained relatively intact as compared to the control group (Fig. 3A and B). Using the OARSI scoring system, a significant decrease in the total score was found in rats received intra-articular injections of $MgCl_2$ at 2 ($P = 0.016$), 4 ($P = 0.030$), 8 ($P = 0.039$), 12 ($P = 0.002$) and 16 weeks ($P = 0.032$) after treatments (Fig. 3C). The degradation of Col-2a1 as shown in IHC staining was not obvious, yet the abrasion and full thickness loss of cartilage in the control group were profound, especially at later time points (12 and 16 weeks after treatments). In contrast, the integrity of cartilage was well-protected in the Mg^{2+} treated group (Fig. 4).

Mg^{2+} inhibited synovitis

H&E staining showed a mid-grade inflammatory response as mainly indicated by the slightly to moderately increased lining cell layer and resident cell density in the synovial tissue from week 2–16 in the control group (Fig. 5A). In contrast, Mg^{2+} supplementation could alleviate these pathological changes (Fig. 5A). In addition, histological grading of the synovitis also revealed that $MgCl_2$ could decrease the total score as compared to the control group at 2 weeks ($P = 0.008$), 4 weeks ($P = 0.033$), 8 weeks ($P = 0.013$), 12 weeks ($P = 0.002$) and 16 weeks ($P = 0.095$) after the treatment (Fig. 5B).

Mg^{2+} reduced the expression of inflammatory markers

We next determined the expression level of IL-6 and MMP-13 in both cartilage and synovium using IHC staining. The results showed that a large number of chondrocytes positively expressed IL-6 and MMP-13 from week 2–16 after intra-articular injections of saline in the control group (Fig. 6A and B, Fig. 7A and B). In the Mg^{2+} group, the percentage of IL-6 and MMP-13 positive chondrocytes in the articular cartilage was significantly decreased at 2 (IL-6, $P = 0.016$; MMP-13, $P = 0.008$), 4 (IL-6, $P = 0.002$; MMP-13, $P = 0.026$), 8 (IL-6, $P = 0.015$; MMP-13, $P = 0.015$), 12 (IL-6, $P = 0.026$; MMP-13, $P = 0.015$) and 16 weeks (IL-6, $P = 0.032$; MMP-13, $P = 0.008$) after treatments. In the synovium, similar results were found. The expression level of IL-6 and MMP-13 at the lining cell layer were reduced in the Mg^{2+} group as compared to the control group at 2 (IL-6, $P = 0.008$; MMP-13, $P = 0.008$), 4 (IL-6, $P = 0.002$; MMP-13, $P = 0.002$), 8 (IL-6, $P = 0.015$; MMP-13, $P = 0.015$), 12 (IL-6, $P = 0.026$; MMP-13, $P = 0.015$) and 16 weeks (IL-6, $P = 0.032$; MMP-13, $P = 0.016$) after treatments (Fig. 6A and B, Fig. 7A and B).

Mg^{2+} enhanced HIF-1α and Sox-9 expression but reduced NFκB expression in cartilage

We further evaluated the regulatory roles of Mg^{2+} on the expression of HIF-1α, Sox-9 and NFκB in cartilage. The qPCR results showed that the supplementation of Mg^{2+} significantly elevated the expression of both *HIF-1α* and *Sox-9* as compared to the control group (*HIF-1α*, $P = 0.002$; *Sox-9*, $P = 0.006$) or under IL-1β induction (*HIF-1α*, $P = 0.041$; *Sox-9*, $P = 0.010$) in the cartilage explants (Fig. 8A). Consistently, IHC staining showed that the expression of both HIF-1α and Sox-9 were significantly enhanced at 2 and 4 weeks after intra-articular injections of Mg^{2+} (Fig. 8B). Previous studies reported that HIF-1α is an anabolic signal in articular

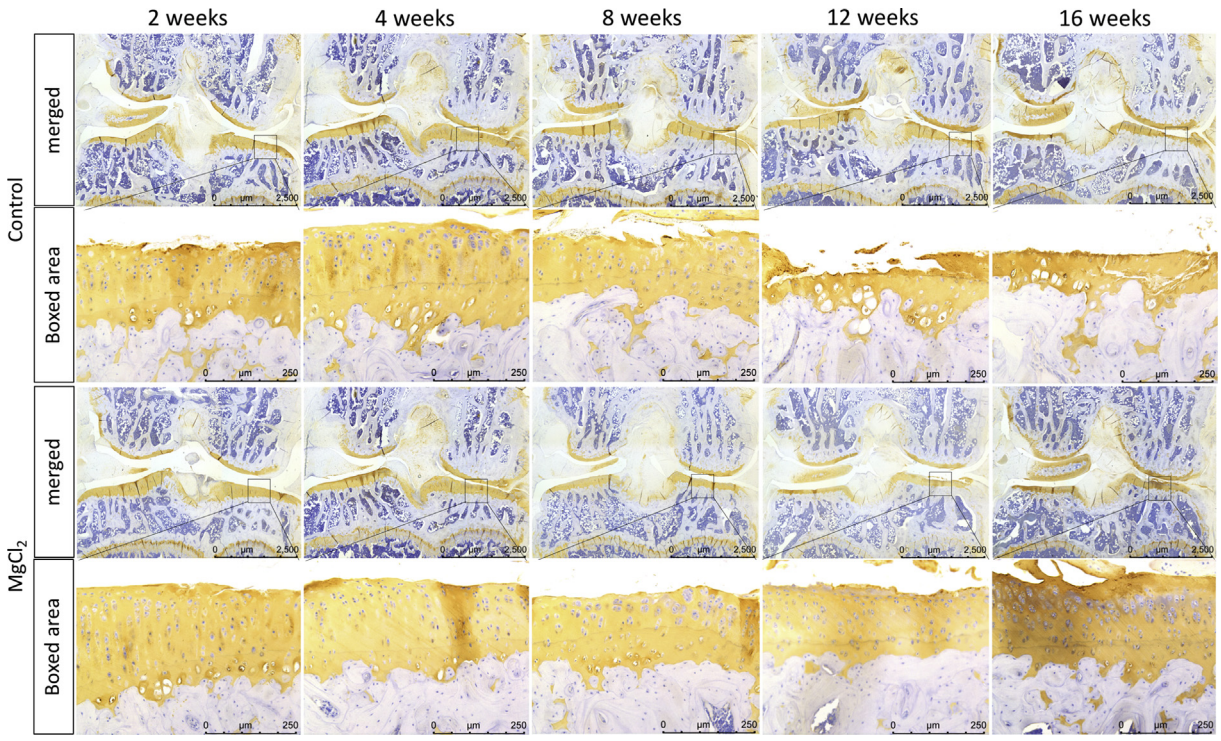

Fig. 4. Representative IHC staining showed the expression and distribution of Col-2a1 in cartilage after treatment.

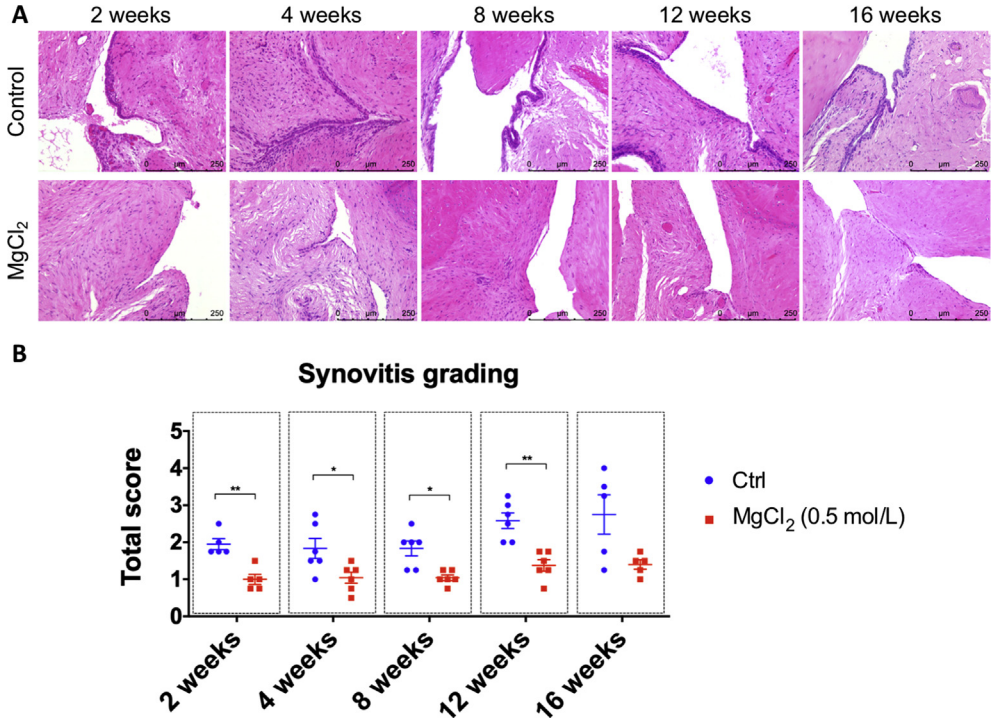

Fig. 5. Intra-articular injections of $MgCl_2$ inhibited the development of synovitis. (A) Representative H&E staining showed a decrease in lining cells and a lower density of resident cells in the synovium in the group treated with Mg^{2+} as compared to the control group. (B) The synovitis score of the synovium in the medial and lateral compartment of the knee (*$P < 0.05$, **$P < 0.01$).

cartilage by promoting the synthesis of cartilage matrix[27,28]. An enhanced expression of HIF-1α can prevent the degradation of articular cartilage in OA[29,30]. In addition, Mg^{2+} was reported to enhance the expression of HIF-1α in human BMSCs[31]. Based on our present data, Mg^{2+} could enhance the expression of HIF-1α in the articular cartilage and facilitate the synthesis of cartilage matrix. We also found that the expression of *NFκB* was significantly suppressed in the Mg^{2+} treated group as compared to the control

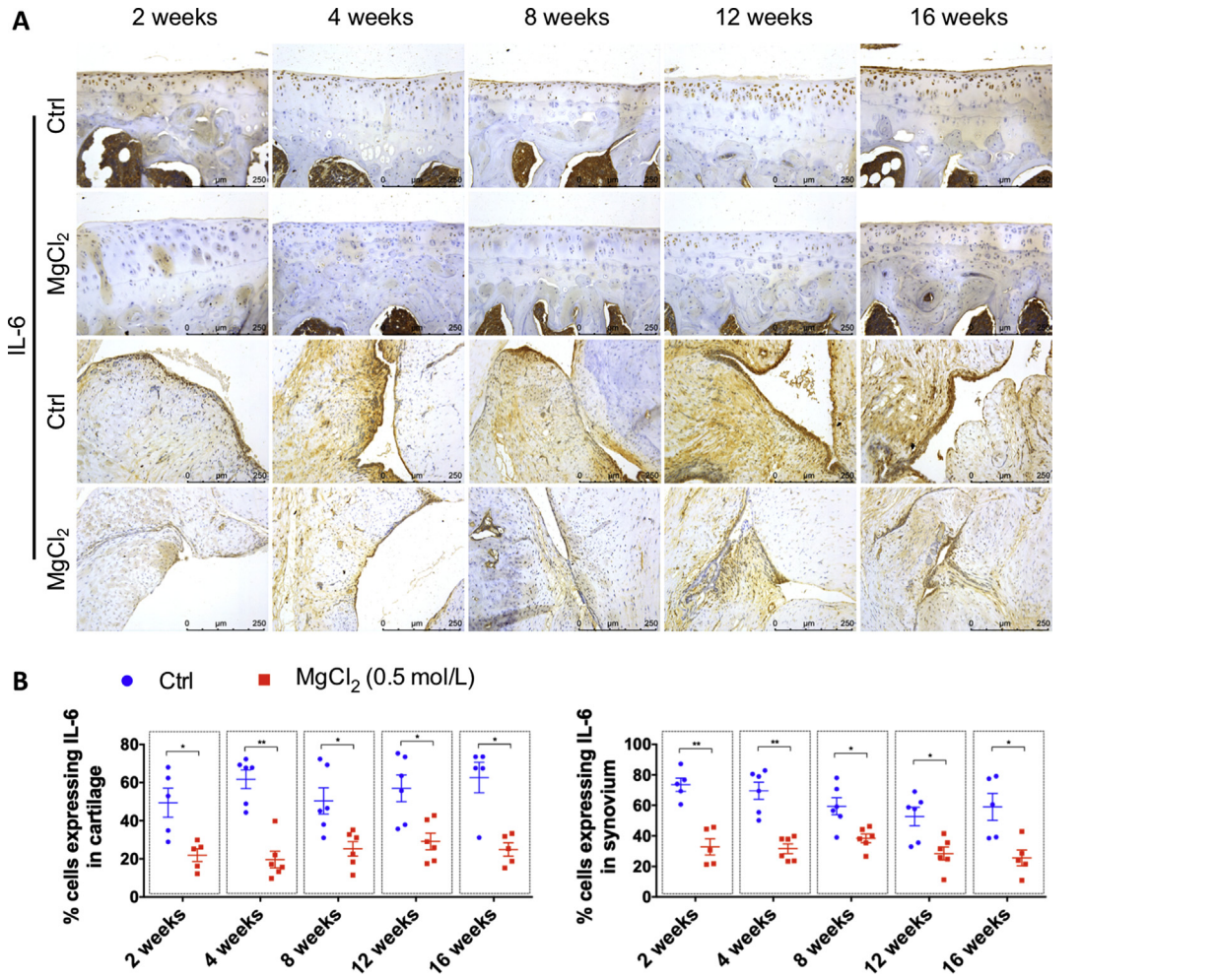

Fig. 6. The change of IL-6 expression in cartilage and synovium after intra-articular injection of $MgCl_2$. (A) Representative IHC staining showed the expression and distribution of IL-6 in cartilage and synovium; (B) Percentage of IL-6 positive cells in cartilage or synovium was calculated and compared between the control group and Mg^{2+} group ($^{*}P < 0.05$, $^{**}P < 0.01$).

group ($P < 0.001$) or under IL-1β induction ($P < 0.001$) in the cartilage explants (Fig. 8A).

Discussion

Although Mg^{2+} deficiency is associated with the progression of OA[14–17,32], the specific effects and underlying mechanisms of Mg^{2+} supplementation for OA treatment have not been fully investigated. A previous study conducted by Lee *et al.*[33] reported that intra-articular injections of $MgSO_4$ could attenuate the progression of collagenase-induced OA in rats. Their work indicated the beneficial effects of intra-articular injection of Mg^{2+} on improving the pain-related behaviour and inhibiting the apoptosis of chondrocytes in rats. However, this study did not evaluate the therapeutic effects of Mg^{2+} on the histomorphometric features and cellular pathogenesis in both the cartilage and synovium. In addition, the collagenase-induced OA model cannot elucidate OA comprehensively as the initiating event and pathological changes are not representative of human OA[34].

In the current study, we used the commonly used ACLT + PMM surgery-induced OA model in rats. Both the cartilage degeneration and synovitis have been evaluated after intra-articular injections of Mg^{2+}. More importantly, we had also performed a longitudinal evaluation and comparison between the control group and Mg^{2+} group at multiple time points. Furthermore, the treatment strategy had also been optimized. We had identified the critical dosage of Mg^{2+} *in vivo* as 0.5 mol/L, and the intra-articular injections were performed twice per week for 2 weeks rather than for 4 weeks[33]. Our findings demonstrated that $MgCl_2$ (0.5 mol/L) could significantly alleviate the progression of OA and the anti-osteoarthritic effects of Mg^{2+} could be maintained for up to 16 weeks after treatment. Apart from demonstrating the alleviation on cartilage degeneration and synovitis by intra-articular injection of Mg^{2+} *in vivo*, we had also tested the effects of Mg^{2+} on the expression of osteoarthritis-related markers in human tissue explants. Lastly, we confirmed that Mg^{2+} acts at least partially through enhancing the expression of HIF-1α and Sox-9, while inhibiting the expression of NFκB.

In this study, histological results suggested that the degeneration of cartilage matrix in rats was significantly alleviated by Mg^{2+} *in vivo*. However, it remains unknown if Mg^{2+} could promote the synthesis of cartilage matrix. Next, we had conducted *in vitro* experiments to investigate the effects of Mg^{2+} on the expression of Col-2a1 and Acan in human cartilage explants. The Mg^{2+} supplementation could increase the gene expression of *Col-2a1* and *Acan* in the cartilage explants after a stimulation with or without IL-1β for 3 days. This finding is consistent with the IHC staining in the cartilage explants which suggests that the Mg^{2+} supplementation could facilitate the synthesis of cartilage matrix.

In the current animal experiments, there was no significant degradation of Col-2a1 in the ACLT + PMM model that may confound the effect of intra-articular injection of $MgCl_2$ on the

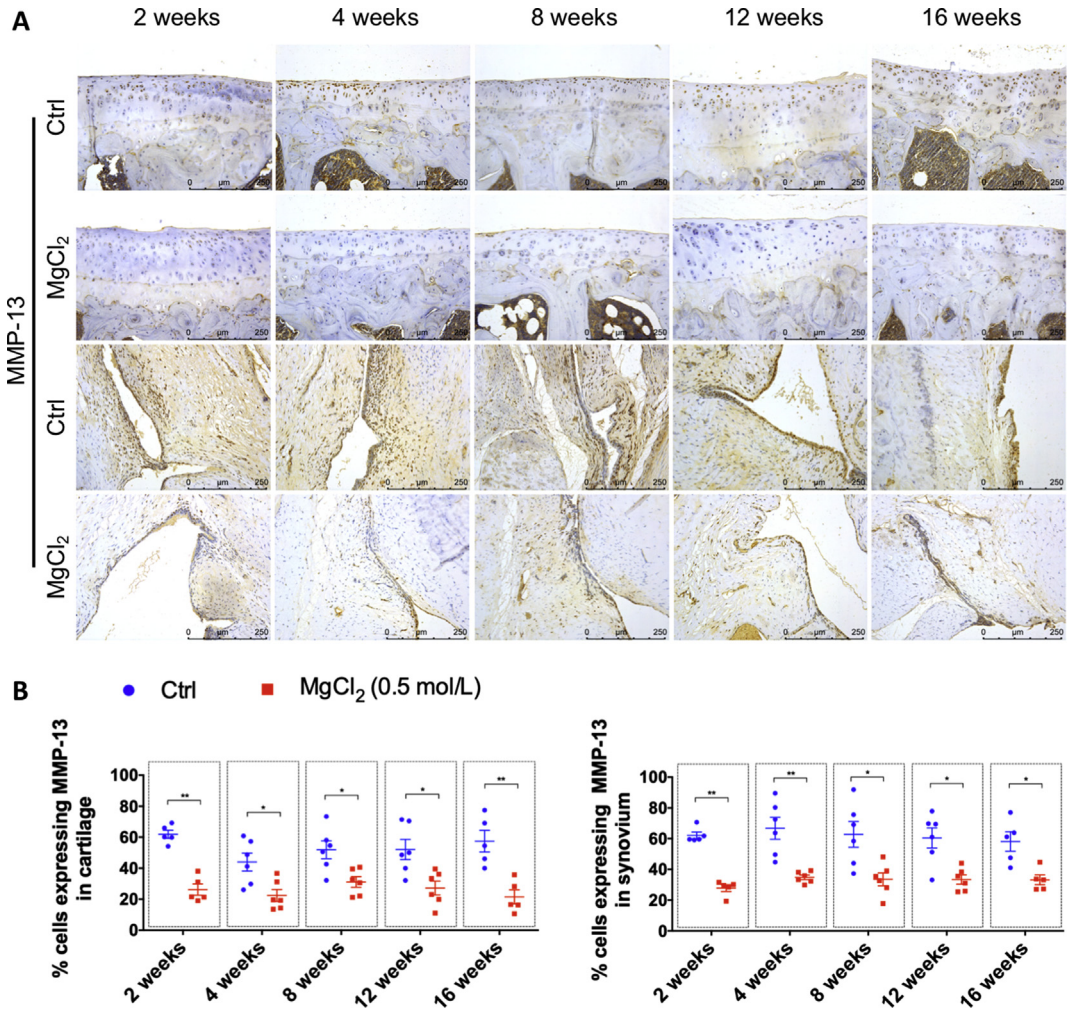

Fig. 7. The change of MMP-13 expression in cartilage and synovium after intra-articular injection of $MgCl_2$. (A) Representative IHC staining showing the expression and distribution of MMP-13 in cartilage and synovium. (B) Percentage of MMP-13 positive cells in cartilage or synovium was calculated and compared between the control group and Mg^{2+} group (*$P < 0.05$, **$P < 0.01$).

expression of Col-2a1. According to previous studies[35,36], apart from the known fact that the half-life of Col-2a1 is longer than that of Acan, this also may be owing to the activated metabolism of chondrocytes after the ACLT + PMM surgery which the expression of Col-2a1 and Acan increase secondary to the surgery. However, a progressive imbalance on the expression of Col-2a1 and Acan was identified, where the increased expression of Col-2a1 dramatically exceeded the increase of Acan which can be explained by the mild degradation of Col-2a1 in the ACLT + PMM model.

Furthermore, we found that Mg^{2+} could stimulate the expression of *HIF-1α* in human cartilage explants obtained from OA patients. HIF-1α was a key regulator of Sox-9, which was an important transcriptional factor in chondrogenesis[37,38]. We also determined the expression of *Sox-9* in the human cartilage explants and found that the expression of *Sox-9* was significantly increased concomitantly by the supplementation of Mg^{2+}. HIF-1α is also known as a survival factor for chondrocytes, catabolic-stress, such as stimulation with IL-1β or oxidative-stress, could induce the expression of HIF-1α in chondrocytes to mediate the anti-catabolic response[28,39]. In our present study, IL-1β increased the expression of HIF-1α in human cartilage explants and the expression of Sox-9 was also enhanced subsequently. Moreover, Mg^{2+} could further stimulate the expression of HIF-1α and Sox-9 in the cartilage explants after IL-1β induction which indicates that Mg^{2+} may facilitate the survival adaptation of chondrocytes. Consistent to our *in vitro* study, the expression of HIF-1α and Sox-9 were enhanced in the cartilage in rats after treatments as determined by IHC staining. On the other hand, the expression of NFκB was also determined in the human cartilage explants in the present study. NFκB involves in numerous catabolic reactions in OA. The RT-qPCR results showed that Mg^{2+} suppressed the expression of NFκB in human cartilage explants even after IL-1β induction.

Taken together, our findings suggest that Mg^{2+} promotes the synthesis of cartilage matrix and suppresses the expression of inflammatory cytokines and proteinases in the articular cartilage through regulating HIF-1α and NFκB, which likely contributes to the attenuation of cartilage degeneration in OA.

Synovitis is also responsible for clinical symptoms and structural destructions in OA. Activated synovial cells secrete numerous inflammatory cytokines and proteinases which promote cartilage degeneration. In addition, products of cartilage breakdown released into the synovial fluid would further activate synovial cells and amplify synovitis in a vicious positive feedback cycle[40,41]. The anti-inflammatory effect of Mg^{2+} has been proven in other diseases, however, it has seldom been investigated in OA[42–44]. Under IL-1β induction, we found that *MMP-9* and *MM-13* were suppressed by Mg^{2+} in synovium explants. In addition, the synovitis score was lower in the Mg^{2+} group as compared to that of the control group from week 2 to week 16 *in vivo*. Therefore, we conclude that intra-

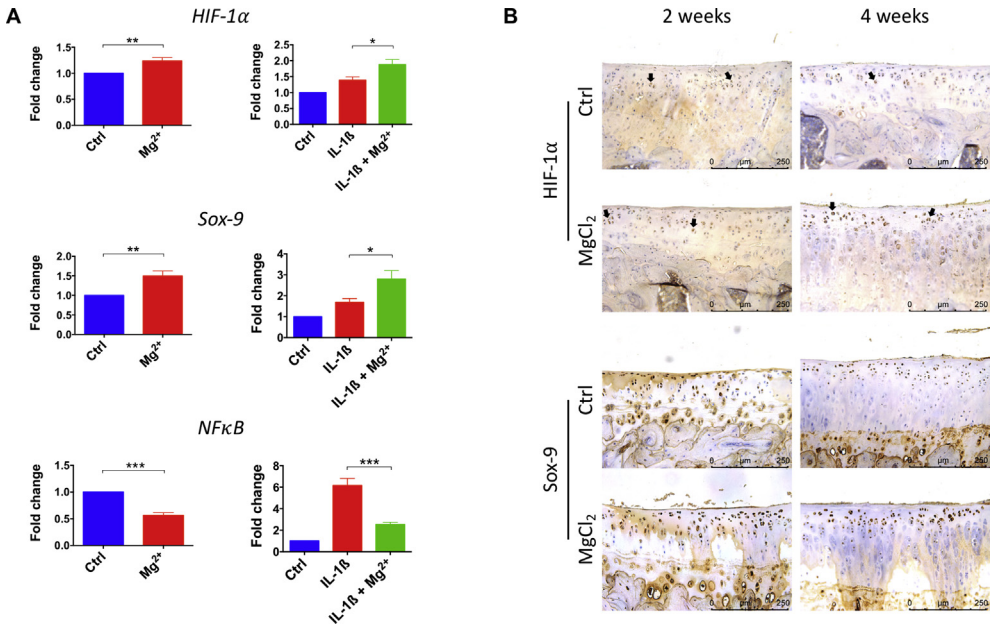

Fig. 8. (A) The expression of *HIF-1α*, *Sox-9* and *NFκB* in human cartilage tissue explants, after incubating with either Mg^{2+} (20 mmol/L), IL-1β (10 ng/ml) or IL-1β (10 ng/ml) + Mg^{2+} (20 mmol/L), determined by PCR (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (B) Representative IHC staining showed the expression and distribution of HIF-1α (solid arrow) and Sox-9 in cartilage after treatment in rats.

articular injections of Mg^{2+} could inhibit the inflammatory response in the synovium in OA. However, the underlying mechanisms require further investigations.

Increasing evidence showed that IL-6 is an important cytokine in OA development, which indicates that a low expression level IL-6 associates with less severe OA[40,45–47]. Another study further provided a direct evidence that the blockade of IL-6/Stat3 signalling pathway could attenuate OA in mice[48]. In the present study, we found the expression of IL-6 was significantly inhibited by Mg^{2+} in both the cartilage and synovium. However, whether Mg^{2+} would inhibit inflammation in OA through a similar or the same regulatory pathway of IL-6 requires further exploration.

Of note, the present study has some limitations. Firstly, the ACLT + PMM rat model adopted in the study can only partially recapitulate OA in human. The constant instability in the ACLT + PMM knee joint may mask with the effects of Mg^{2+}. Secondly, the sample size of human tissue explants in our *in vitro* study was limited. Hence, it may be worthy to test the efficacy of intra-articular injections of Mg^{2+} in other OA animal model(s), for example, the spontaneous OA model in the guinea pig[49]. Meanwhile, more human samples would be accumulated for our further assessments.

Conclusion

In the current study, we confirmed that the anti-osteoarthritic effects of intra-articular injections of Mg^{2+} on an ACLT + PMM induced OA model in rats. The effect of Mg^{2+} lasted for 16 weeks after the last drug delivery. Multiple actions of Mg^{2+} in OA treatment involve the synthesis of cartilage matrix while suppressing the inflammatory response through regulating the expression of HIF-1α and NFκB that could significantly attenuate the progression of OA. Mg^{2+} can potentially regulate other signalling pathways that participate in the progression of OA, which needs further exploration. However, the current protocol requires repeated injections, which limits its further translation to bedside applications. Thus, a delivery system that provides sustained and controlled release of Mg^{2+} are desirable for future development to improve its long-term treatment efficacy.

Contributions

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Final approval of the article: Hao Yao, Jian Kun Xu, Ki Wai Ho, Ling Qin.

Provision of the study material or patients: Ki Wai Ho, Ling Qin.

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Obtaining of funding: Ki Wai Ho, Ling Qin.

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Competing interest statement

There is no conflict of commercial interest for any of the authors with the publication of the manuscript.

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Ethics approval of research on humans or animals

Collection of human tissue explants was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee, Ref. No. 2017.053.

Animal experiments were approved by the Animal Experimentation Ethics Committee, The Chinese University of Hong Kong, Ref. No. 17-060-GRF.

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Supplementary data

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