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Combination of magnesium ions and vitamin C alleviates synovitis and osteophyte formation in osteoarthritis of mice

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ABSTRACT

Introduction: We previously demonstrated that magnesium ions (Mg^{2+}) was a novel therapeutic alternative for osteoarthritis (OA) through promoting the hypoxia inducible factor-1α (HIF-1α)-mediated cartilage matrix synthesis. However, oxidative stress can inhibit the expression of HIF-1α, amplify the inflammation that potentially impairs the therapeutic efficacy of Mg^{2+} in OA. Vitamin (VC), a potent antioxidant, may enhance the efficacy of Mg^{2+} in OA treatment. This study aims to investigate the efficacy of combination of Mg^{2+} and VC on alleviating joint destruction and pain in OA.

Material and methods: Anterior cruciate ligament transection with partial medial meniscectomy induced mice OA model were randomly received intra-articular injection of either saline, $MgCl_2$ (0.5 mol/L), VC (3 mg/ml) or $MgCl_2$ (0.5 mol/L) plus VC (3 mg/ml) at week 2 post-operation, twice weekly, for 2 weeks. Joint pain and pathological changes were assessed by gait analysis, histology, western blotting and micro-CT.

Results: Mg^{2+} and VC showed additive effects to significantly alleviate the joint destruction and pain. The efficacy of this combined therapy could sustain for 3 months after the last injection. We demonstrated that VC enhanced the promotive effect of Mg^{2+} on HIF-1α expression in cartilage. Additionally, combination of Mg^{2+} and VC markedly promoted the M2 polarization of macrophages in synovium. Furthermore, combination of Mg^{2+} and VC inhibited osteophyte formation and expressions of pain-related neuropeptides.

Conclusions: Intra-articular administration of Mg^{2+} and VC additively alleviates joint destruction and pain in OA. Our current formulation may be a cost-effective alternative treatment for OA.

1. Introduction

Osteoarthritis (OA) is the most common degenerative joint disorder that will affect around 78 million people worldwide by 2040 [1]. It remains a grand challenge to treat OA [2,3]. Efforts for developing new approaches have been made. For example, platelet-rich plasma (PRP) or mesenchymal stem cells (MSCs) therapies showed some potentials on cartilage repair [4,5]. However, the cost, potential risks, lack of standardization in preparations of PRP and MSCs are concerns for further clinical applications [2,3]. Nutritional supplements have been developed as non-pharmacological treatment. For example, supplementation of olive tree compound and vitamin D has shown protective effect on articular cartilage [6,7]. Recently, magnesium (Mg)-based biomedical devices have shown great translational potential in orthopaedics [8].

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Mg-based alloys exhibit good tissue compatibility, pro-osteogenic property after implantation [9]. Proteome analysis has also indicated that Mg-based biomaterials is beneficial for cartilage regeneration [10]. Moreover, our previous study has demonstrated that magnesium ions (Mg^{2+}) alleviates cartilage degeneration in OA by regulating hypoxia inducible factor-1α (HIF-1α)-mediated cartilage matrix synthesis [11]. With regards to the cost-effective and safety properties, Mg^{2+} shows a great potential for clinical application. However, this benefit of Mg^{2+} alone is diminished in a relatively short period after discontinuing the therapy, likely attributed to the fact that OA is a multifactorial disease. Therefore, reagents which can eliminate detrimental factors may help clear the obstacles limiting the efficacy of Mg^{2+} in OA treatment.

Articular cartilage is an avascular tissue favoring a hypoxic environment. HIF-1α is a key mediator that promotes the chondrocyte adaptation in the hypoxic environment and exerts anabolic and anti-catabolic effects in the chondrocytes of OA [12–15], suggesting that manipulating HIF-1α may have potential therapeutic benefits in OA. We previously reported that Mg^{2+} could enhance the cartilage matrix synthesis through promoting the expression of HIF-1α [11]. However, previous study indicated that oxidative stress negatively affected oligodendroglial development through inhibiting the expression of HIF-1α [16]. Given the fact that overactivation of oxidative stress has critical role in initiating and potentiating the progression of OA [17]. As a result, oxidative stress may impair the promotive effect of Mg^{2+} on the expression of HIF-1α in cartilage. Vitamin C (VC) is not only a potent antioxidant, but also a key factor participates in post-translational modification of collagen synthesis and the stabilization of collagen network [18–22]. Whether combining with VC could enhance the promotive effect of Mg^{2+} on the HIF-1α-mediated cartilage matrix synthesis is worthy to be investigated.

Synovitis is a recognized contributing factor to joint pathology and pain. Released inflammatory cytokines from synoviocytes (for example, macrophage and fibroblast-like synoviocyte) promote cartilage degradation, also induce hyperalgesia by sensitizing nociceptive nerve in the synovium of OA [23–25]. Therefore, alleviating synoviocyte dysfunction is crucial for OA treatment [26,27]. Moreover, overactivated oxidative stress can amplify the inflammation and further aggravate the pathological changes and pain in OA [28,29]. Therefore, VC supplementation is essential for controlling inflammation combined with Mg^{2+} [11,30]. On the other hand, clinical data showed that osteophyte formation is also in relation to joint pain in OA [31–33]. However, it remains to be investigated how osteophyte induces pain in OA, and whether Mg^{2+} plus VC could ameliorate osteophyte formation in OA.

In this study, we evaluated the efficacy of intra-articular injection of Mg^{2+} and VC on alleviating joint destruction and pain in surgical induced OA model in mice. We investigated whether VC could enhance the promotive effect of Mg^{2+} on the expression of HIF-1α in cartilage. We further clarified whether combination of Mg^{+} and VC could ameliorate pain in OA through the possible underlying mechanisms of inhibiting the inflammation, productions of pain-related neuropeptides and osteophyte formation.

2. Material and methods

2.1. Establishment of OA model, grouping and treatments

Male C57/BL6 mice (3-month-old) were purchased for this study. The animal experiment was approved by the Animal Ethics and Experimentation Committee of the Chinese University of Hong Kong (Reference No:18/071/MIS-5-C) and conducted following the ARRIVE guidelines. OA was induced by performing anterior cruciate ligament transection with partial medial meniscectomy (ACLT + PMM) surgery in mice [11,34]. From day 1 to day 3 post-operation, the mice received temgesic (0.3 mg/kg of body weight) once per day. The mice were allowed free to food, water and cage activities after the operation. At week 2 post-operation (relative early stage of OA), the mice were randomly assigned to four different groups (n = 6 per group per time point) and received 10 μl intra-articular injection of either saline (control group), 3 mg/ml VC (A5960, Sigma Aldrich, US) (VC group), 0.5 mol/L $MgCl_2$ (M8266, Sigma Aldrich, US) (Mg group) or 0.5 mol/L $MgCl_2$ + 3 mg/ml VC (Mg + VC group) twice per week for two consecutive weeks. The optimal doses of $MgCl_2$ and VC were selected according to our previous studies [11,35]. The grouping of the mice in this study was summarized in Supplementary Table 1.

2.2. Pain assessment by gait analysis

At day one pre-treatment, day 3–84 post-treatment, gait analysis was performed to evaluate the pain-related behaviors of mice by using Catwalk XT 9.0 system (Noldus Information Technology, Wageningen, the Netherlands) [36,37]. Each mouse was placed individually onto a glass walkway (width, 5 cm) and allowed to walk voluntarily back and forth. The footprint was recorded by a high-speed video camera that was positioned (34 cm from the glass walkway) under the glass walkway. Gait parameters (Duty cycle, Intensity, Print area, Single stance, Stand, Swing, Swing speed) of all mice were recorded as baseline one day before treatments [37–39]. At day 3–84 post-treatment, these gait parameters of the mice in all groups were further recorded. The ratio of left hind to right hind (LH/RH) of the parameters was calculated. Limb Idleness Index (LII) was also calculated according to our previous description [37]. Finally, ΔLH/RH (ΔLH/RH = LH/RH post-treatment - LH/RH at baseline) and ΔLII (ΔLII = LII post-treatment - LII pre-treatment) were used for statistical analysis.

2.3. Histomorphometric analysis

At week 6 and 12 post-treatment, knee samples were prepared for fixation in 10% buffered formalin solution and decalcified in 9% formic acid. Paraffin sections were prepared for Safranin O/Fast green or Hematoxylin and Eosin (H&E) staining. The severity of cartilage degeneration and synovitis was evaluated using OARSI scoring system and Krenn scoring system [40,41]. Subchondral bone damages and osteophytes thickness were also evaluated accordingly to previous descriptions [40,42].

2.4. Immunohistochemical (IHC) and immunofluorescence (IF) staining

Primary antibodies were applied and incubated overnight at 4 °C. Horseradish peroxidase (HRP) conjugated secondary antibodies were applied and incubated for 1 h at room temperature. For IHC staining, the signal was developed with DAB kit (TA-060-QHDX, Thermo Fisher scientific) and counterstained with Hematoxylin. For IF staining, DAPI (D1306, Life Technology) was used to stain the nuclei. The antibodies used were listed in the Supplementary Table 2.

2.5. Western blot

At week one post-treatment, four mice from each group were sacrificed for dissecting cartilage tissues from the tibial plateau and divided into two separate samples. The samples were smashed into powder and lysed with RIPA buffer (Cat#89901, Thermo Fisher Scientific). After electrophoresis (protein ladder, BIO-RAD, 1610374), the proteins were electroblotted onto polyvinylidene fluoride membranes. The membranes were blocked and incubated with primary antibodies (HIF-1α, MW 120 kDa; COL2A1, MW 142 kDa; SOX-9, MW 56 kDa; GAPDH, MW 37 kDa) at 4 °C overnight. Subsequently, the membranes were incubated with HRP conjugated secondary antibodies for 1 h at room temperature. Protein bands were detected using an enhanced chemiluminescence kit (Cat#1705062, BIO-RAD). The antibodies were listed in the Supplementary Table 2.

2.6. μCT scanning

At week 12 post-treatment, the fixed knee samples were scanned by μCT (μCT 40, SCANCO MEDICAL, Brttisellen, Switzerland, Voltage = 70 kV, Current = 113 μA; Voxel size = 15 μm; Threshold = 200). Twenty consecutive sagittal images from medial compartment of proximal tibial epiphysis were used to perform 3-D morphometric analyses. Bone volume/total tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) were used to evaluate the subchondral bone changes [43].

2.7. Statistical analysis

Sample size was estimated by power calculation based on our pilot study. Six mice per group was sufficient to provide a 30% difference in the OARSI score (average total score of medial tibial plateau and femoral condyle, $\alpha = 0.05$, power > 0.85) at week 6 post-treatment. Two mice were excluded from our experiments due to anesthetic accidents. Histological semi-quantitative analysis was performed by two colleagues blindly based on three sections from different regions in each sample. In cases of discrepancies, opinions from a third party was sought. Differences between each treatment and control were analyzed using two-way ANOVA and Turkey's multiple comparisons test using the GraphPad Prism software (Version 8.2) [44]. The significance was defined as $P < 0.05$.

3. Results

3.1. Intra-articular injection of Mg^{2+} and VC alleviated structural degeneration in OA

At week 6 post-treatment, in the control group, obvious abrasion on the cartilage surface and focal loss of full-thickness cartilage resulted in an increased OA score. VC only provided a moderate alleviation on cartilage degeneration. Mg^{2+} alone and Mg combined with VC largely alleviated the cartilage degeneration. Moreover, the mice from Mg + VC group showed the lowest OA score (tibial plateau, $P = 0.011$; femoral condyle, $P = 0.184$, average score of tibial plateau and femoral condyle, $P = 0.004$) compare to the control (Fig. 1A). At week 12 post-treatment, we found severe diffuse cartilage degeneration characterized by absence of entire cartilage at both medial tibial plateau and femoral condyle and resulted in higher OA scores in control and VC groups. Mg^{2+} slightly ameliorated the cartilage degeneration at this time point. Intriguingly, combination of Mg^{2+} and VC could still protect the integrity of the cartilage. The OA score was significantly decreased in Mg + VC group (tibial plateau, $P = 0.005$; femoral condyle, $P = 0.010$; average score of tibial plateau and femoral condyle, $P = 0.001$) when comparing to that in the control group (Fig. 1A).

At week 6 and 12 post-treatment, we found mid-grade inflammatory response in synovium characterized by increased lining cell layer and focal inflammatory cells infiltration in the control group. The synovitis was less severe in all treatment groups, while combination of Mg^{2+} and VC provided better efficacy on alleviating the synovitis with the lowest synovitis score over other treatments when comparing to the control (week 6, $P = 0.014$; week 12, $P = 0.018$) (Supplementary Fig. 1A). In addition, we found a progressively increased thickness of osteophyte in both medial tibial plateau and femoral condyle of the mice in the control group, either Mg^{2+} or VC could partially inhibit the osteophyte formation, combination of Mg^{2+} and VC could significantly reduce the thickness of osteophyte compared to the control at week 6 (tibial plateau, $P = 0.039$; femoral condyle, $P = 0.025$; average score of tibial plateau and femoral condyle, $P = 0.016$) and 12 (tibial plateau, $P < 0.001$; femoral condyle, $P = 0.061$; average score of tibial plateau and femoral condyle, $P < 0.001$) (Fig. 1A). Moreover, the mice in the control group showed an increased thickening of subchondral bone in proximal tibia, resulting in increased subchondral bone score (based on OARSI scoring system) at week 6 post-treatment. VC and Mg^{2+} alone only partially alleviated the subchondral bone changes. Whereas, combination of Mg and VC could significantly alleviate the subchondral bone changes compared to the control ($P = 0.026$) (Fig. 1A). At week 12 post-treatment, apart from the progressively increased thickening of subchondral bone, we also found that the articular cartilage had collapsed into the epiphysis in both the control and VC groups. Mg^{2+} provided limit potential on alleviating these changes at this time point. However, the results still showed significantly decreased subchondral bone score in Mg + VC group when comparing to that in the control group ($P = 0.004$) (Fig. 1A). Consistent to the histological findings, at week 12 post-treatment, the results of μCT scanning showed that Mg^{2+} + VC significantly alleviated the subchondral bone changes with decreased BV/TV ($P = 0.019$) and Tb.Th ($P = 0.015$), increased Tb.N ($P = 0.065$) and Tb.Sp ($P = 0.132$) compared to the control (Supplementary Fig. 1B).

3.2. Mg^{2+} and VC prevented the cartilage degeneration via regulating HIF-1α

To investigate the efficacy of Mg^{2+} + VC on prevention of the cartilage matrix degeneration, we evaluated the content of collagen type 2A1 (COL2A1) in cartilage using IHC staining. The results showed no marked difference on COL2A1 between groups. However, the integrity of cartilage in Mg + VC group was well protected compared to that in the control group at week 6 and 12 post-treatment (Supplementary Fig. 2A). We next dissected the cartilage tissue from the mice at week 1 post-treatment, Western blot showed that the expression of COL2A1 in cartilage tissue of Mg + VC group was significantly higher than that of the control group (Fig. 1B). Furthermore, we found that the expression levels of HIF-1α and its downstream marker, transcription factor sex determining region Y-box 9 (SOX-9), were markedly elevated in Mg + VC group with respected to that in the control group (Fig. 1B). We next confirmed the expressions of HIF-1α and SOX-9 in cartilage at week 6 post-treatment using IHC staining. Similarly, we found that Mg^{2+} and VC had an additive effect to significantly promote the expressions of HIF-1α in articular cartilage (Ctrl vs Mg + VC, $P < 0.001$, interaction effect, $P = 0.544$). The expression of SOX-9 in hyaline cartilage layer was also markedly increased after injection of Mg^{2+} and VC (Ctrl vs Mg + VC, $P < 0.001$) (Fig. 1D). However, there was no significant difference on the expression of HIF-1α in synovium between the groups (Supplementary Fig. 2B).

3.3. Mg^{2+} and VC suppressed the expressions of OA markers

The IHC staining showed increased expression levels of matrix metalloproteinase-13 (MMP-13), interleukin-6 (IL-6) and inducible nitric oxide synthases (iNOS) in both lining cell layer and resident cells in synovium of control group at week 6 and 12 post-treatment (Supplementary Fig. 3). Both VC and Mg^{2+} reduced the percentage of cells expressing MMP-13, IL-6 and iNOS in the synovium, respectively. The MMP-13, IL-6 and iNOS positive cells in synovium was significantly reduced in Mg^{2+} + VC group (MMP-13, week 6, $P < 0.001$; week 12, $P < 0.001$. IL-6: week 6, $P < 0.001$; week 12, $P < 0.001$; iNOS, week 6, $P < 0.001$; week 12, $P < 0.001$) compared to that of the control group (Supplementary Fig. 3). We also found similar results in cartilage, the percentage of either MMP-13 or IL-6 positive chondrocytes slightly decreased in VC or Mg groups while significantly reduced in Mg + VC group (MMP-13, week 6, $P = 0.003$; week 12, $P < 0.001$. IL-6: week 6, $P = 0.003$; week 12, $P < 0.001$) with respected to the control group (Supplementary Fig. 4). The percentage of chondrocytes expressing collagen type 10A1 (COL10A1) was profoundly reduced in Mg + VC group at week 6 ($P < 0.001$) and week 12 ($P < 0.001$) post-treatment when comparing to the control group (Supplementary Fig. 4).

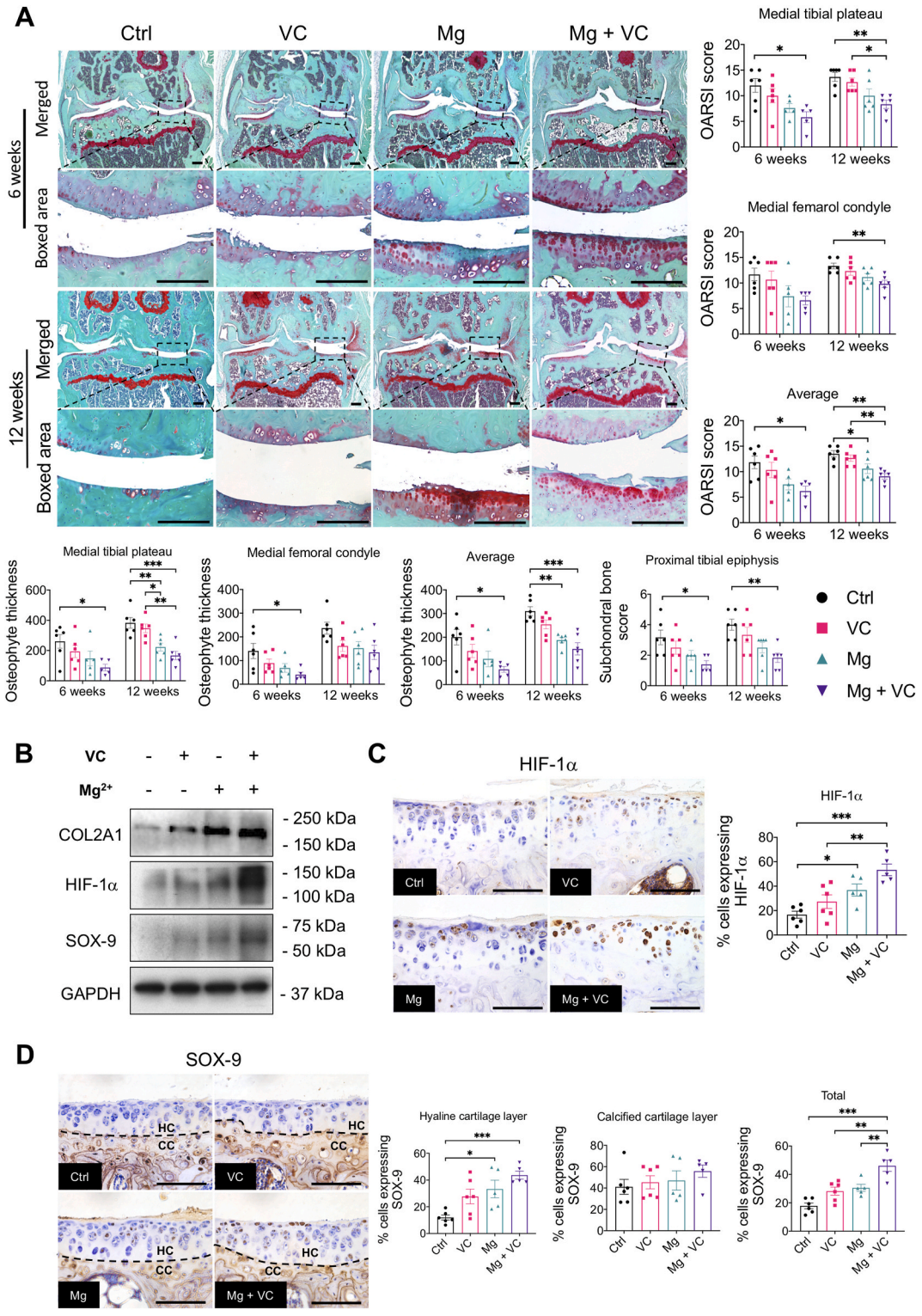

Figure 1. The structural degeneration of OA was significantly attenuated by intra-articular injection of Mg^{2+} and VC. (A) Left, Safranin O/Fast green staining showed the changes on cartilage in each group at week 6 and 12 post-treatment. Scale bar, 250 μm. Right and bottom, cartilage degeneration, subchondral bone changes and osteophyte development were quantified by OARSI scoring system at the indicated time points. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 5–6 animals per group. (B) Western Blotting showed the expressions of COL2A1, HIF-1α and SOX-9 in dissected cartilage tissue at week 1 post-treatment. (C) Left, IHC staining showed the expressions of HIF-1α in articular cartilage at week 6 post-treatment. Scale bar, 100 μm. Right, quantification of HIF-1α positive cells in cartilage at week 6 post-treatment. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. n = 5–6 animals per group. (D) Left, IHC staining showed the expression of SOX-9 in hyaline cartilage (HC) and calcified cartilage (CC) layer at week 6 post-treatment. Right, quantification of SOX-9 positive cells in either HC or CC layer. Scale bar, 100 μm. Dash line, tide mark. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. n = 5–6 animals per group.

3.4. Mg^{2+} and VC alleviated pain-related animal behaviors

Gait analysis (Catwalk) was used to evaluate the pain-related behaviors of mice. Both VC and Mg^{2+} reduced OA pain, respectively, at the relatively early time point post-treatment. Intriguingly, only Mg^{2+} + VC still provided a significant amelioration on the pain-related behaviors over other treatments at the later time points post-treatment (Figs. 2 and 3). According to the time-course analysis of gait analysis, the ΔLII and Δratios of LH/RH on duty cycle, stand, single stance, intensity and swing speed were deceased, while the Δratio of LH/RH on swing was increased after injection of Mg^{2+} + VC from day 3–84 post-treatment compared to the control. These data suggested that intra-articular injection of Mg^{2+} + VC efficaciously alleviated pain-related behaviors (Figs. 2 and 3).

3.5. Mg^{2+} and VC might ameliorate inflammation-induced pain via regulating synovial macrophage polarization in OA

Macrophage infiltration in synovium plays an essential role in triggering the inflammation in OA [24]. Released inflammatory cytokines could sensitize nociceptive nerve terminals in synovium and induce inflammatory OA pain [25]. To investigate the regulatory effects of Mg^{2+} and VC on synovial macrophage in OA, we identified the phenotypic characterization of macrophage in synovium at week 6 post-treatment. The results of immunofluorescent staining showed a marked elevation of CD86 (M1-like macrophage marker) in F4/80 (macrophage marker) positive cells in synovial tissue of the control group, the percentage of CD86 expressing cells in F4/80 positive cells was decreased in VC ($P < 0.001$), Mg ($P < 0.001$) and Mg + VC ($P < 0.001$) groups compared to the control group (Fig. 4A). Meanwhile, the proportion of cells positive for CD206 (M2-like macrophage marker) in F4/80 positive cells was increased in groups of VC ($P = 0.006$), Mg ($P <$

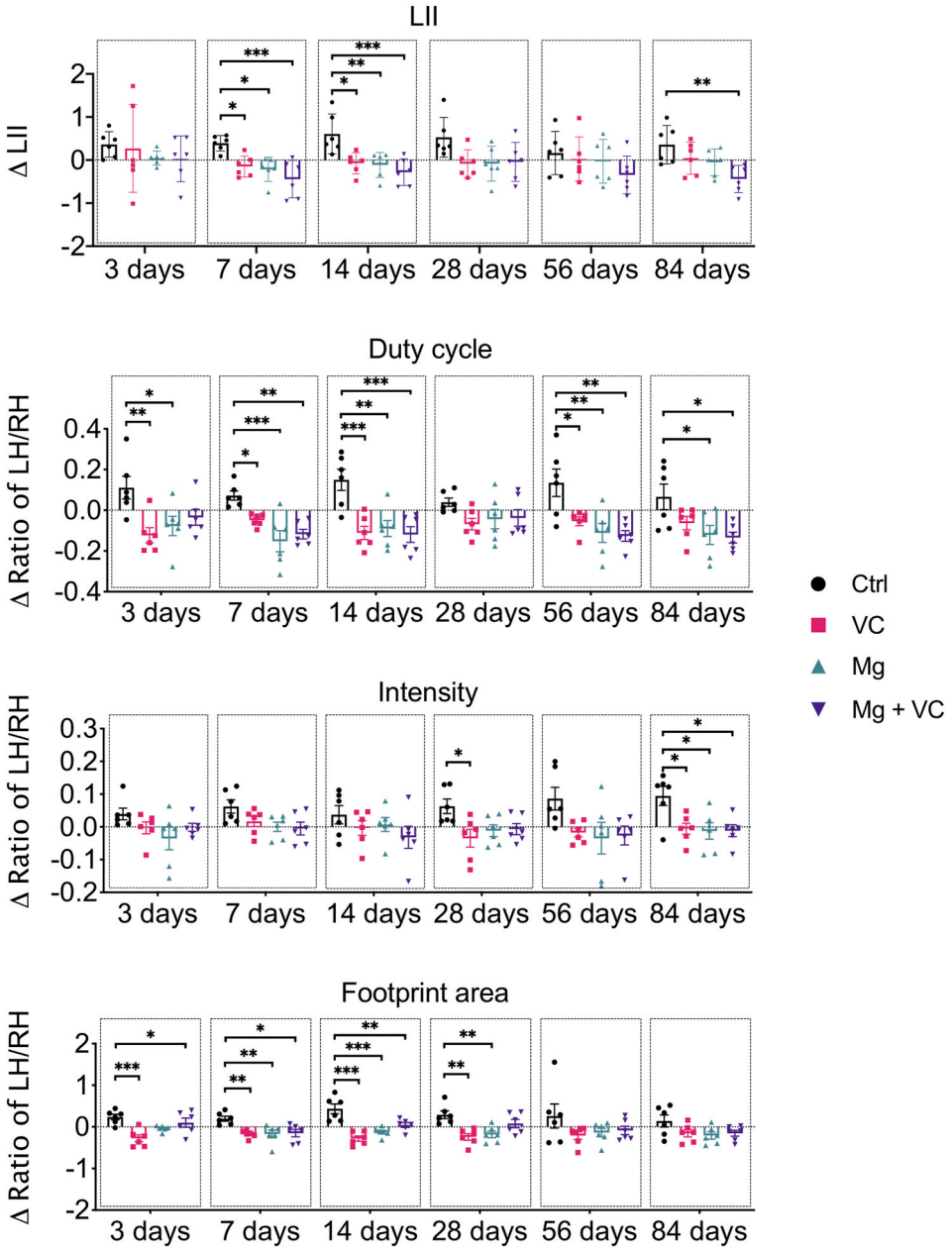

Figure 2. Time-course analysis on gait parameters of LII, Duty cycle, Intensity, Footprint area showed the alterations of pain related animal behaviors from day 3–84 after intra-articular injection of Mg^{2+} + VC. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 6 animals per group.

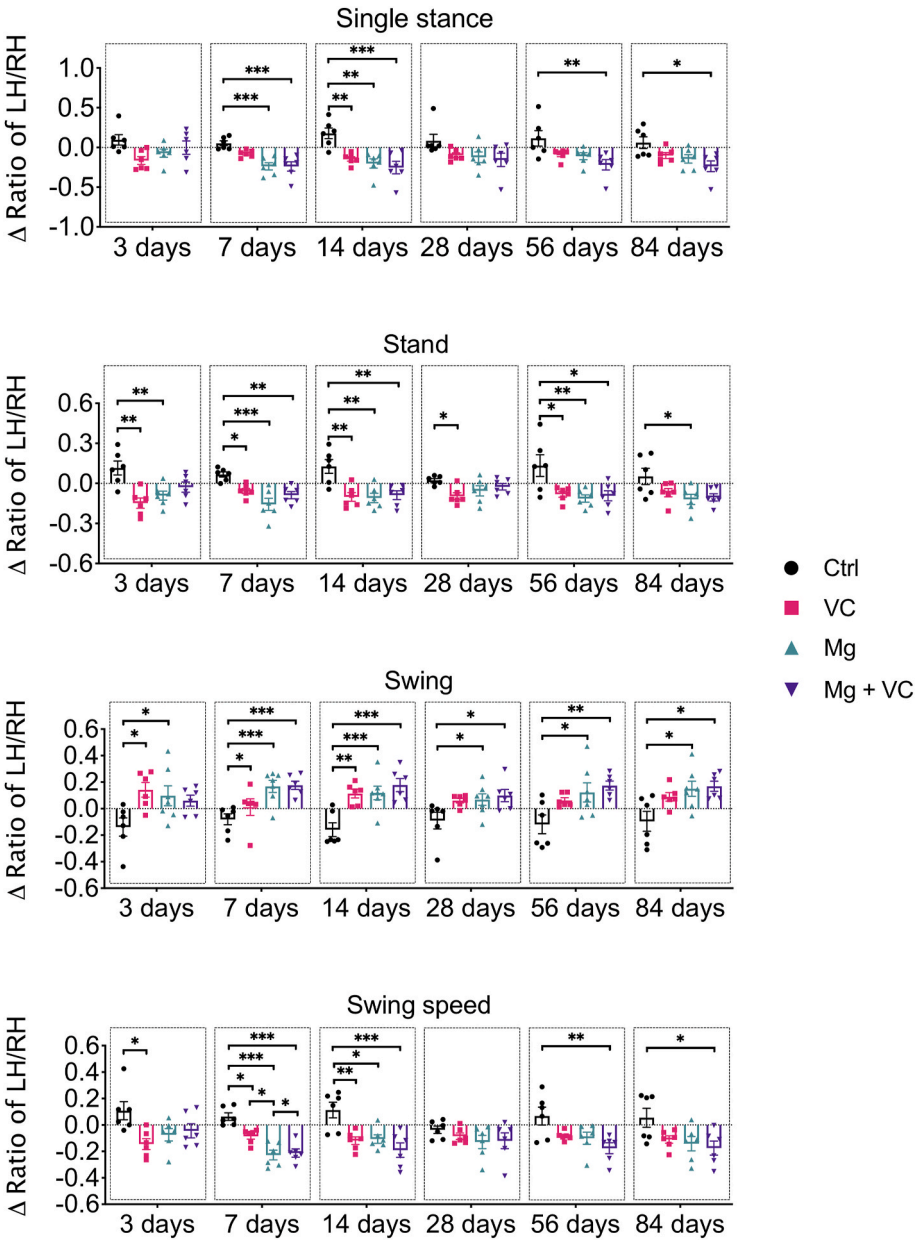

Figure 3. Time-course analysis on gait parameters of Single stance, Stand, Swing, Swing speed showed the alterations of pain related animal behaviors from day 3–84 after intra-articular injection of Mg^{2+} + VC. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 6 animals per group.

0.001) and Mg + VC ($P < 0.001$) compared to that of the control group (Fig. 4B). Semi-quantitative analysis indicated that Mg^{2+} and VC had an additive effect to significantly promote the M2 polarization of macrophage in synovium at weeks 6 post-treatment (CD86, interaction effect, $P = 0.070$; CD206, interaction effect, $P = 0.285$). Meanwhile, as expected, Mg^{2+} and VC also additively suppress the expressions of inflammatory cytokines over other treatments. The results of IHC staining showed that the percentages of tumor necrosis factor-α (TNF-α) and prostaglandin E2 (PGE2) positive cells were significantly decreased in synovial tissues of Mg + VC group compared to that of the control group at week 6 (TNF-α, $P < 0.001$; PGE2, $P < 0.001$) and 12 (TNF-α, $P < 0.001$; PGE2, $P < 0.001$) post-treatment (Fig. 5A). Additionally, we also found similar results that the expressions of TNF-α and PGE2 were suppressed in cartilage after injection of Mg^{2+} and VC (Supplementary Fig. 5). To determine the nerve sensitization in synovium after treatments, we further assessed the expressions of substance P (SP) and calcitonin gene-related peptide (CGRP) in synovium. The IHC staining showed that either VC or Mg^{2+} could reduce the expressions of SP and CGRP. Combination of Mg^{2+} and VC had larger effect on suppressing the expressions of SP and CGRP than other treatments compared to the control at week 6 (SP, $P = 0.005$; CGRP, $P = 0.001$) and 12 (SP, $P < 0.001$; CGRP, $P < 0.001$) post-treatment (Fig. 5B). These results indicated that the nerve sensitization in synovium might be reduced [25].

3.6. Mg^{2+} and VC might alleviate pain through inhibiting osteophytes formation in OA

Our histomorphometric analysis showed that intra-articular injection of Mg + VC limited the thickness of osteophytes (Fig. 1A). It is known that osteophyte formation also occurred through the process of endochondral ossification [45]. In this study, we determined the expressions of MMP-13, COL10A1 and PGE2 in osteophytes using IHC staining. We found significant elevations of MMP-13, COL10A1 or PGE2 positive hypertrophic chondrocytes in osteophytes of the control group

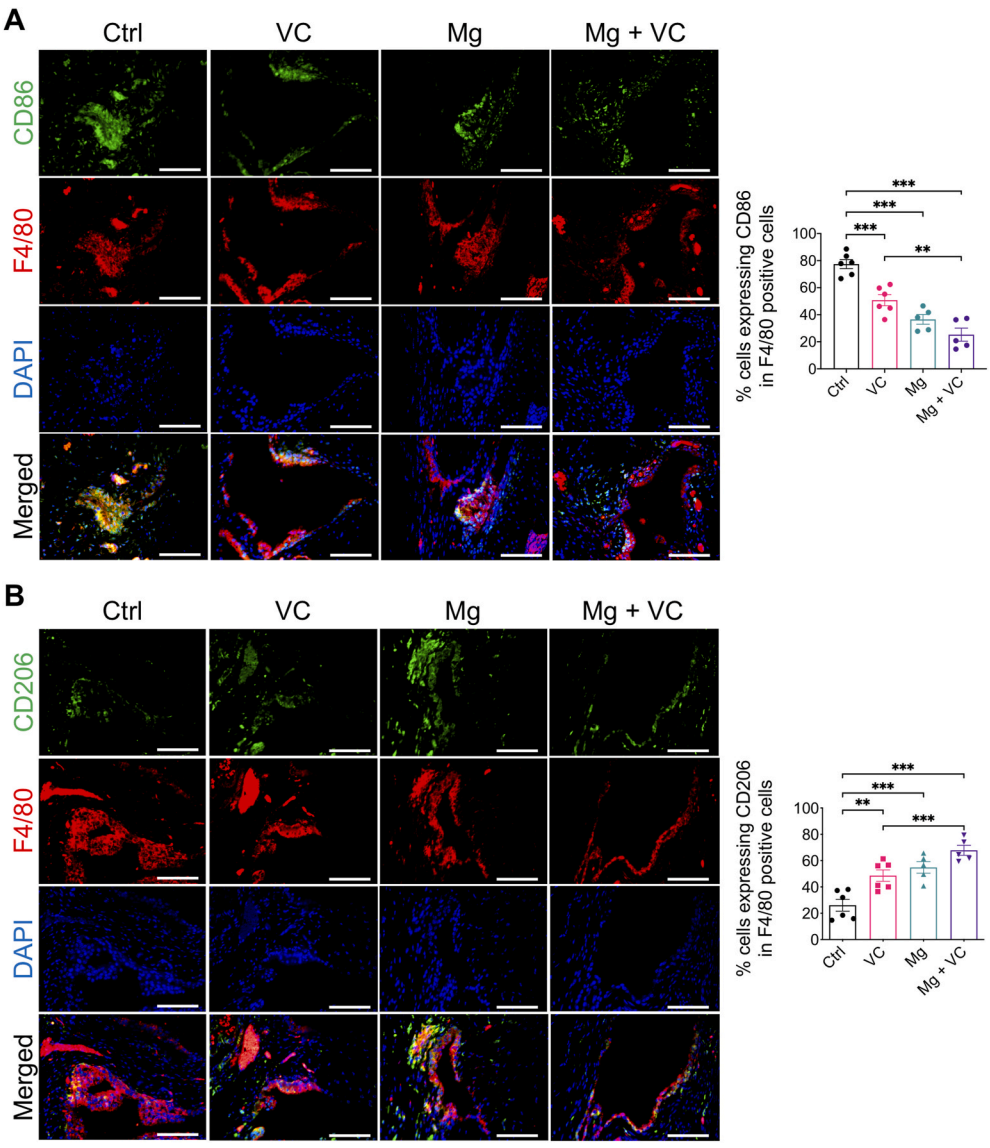

Figure 4. Intra-articular injection of Mg^{2+} combined with VC promoted the M2 synovial macrophage polarization. (A) Left, IF staining showed the expressions of CD86 (M1 macrophage marker) and F4/80 (macrophage marker) in synovium at week 6 post-treatment. Scale bar, 100 μm. Right, quantification of CD86 in F4/80 positive cells in synovium at week 6 post-treatment. $P < 0.01$, $^{***}P < 0.001$. n = 5–6 animals per group. (B) Lift, IF staining showed the expressions of CD206 (M2 macrophage marker) and F4/80 in synovium at week 6 post-treatment. Scale bar, 100 μm. Right, quantification of CD206 in F4/80 positive cells in synovium at week 6 post-treatment. $^{**}P < 0.01$, $^{***}P < 0.001$. n = 5–6 animals per group.

at week 6 post-treatment (Fig. 6A). In contrast, combination of Mg^{2+} and VC significantly reduced the proportion of MMP-13 ($P < 0.001$), COL10A1 ($P < 0.001$) and PGE2 ($P = 0.002$) expressing cells in osteophytes (Fig. 6A). At week 12 post-treatment, along with the osteophyte development, the hypertrophic chondrocytes were replaced by bony tissue thus resulted in a reduced proportion of MMP-13, COL10A1 or PGE2 positive cells in osteophytes of the control group (Fig. 6A). However, combination of Mg^{2+} and VC could still suppress the expressions of MMP-13 ($P = 0.006$), COL10A1 ($P = 0.043$) and PGE2 ($P < 0.001$) in osteophytes with respected to the control at this time point (Fig. 6A). Previous studies demonstrated that hypertrophic chondrocytes in fracture healing callus could produce neuropeptides including SP [46]. The results from this study showed high expressions of SP and CGRP in hypertrophic chondrocyte in osteophyte of the control group at week 6 and 12 post-treatment, whereas the percentages of SP and CGRP expressing cells (SP, week 6, $P < 0.001$, week 12, $P < 0.001$; CGRP, week 6, $P = 0.001$, week 12, $P = 0.012$) in osteophyte were significantly reduced in Mg + VC group when comparing to that of the control group (Fig. 6B).

4. Discussion

In this study, we demonstrated that intra-articular injection of Mg^{2+} and VC could efficaciously alleviate the joint pathology and pain-related behaviors in ACLT + PMM induced mice OA model. The efficacy of such combination therapy could sustain for 3 months after the last treatment. We demonstrated that VC significantly enhanced the promotive effect of Mg^{2+} on the expression of HIF-1α in cartilage. In addition, intra-articular injection of Mg^{2+} and VC markedly promoted the M2 polarization of synovial macrophages. Moreover, combination of Mg^{2+} and VC also inhibited the osteophyte formation and the productions of pain-related neuropeptides (SP and CGRP).

HIF-1α serves as a key factor for maintenance of hyaline chondrocyte phenotype by promoting the synthesis of type II collagen and aggrecan [47–51]. Depletion of HIF-1α can induce chondrocyte apoptosis and aggravate cartilage degeneration in OA [52–54]. Yet, clinical study has shown that the increased expression of HIF-1α in cartilage is closely associated with severity of OA and indicated HIF-1α may be a harmful factor in OA progression [55]. In fact, HIF-1α plays essential roles in promoting chondrocyte adaptation to survival [56]. In OA, catabolic-stress (for example the inflammatory cytokines, IL-1β) stimulates the expression of HIF-1α in chondrocyte to mediate the

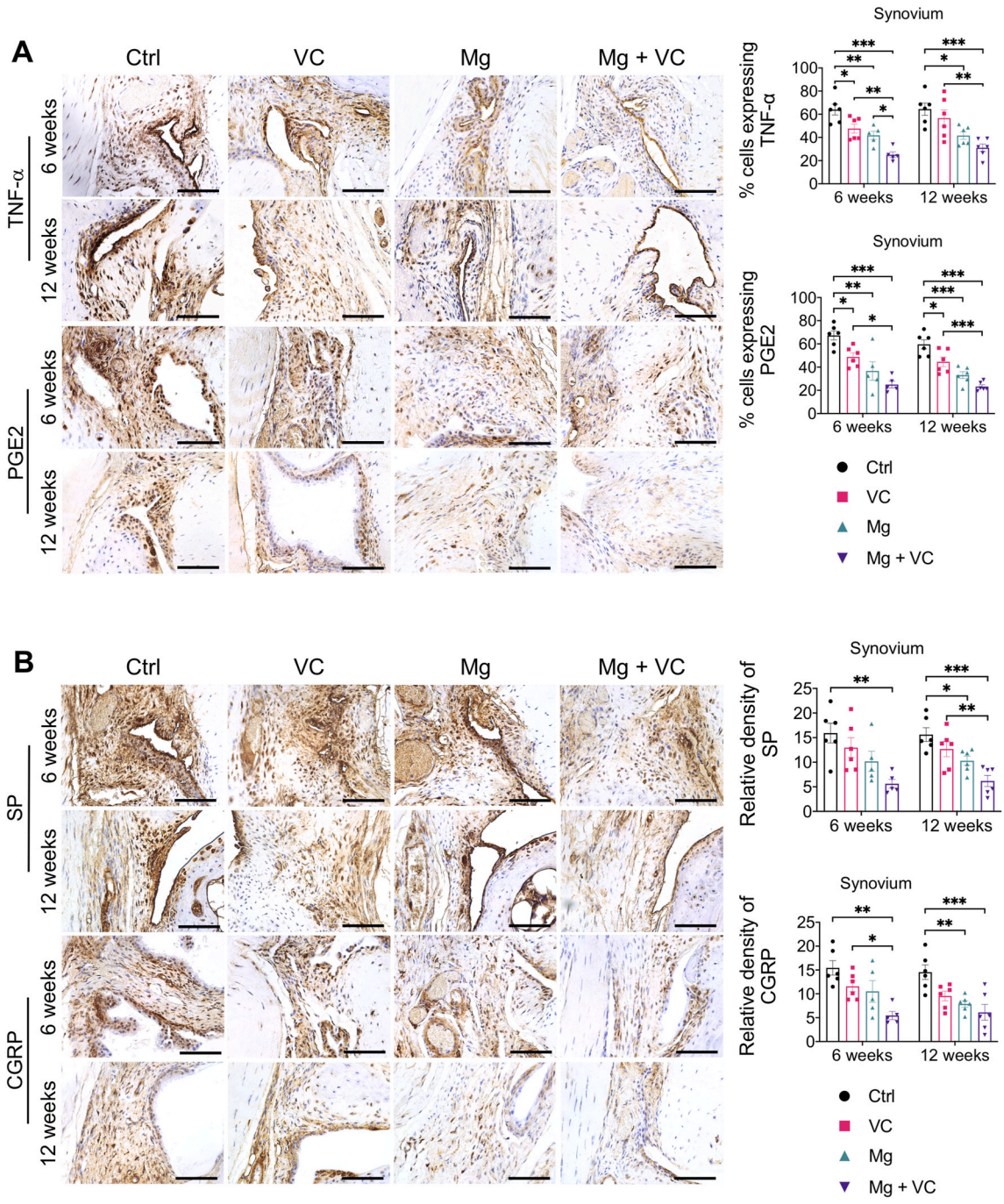

Figure 5. Intra-articular injection of Mg^{2+} combined with VC reduced the productions of inflammatory cytokines (TNF-α and PGE2) as well as the pain related neuropeptides (SP and CGRP) in the synovium. (A) Left, IHC staining showed the expressions of TNF-α and PGE2 in synovium at week 6 and 12 post-treatment. Scale bar, 100 μm. Right, quantification of TNF-α and PGE2 positive cells in synovium at the indicated time points. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n = 5–6 animals per group. (B) Left, IHC showed the expressions of SP and CGRP in synovium at week 6 and 12 post-treatment. Scale bar, 100 μm. Right, quantification of SP and CGRP positive cells in synovium at the indicated time points. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n = 5–6 animals per group.

anti-catabolic responses [48,53]. Our previous findings also have shown that Mg^{2+} can further stimulate the HIF-1α expression in human cartilage tissue explants after IL-1β induction *in vitro* [11]. The Mg^{2+} induced HIF-1α expression in chondrocyte may contribute to the enhanced matrix synthesis of cartilage and the alleviation of OA progression [11]. Nevertheless, the enhanced oxidative stress in OA may counteract the promotive effect of Mg^{2+} on the expression of HIF-1α [16]. Apart from confirming our previous finding that Mg^{2+} promotes the expression of HIF-1α [11], here we further found that intra-articular injection of Mg^{2+} and VC additively enhanced the expression of HIF-1α in cartilage. Additional supplementation of VC may eliminate the negative effect of oxidative stress thus significantly enhanced the promotive effect of Mg^{2+} on the expression of HIF-1α in cartilage [16]. Meanwhile, this combination therapy markedly elevates the expressions of SOX-9 and COL2A1 in cartilage. Therefore, the results collectively indicate that VC can enhance the promotive effect of Mg^{2+} on the HIF-1α-mediated cartilage matrix synthesis, in turn, attenuate the cartilage degeneration in OA. In the future study, experiments are still needed to block the HIF-1α pathway in presence of Mg^{2+} and VC to consolidate our findings.

As known that pain runs throughout the whole course of OA and is

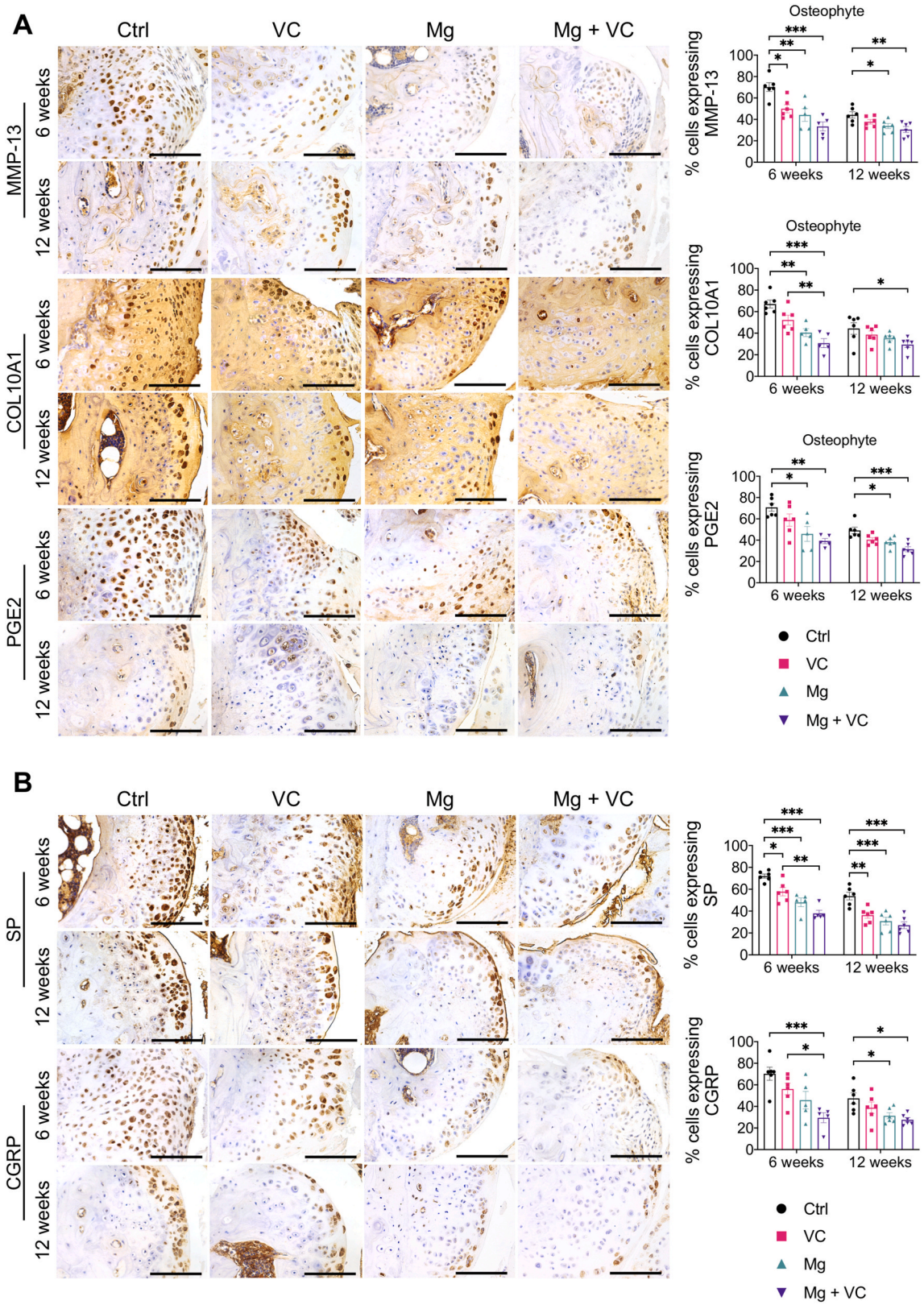

Fig. 6. Intra-articular injection of Mg^{2+} combined with VC reduced the expressions of hypertrophic markers (MMP-13, COL10A1 and PGE2) and subsequent productions of the pain related neuropeptides (SP and CGRP) in osteophyte. (A) Left, IHC staining showed the expressions of MMP-13, COL10A1 and PGE2 in osteophyte at week 6 and 12 post-treatment. Scale bar, 100 μm. Right, quantification of MMP-13, COL10A1 and PGE2 positive cells in osteophyte at the indicated time points. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 5–6 animals per group. (B) Left, IHC staining showed the expressions of SP and CGRP in osteophyte at week 6 and 12 post-treatment. Scale bar, 100 μm. Right, quantification of SP and CGRP positive cells in osteophyte at the indicated time points. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 5–6 animals per group.

the main complaint of the patients. Multiple factors are involved in causing OA pain. Except for inflammation that plays essential role in pain development of OA [24,25]. Several clinical data show a closed relationship between osteophyte formation and pain in OA [31–33]. In this study, we assessed the efficacy of intra-articular administration of Mg^{2+} and VC on ameliorating pain in the OA model and further elucidated the possible underlying mechanisms.

According to previous studies on OA pain, Philip G et al. [25] has suggested a concept of inflammatory OA pain which describes a bidirectional crosstalk between immune and nervous system regulates OA pain. Specifically, in the inflamed synovial tissue in OA, activated immune cells (for example, macrophage) secrete number of cytokines include TNF-α and PGE2 which can be recognized by nociceptive nerve terminals in synovium [57,58]. The sensitized nerve terminals, in turn, can produce SP and CGRP which further promote nerve sensitization, vasodilation and extravasation of immune cells [59]. Among the immune cells, macrophage is reported as one of the main cell types triggering the inflammation in OA [24]. Previous studies have indicated that both Mg^{2+} and VC may have possible roles on regulating the polarization of macrophage [60–62]. In this study, we have found that either Mg^{2+} or VC has an independent effect to promote the M2 polarization while inhibit M1 polarization of macrophage in synovium. More importantly, Mg^{2+} and VC acts additively to promote the M2 polarization of synovial macrophage. Consequently, the expression levels of TNF-α and PGE2 are significantly reduced. As aforementioned, these released inflammatory cytokines can enhance the sensitization of nerve terminals in synovium and induce hyperalgesia with increased expressions of neuropeptides [25]. Our results have further showed that Mg^{2+} and VC can decrease the expressions of CGRP and SP in synovium additively. These results indicate that the nerve sensitization may be reduced after the treatment. Previous studies have shown that Mg^{2+} deficiency induces raised levels of neuropeptides (CGRP and SP) in peripheral blood [63,64]. We have also reported that Mg^{2+} can promote the production of CGRP in dorsal root ganglia (DRG) [65]. However, whether local supplementation of Mg^{2+} could directly influence the expression of CGRP in the nerve terminals of synovium in OA is unclear. In the present study, Mg^{2+} may inhibit the releases of several inflammatory cytokines through promoting the M2 polarization of synovial macrophage, in turn, ameliorates the inflammatory cytokines induced nerve sensitization by decreasing expressions of CGRP and SP. Meanwhile, supplementation of VC may also exert similar functions with Mg^{2+}. Previous study has indicated that the elevated expressions of CGRP and SP in sciatic nerve after fracture is attributed to oxidative stress [66]. Treatment with VC can inhibit oxidative stress to down-regulate the productions of CGRP and SP, subsequently alleviates the nociceptive features post-fracture [66]. Therefore, VC may enhance the efficacy of Mg^{2+} on the alleviation of OA pain.

Previous clinical data has indicated that osteophyte formation may be another source of pain in OA [31–33]. However, the mechanism of osteophyte on triggering the OA pain is not fully understood. In this study, we have found marked elevated proportion of hypertrophic chondrocytes with expressions of MMP-13, COL10A1 and PGE2 in osteophyte of the OA model in the control group which is consistent to previous reports [45]. Whereas combination of Mg^{2+} and VC can significantly inhibit the osteophyte formation with a decreased osteophyte size and reduced hypertrophic markers. It is worth bearing in mind that hypertrophic chondrocytes in fracture healing callus could produce SP and CGRP which facilitated osteogenesis and fracture healing reported by previous studies [46,67]. In our present study, for the first time, we demonstrate that hypertrophic chondrocytes in osteophyte of OA also produce SP and CGRP. These neuropeptides (SP and CGRP) can promote the nerve sensitization and cause the hyperalgesia in OA [68–70]. This evidence provides us a concept that the released SP and CGRP from osteophyte can be recognized by nociceptive nerve terminals distributed in the synovium or even subchondral bone thus promote the OA pain. The results in this study indicate another possible mechanism provided by combination of Mg^{2+} and VC on pain control in OA.

Our current intra-articular injectable treatment still has limitation. As known that repeated administrations are still needed in this study. In the future study, we will develop a delivery system with controlled release of Mg^{2+} and VC which can help to minimize the administration frequency. Other relevant assessments (for example, detection of serum concentration of Mg^{2+} and VC at multiple time points after the treatment) will be also conducted to evaluate the efficiency of drug delivery system on releasing of Mg^{2+} and VC.

5. Conclusions

In the current study, we demonstrated that combined intra-articular injection of Mg^{2+} and VC can efficaciously attenuate the joint destruction and pain in a surgical induced OA model in mice. Mg^{2+} and VC acts additively on promoting the HIF-1α-mediated cartilage matrix synthesis. In addition, Mg^{2+} and VC have additive effect to significantly promote the M2 polarization of macrophage in synovium, may subsequently alleviate inflammatory OA pain. Furthermore, combination of Mg^{2+} and VC also inhibits the osteophyte formation and productions of CGRP and SP. The multiple actions of Mg^{2+} combined with VC injection thus explain the long-term beneficial outcomes in OA. Additionally, the safe and cost-effective properties of this current formulation shed light on the great translational potential. Our study provides essential preclinical evidence for coming clinical trials and bedside applications.

CRediT authorship contribution statement

Hao Yao: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. **Jiankun Xu:** Conceptualization, Methodology, Data curation, Validation, Writing - review & editing. **Jiali Wang:** Conceptualization, Methodology, Validation. **Yifeng Zhang:** Conceptualization, Methodology, Validation. **Nianye Zheng:** Methodology, Investigation. **Jiang Yue:** Methodology, Investigation. **Jie Mi:** Methodology, Investigation. **Lizhen Zheng:** Methodology, Investigation. **Bingyang Dai:** Methodology, Investigation. **Wenhan Huang:** Methodology, Investigation. **Shuhang Yung:** Methodology, Resources. **Peijie Hu:** Methodology. **Yechun Ruan:** Methodology. **Qingyun Xue:** Methodology. **Kiwai Ho:** Supervision, Writing - review & editing. **Ling Qin:** Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2020.10.016.

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