Intra-articular magnesium sulfate (MgSO₄) reduces experimental osteoarthritis and nociception: association with attenuation of N-methyl-D-aspartate (NMDA) receptor subunit 1 phosphorylation and apoptosis in rat chondrocytes


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Summary

Objective: To study the effects of intra-articular injection of magnesium sulfate (MgSO₄) on the development of osteoarthritis (OA) and to examine concomitant changes in the nociceptive behavior of rats.

Methods: OA was induced in Wistar rats with intra-articular injection of collagenase (500 U) in the right knee; the left knee was left untreated. In the OA + MgSO₄ group (n = 7), the treated knee was injected with 500-mg 0.1-ml MgSO₄ twice a week for 5 consecutive weeks starting at 1 week after collagenase injection; in the OA group (n = 7), the same knee was injected with the same amount of physiological normal saline. In the MgSO₄ group (n = 6), naive rats received only MgSO₄ injections; in the control group (n = 6), naive rats received only physiological normal saline injections. Nociceptive behavior (mechanical allodynia and thermal hyperalgesia) on OA development was measured before and at 1, 2, 4, 6, and 8 weeks after collagenase injection, following which the animals were sacrificed. Gross morphology and histopathology were examined in the femoral condyles, tibial plateau, and synovia. Immunohistochemical analysis was performed to examine the effect of MgSO₄ on N-methyl-D-aspartate (NMDA) receptor subunit 1 phosphorylation (p-NR1) and apoptosis in the articular cartilage chondrocytes.

Results: OA rats receiving intra-articular MgSO₄ injections showed a significantly lower degree of cartilage degeneration than the rats receiving saline injections. MgSO₄ treatment also suppressed synovitis. Mechanical allodynia and thermal hyperalgesia showed significant improvement in the OA + MgSO₄ group as compared to the OA group. Moreover, MgSO₄ attenuated p-NR1 and chondrocyte apoptosis in OA-affected cartilage.

Conclusions: Our results indicate that local intra-articular administration of MgSO₄ following collagenase injection in an experimental rat OA model (1) modulates chondrocyte metabolism through inhibition of cell NMDA receptor phosphorylation and apoptosis, (2) attenuates the development of OA, and (3) concomitantly reduces nociception.

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Key words: Magnesium sulfate, Osteoarthritis, Nociception, NMDA, Chondrocyte apoptosis.

Introduction

Osteoarthritis (OA), the most common cause of pain and disability in the elderly, is considered to be caused by long-term mechanical disturbance the aging process. Recent studies have revealed the role of inflammation in the pathogenesis of OA, and potential mediators have been described. Currently available pharmacological therapies for OA mainly target palliation of pain and include analgesics, intra-articular therapy, and topical treatment. Pain inhibitors targeting both joint pain and secondary hyperalgesia and allodynia may therefore represent the optimal approach for reducing OA-induced pain. Magnesium, the fourth-common cation in the body, has numerous physiological activities, including activation of many enzymes involved in energy metabolism and protein synthesis. Magnesium sulfate (MgSO₄) is used as a pharmacological agent in a variety of clinical situations: tachyarrhythmia, myocardial and neuronal ischemia, asthma, spasmophilia, preeclampsia, tocolysis, and post-anesthesia shivering. Magnesium has been shown to exert...
a physiological block of the ion channel on the N-methyl-D-aspartate (NMDA) receptor, preventing extracellular calcium ions from entering the cell and contributing to secondary neuronal changes. The NMDA receptor subunit 1 (NR1) is considered an essential component of all functional NMDA receptors. Increased phosphorylation of NR1 (p-NR1), occurring via intracellular signaling pathways, has been recognized as a major mechanism contributing to the regulation of NMDA receptor function. Bondok and Eli-Hady showed that intra-articular magnesium reduces the postoperative analgesic requirement of patients after arthroscopic knee surgery. MgSO4 is an old and well-known therapeutic agent used in clinical practice. However, the role of MgSO4 in the development of OA and OA-induced nociceptive behavior has not been well elucidated. In the present study, we investigated the effects of intra-articular MgSO4 injection on cartilage degeneration and nociception in a rat model of collagenase-induced OA. Immunohistochemical examination was also performed to determine the effect of MgSO4 on p-NR1 and chondrocyte apoptosis in the articular cartilage.

Methods

ANIMAL MODEL (COLLAGENASE INJECTION FOR OA INDUCTION)

The experimental protocol was approved by the Animal Care and Use Committee of National Sun Yat-Sen University and conformed to the National Institutes of Health guidelines for the care and use of animals in research. Two-month-old male Wistar rats (body weight 275–310 g) were used. OA was induced by intra-articular injection of collagenase (Cladosporium histolyticum type II, enzyme activity 333 U/mg; Sigma, St. Louis, MO) into the right knee joint. The enzyme was dissolved in saline and filtrated with a 0.22-μm membrane, and administered in a volume of 0.1-ml using a 27-gauge, 0.5-inch needle. After shaving and sterilizing, the right knee joint was injected intra-articularly with 0.1-ml of collagenase (500 U) or saline solution. Injection was performed twice on Days 1 and 4 according to the schedule. Na+ and interval scale. The withdrawal threshold was determined by Chaplan’s "up-down" method involving the use of alternate large and small fibers to determine the 50% withdrawal threshold. Each von Frey hair was applied to the plantar surface of the paw for 5 s. Briefly, when the rat lifted its paw in response to the pressure, the filament size was recorded, and a weaker filament was used subsequently. Conversely, in the absence of a response, a stronger stimulus was used. A sequence of such responses was thereby generated, and the 50% response threshold was calculated using a response variable spreadsheet. The von Frey filament was applied to each paw for five trials at approximately 3-min intervals.

THERMAL HYPERALGESIA

Thermal hyperalgesia was assessed by placing the hind paw on a radiant heat source; the paw-withdrawal latency at low-intensity heat was measured with an IITC analgesiometer (IITC Inc., Woodland Hills, CA) using a previously described method. The heat stimulus was applied until the animal withdrew, defined as lifting, licking, or flinching of the paw, was observed. The latency in foot withdrawal response to heat was recorded. A 30-s cutoff was used to prevent soft tissue damage in the absence of a response. For each rat, five stimuli were applied with a stimulus interval of 10 min.

INFLAMMATION, GROSS MORPHOLOGY, AND HISTOPATHOLOGICAL EXAMINATION OF THE KNEE JOINTS

The severity of knee joint inflammation was reflected by an increase in the hind-limb knee joint width. The width of the bilateral hind-limb knee joints was measured from the medial to the lateral aspect of the joint line by using calipers before (baseline) and 1, 2, 4, 6, and 8 weeks after the collagenase injection. The gross morphological changes in the cartilage of the femoral condylar and tibial plateau were examined according to previously described methods. A macroscopic total joint score was also obtained by adding the mean scores of the cartilage lesions from the medial and lateral femoral condyles together with those from the medial and lateral tibial plateaus. The joints were sectioned 0.5 cm above and below the joint line, fixed in 10% neutral buffered formalin for 3 days, and then decalcified for 2 weeks in buffered 12.5% ethylenediaminetetraacetic acid (EDTA) and formalin solution. The tissues were then sectioned mid-sagittally, washed under running tap water, and paraffin-embedded in an automatic processor (Autotechnicon Mono 2; Technion Co., Chauncey, NY). The cartilage was stained with hematoxylin–eosin (H & E) and Safranin-O/fast green stains to assess the general morphology and matrix proteoglycans. Microscopic examination of the articular cartilage of the medial and lateral femoral condyles and the tibial plateau were graded according to Mankin's grading system. A representative specimen of the synovial membrane from the medial and lateral compartments of the knee was dissected from the underlying tissues for histological examination, as previously described.

IMMUNOHISTOCHEMISTRY FOR p-NR1

Cartilage specimens were processed for immunohistochemical analysis as described in previous studies. Briefly, sections (2 μm) of the paraffin-embedded specimens were rehydrated, deparaffinized with xylene, and dehydrated in a graded series of ethanol, following which the endogenous peroxidase activity was quenched by 30-min incubation in 0.3% hydrogen peroxide. The antigen was retrieved by enzymatic digestion with proteinase K (20 μm; Sigma) in phosphate-buffered saline (PBS) for 20 min. The slides were incubated with the primary antibody against either rabbit anti-p-NR1 antibody (1:1000; Upstate, Lake Placid, NY) at 4 °C for 48 h in a humidified chamber. Thereafter, the sections were treated with the avidin–biotin complex (ABC) technique by using an ABC kit (Vectastain ABC kit; Vector Labs, Burlingame, CA). The images were viewed using a Leica DM-2500 microscope (Leica, Heiderberg, Germany) and captured using a SUT 17 CCD RT-silder camera (Diagnostic Instruments Inc., Sterling Heights, MI). For the statistical analysis of the immunohistochemical findings, the presence of different antigens in the cartilage was detected by a modification of the method of Pelletier et al. Prior to evaluation, it was ensured that each OA specimen had an intact cartilage surface that could be used and detected as a marker for the validation of morphometric analysis. The data obtained from the medial and lateral femoral condyles and medial and lateral tibial plateaus were considered together for the purpose of statistical analysis. Each slide was reviewed by two independent readers (Lee CH and Hsiue SP) who were blinded to the treatment groups.

CHONDROCYTE APOPTOSIS IN THE ARTICULAR CARTILAGE

Apoptotic chondrocytes were detected by terminal deoxynucleotidyl trans-ferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assays. Reaction, labeling, and detection of all samples were performed by using In Situ Cell Death Detection Kits, AP (Roche, Indianapolis, IN, USA) according to the manufacturer’s protocols. The number of positive TUNEL-positive chondrocytes was counted using a microscope. The number of TUNEL-positive chondrocytes in the articular cartilage was detected with the method proposed by Diaz-Gallego. After TUNEL staining, the sections were subjected to the following...
Results

On sacrifice, all collagenase-injected knees showed OA. No signs of drug toxicity such as ataxia, muscle weakness, and lethargy were noted in the rats treated with MgSO4.

NOCITIVE BEHAVIORS IN OA (MECHANICAL ALLODYNYA AND THERMAL HYPERALGESIA)

The force required for hind-paw withdrawal in the OA + MgSO4 group was significantly increased compared with the OA group (P < 0.05) at 6 and 8 weeks (7.5 ± 0.6 vs 4.4 ± 0.5 g and 6.2 ± 0.3 vs 4.1 ± 0.4 g, respectively) after collagenase-induced OA (Fig. 1). The OA + MgSO4 group showed increased von Frey thresholds at 6 and 8 weeks after collagenase injection compared with the OA group (Fig. 1). Eight weeks after the induction of OA, the mechanical threshold in the hind paw of the side contralateral to the side of intra-articular injection was 12.1 ± 1.7, 12.8 ± 0.7, 11.8 ± 0.7, and 12.2 ± 1.2 g in the OA, OA + MgSO4, MgSO4, and control groups, respectively. Before intra-articular collagenase injection (baseline values), the mechanical threshold in the hind paw on the side contralateral to the side of intra-articular injection was 12.3 ± 0.9, 12.4 ± 0.5, 12.6 ± 0.4, and 12.4 ± 0.8 g for the OA, OA + MgSO4, MgSO4, and control groups, respectively. No significant difference was detected in the mechanical allodynia in the contralateral limbs among the four experimental groups. In the thermal hyperalgesia test, the paw-withdrawal latency in the right hind paw was reduced at 1, 2, 4, 6, and 8 weeks after collagenase injection as compared to the control groups (Fig. 2). The OA + MgSO4

<table>
<thead>
<tr>
<th>Group</th>
<th>Macroscopic score</th>
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<tr>
<td>OA (n = 7)</td>
<td>2.68 ± 0.08*</td>
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<tr>
<td>OA + MgSO4 (n = 7)</td>
<td>1.45 ± 0.23*†</td>
</tr>
<tr>
<td>Control (n = 6)</td>
<td>0.18 ± 0.03</td>
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</table>

Data are expressed as the mean ± S.E.M. For the macroscopic score, refer to Methods. OA: collagenase-induced OA knee treated with normal saline; OA + MgSO4: collagenase-induced OA knee treated with MgSO4; control: saline injection in naïve rat knee. *P < 0.05 vs the control group. †P < 0.05 vs the OA group.
group showed increased paw-withdrawal latency as compared to the OA group at 4, 6, and 8 weeks after collagenase injection (22.1 ± 0.3 vs 17.2 ± 0.5 s, 23.5 ± 0.5 vs 18.3 ± 0.4 s, and 24.7 ± 0.1 vs 20.5 ± 0.6 s, respectively; Fig. 2). Eight weeks after the induction of OA, the hind-paw-withdrawal latency on the side contralateral to the side of intra-articular injection was 25.8 ± 0.9, 26.7 ± 1.8, 27.5 ± 1.6, and 27.1 ± 0.8 s in the OA, OA + MgSO₄, MgSO₄, and control groups, respectively. Before intra-articular collagenase injection (baseline values), the hind-paw-withdrawal latency on the side contralateral to the side of intra-articular injection was 26.1 ± 1.2, 26.4 ± 0.8, and 25.8 ± 1.0 s in the OA, OA + MgSO₄, and control groups, respectively.
The severity of cartilage damage was seen in the OA group. Erosion and ulcer formation, and osteophyte formation, were characteristics of cartilage degeneration, such as fibrillation, among the four experimental groups. There was no significant difference in mechanical allodynia and thermal hyperalgesia between the MgSO4 and control groups at any time point during the study (Figs. 1 and 2). P < 0.05 vs the control group. *P < 0.05 vs the control group.

**KNEE JOINT WIDTH AND GROSS MORPHOLOGIC CHANGES**

An increase in the width of the hind-limb knee joint was significant at 1, 2, 4, 6, and 8 weeks after the collagenase injection in the OA + MgSO4 and OA groups. As shown in Fig. 3, with a significant difference (P < 0.05) between the control group and both OA + MgSO4 and OA groups as well as between the OA + MgSO4 group and the OA group. The OA + MgSO4 group showed a greater decrease in joint inflammation than the OA group (Fig. 3). In the OA group, gross characteristics of cartilage degeneration, such as fibrillation, erosion and ulcer formation, and osteophyte formation, were found in the femoral condyle and tibial plateau. Markedly less severity of cartilage damage was seen in the OA + MgSO4 group. In the control and MgSO4 groups, the cartilage of the femoral condyle and tibial plateau was macroscopically normal, with a glistening, smooth surface, and no cartilage defects or osteophytes were observed. Table I lists the gross evaluation scores for each group. A significant difference (P < 0.05) in gross morphologic score was found between the OA group and both OA + MgSO4 and control groups (Table I), but not between the control group and the MgSO4 group (P = 0.49). The grade of cartilage damage in the OA + MgSO4 group was significantly lower than that in the OA group. Synovia from the OA group were hypertrophic and showed a reddish-yellow discoloration, whereas in the OA + MgSO4 group, they were thinner and the discoloration was less intense. Synovia from the control and MgSO4 groups had a white luster and transparent appearance, with no hyperemia or evidence of synovitis.

**MICROSCOPIC FINDINGS**

The cartilage of the control and MgSO4 groups had a normal histological appearance. A thin, glistening, smooth lamina filled with flattened chondrocytes was observed, and no loss of proteoglycan was seen in the matrix on Safranin-O staining [Fig. 4(A and D)]. Specimens from the OA group showed obvious histological changes, including complete disorganization, moderate-to-severe hypocellularity, proteoglycan reduction on Safranin-O fast green staining, and denudation of articular surface and fissures extending into the deep zones [Fig. 4(B)]. Osteophytes were present at the medial margins of the femoral condyle and tibial plateau [Fig. 4(E)]. In the OA + MgSO4 group, there was marked reduction in the severity of the femoral condyle and tibial plateau lesions: only fibrillation and fissures extending into the superficial layer of cartilage were observed [Fig. 4(C)]. The average scores obtained for the above findings based on the evaluation criteria are shown in Table II. Significant differences (P < 0.05) were found between the control and both OA and OA + MgSO4 groups (Table II). Mankin’s score for the OA + MgSO4 group was significantly lower than that for the OA group. Cartilage degeneration was more severe at the medial sides than at the lateral sides of the femoral condyle and tibial plateau in the OA group (Table II). In the present study, the gross and histopathological appearance of both the hip and ankle joints (n = 4) did not show any significant changes among the experimental groups at 8 weeks after collagenase injection (data not shown). Synovia from the OA group were thick, had focal villi, and showed hyperplasia of the lining cells and moderate infiltration of mononuclear inflammatory cells [Fig. 4(F)]. The histology of the synovia from the control and MgSO4 groups was within normal limits. The synovitis scores on microscopic evaluation are shown in Table II; significant differences (P < 0.05) were found between the control group and both OA and OA + MgSO4 groups, and between the OA + MgSO4 group and the OA group (Table II). The synovitis score was lower for the OA + MgSO4 group than the OA group, suggesting that synovial inflammation was less severe in the OA + MgSO4 group.

**IMMUNOHISTOCHEMISTRY OF p-NR1 IN ARTICULAR CARTILAGE**

The immunolocalization of p-NR1 protein expression in cartilage specimens from the control, OA, OA + MgSO4, and MgSO4 groups, and the negative control were examined [Fig. 5(A–E)]. Little or no p-NR1 protein was observed in chondrocytes of specimens from the control and MgSO4 groups [Fig. 5(A and D)]. The p-NR1 protein was seen in chondrocytes of the superficial and transitional cartilaginous zones of the OA group [Fig. 5(B) inset, arrow]. Compared with the OA group, OA + MgSO4 specimens showed a noticeable decrease in the number of p-NR1-positive chondrocytes [Fig. 5(C)]. From the four individual observations, no staining was observed in the negative control [Fig. 5(E) inset]. Quantitative analysis showed that MgSO4 significantly reduced the collagenase-induced increase in the number of p-NR1-positive chondrocytes in knee cartilage [Fig. 5(F)].

**INHIBITION OF CHONDROCYTE APOPTOSIS BY MgSO4**

In general, in the cartilage of the control group, only a very small number of cells stained positive on TUNEL staining [Fig. 6(A inset and E)]. Compared with the control group, the OA group showed a noticeable and statistically significant increase in the number of TUNEL-positive chondrocytes [Fig. 6(B inset and E)]. Notably, the collagenase-induced increase in the number of TUNEL-positive chondrocytes in articular cartilage was statistically reduced...
by the MgSO₄ injection [seen in the OA + MgSO₄ group; Fig. 6(C inset and E)]. The MgSO₄ group did not have a significantly changed number of TUNEL-positive cells, compared with the control group [Fig. 6(D and E)].

**Discussion**

To our knowledge, this is the first study to demonstrate that intra-articular MgSO₄ injection can attenuate the development of OA and associated nociceptive behavior (mechanical allodynia and thermal hyperalgesia) in an experimental rat OA model. More interestingly, MgSO₄ inhibited p-NR1 and chondrocyte apoptosis in the articular cartilage in the experimental OA model.

A collagenase injection into the knee joint is thought to not only directly destroy the cartilage but also cause an inflammatory reaction in synovial tissues and accelerates cartilage degeneration. According to the study of van der Kraan et al. and Kikuchi et al., intra-articular administration of collagenase-induced OA of the knee in mice and rabbit models, respectively. In the present study, we demonstrated that intra-articular collagenase administration induced characteristic OA changes, including cartilage degradation, synovial inflammation, and osteophyte formation, in the knee joints of rats (Fig. 4). In the present study, both the macroscopic grading score and the Mankin score were significantly lower in the OA + MgSO₄ group than in the OA group (Fig. 4, Tables I and II). MgSO₄ injection significantly reduced the severity of cartilage degradation in
the OA knee. In OA, synovial inflammation also plays an important role in the disease process\(^2\). In the present study, moderate synovitis was noted in the collagenase-induced OA knee. The synovitis score was lower in the OA\(^+\)MgSO\(_4\) group than in the OA group (Table II). Some authors had found that joint width could be measured to determine the extent of tissue swelling as an index of inflammation\(^14,23\). In the early stage, the increase in the knee joint width is accompanied with joint damage. In the present study, the OA\(^+\)MgSO\(_4\) group showed a greater decrease in the knee joint width as compared to the OA group (Fig. 3).

Animal models of OA have been widely used to study the pathophysiology and progression of joint damage, but with little characterization of the associated pain. Mechanical allodynia and hyperalgesia, i.e., increased touch-evoked sensitivity to harmless and noxious stimuli, respectively, are common symptoms of inflammation\(^24\). While the pain associated with OA is primarily localized to the joint, it is becoming increasingly apparent that a number of patients exhibit increased nociception in adjacent or even remote body areas\(^25,26\). Bradley \textit{et al.}\(^26\) showed that patients with knee OA demonstrated increased sensitivity to pressure pain in the hip as compared to healthy age-matched controls. Fernhough \textit{et al.}\(^14\) reported that both iodoacetate injection and partial meniscectomy in the knee joint of the rats induced histological change, and mechanical allodynia and thermal hyperalgesia pain-related behaviors characteristic of human OA. In the present study, we measured mechanical allodynia

![Image of histological sections and TUNEL staining](https://example.com/image)

**Fig. 6.** Inhibition of chondrocyte apoptosis by MgSO\(_4\) in the articular cartilage of rat knee. (A) In general, in the cartilage of the control and MgSO\(_4\) groups, only a very small number of cells stained positive on TUNEL staining (inset and E). (B) In the OA group, a statistically significant increase was observed in the number of TUNEL-positive chondrocytes (inset and E). (C) In the OA\(^+\)MgSO\(_4\) group, the number of TUNEL-positive chondrocytes was significantly reduced (inset and E). (D) In the MgSO\(_4\) group, no significant change in the number of TUNEL-positive cells was observed when compared with the control group (inset and E). Scale bar = 100 \(\mu\)m. *\(P < 0.05\).
and thermal hyperalgesia as analgesic effects on intra-articular injection of MgSO4 in the OA knee. Mechanical allodynia and thermal hyperalgesia were significantly improved in the OA + MgSO4 group as compared to the OA group.

In the inflamed state of arthritic knees, an increase in glutamate concentration is observed not only in axons in the inflamed region, but also in the synovial fluid. Intra-articular injection of glutamate into the knee joint results in thermal hyperalgesia and mechanical allodynia, which are attenuated by local injection of NMDA or non-NMDA receptor antagonists. Dietary restriction of magnesium intake lowers the mechanical nociceptive thresholds in rats, which is reversed by the NMDA receptor antagonist, MK-801.

MgSO4 treatment in an OA rat model. We proposed that a functional NR1 in chondrocytes can be attenuated by p-NR1 in OA rat knees. This is the first presentation that p-NR1, suggesting that spinal IL-1 blockade depends on Mg2+, may pave the way for further investigations on MgSO4 as a potentially therapeutic agent in the treatment of the inflammatory component of OA. Further research is needed not only to better define the effect of MgSO4 on OA but also to clarify the roles of the NMDA antagonist in OA treatment.

Conflict of interest
The authors acknowledge that there are no conflicts of interest pertaining to this manuscript.

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References