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Short Communication

Impact of walking exercise intensity on cartilage IL-1, TNF-α, IL-4, MMP-13 and pain threshold in osteoarthritis rat models

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Abstract

Pro-inflammatory cytokines produced by chondrocytes play a crucial role in activating matrix metalloproteinase-13 (MMP-13), leading to an imbalance between the synthesis and degradation of the extracellular matrix (ECM) in osteoarthritis (OA). Although regular walking exercise has been shown to reduce inflammatory cytokine levels in OA animal models, the optimal exercise intensity remains underexplored. Therefore, the aim of this study was to investigate the effects of different intensities of regular walking exercise on the levels of pro-inflammatory cytokines (interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α)), anti-inflammatory cytokine (interleukin-4 (IL-4)), as well as MMP-13 expression in cartilage and pain thresholds in an OA animal model. A total of 30 adult male *Rattus norvegicus* (6–8 weeks old) were divided into five groups: (1) healthy control; (2) monosodium iodoacetate (MIA)-induce OA model; (3) OA with light-intensity walking (OA1); (4) OA with moderate-intensity walking (OA2); (5) and OA with high-intensity walking (OA3). The exercise intervention began one week after MIA injection and continued for six weeks. Pain threshold, inflammatory cytokine (IL-1, TNF-α, IL-4) levels, and MMP-13 expression were measured using an analgesymeter, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry (IHC), respectively. The results demonstrated a significant reduction in IL-1 and TNF-α levels, along with decreased MMP-13 expression and increased IL-4 levels, in all exercise groups (OA1, OA2, OA3) compared to the untreated OA group. Additionally, pain thresholds improved following exercise. However, no significant differences were observed among the three exercise intensities in terms of cytokine levels, MMP-13 expression, or pain threshold. This study highlights that the light-intensity regular walking exercise effectively reduces inflammation, MMP-13 expression, and pain in OA. Further research is needed to elucidate the underlying mechanisms of exercise in OA management.

Keywords: Osteoarthritis, cytokines, MMP-13, pain, walking exercise

Introduction

Osteoarthritis (OA) is a prevalent chronic degenerative joint disease worldwide, with its incidence increasing with age [1]. This condition is characterized by chronic pain and progressive joint impairment due to cartilage damage, contributing to increased disability. The pain, often

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triggered by daily activities, leads to mobility limitations and a reduced quality of life among patients [2]. According to the World Health Organization (WHO) database in 2019, approximately 9.6% of men and 18% of women over the age of 60 reported OA symptoms [3]. Among OA patients, 80% experience movement limitations, about 25% are unable to perform activities of daily living, and an estimated one-third suffer from disability [4]. Data from the Basic Health Research of the Indonesian Ministry of Health indicate that the incidence of knee OA is 240 per 100,000 population, with prevalence increasing with age [5]. The distribution of OA is 5% among individuals aged <40 years, 30% among those aged 40–60 years, and 65% among those aged >61 years [5].

The pathological mechanisms underlying joint damage and pain in knee OA have been widely reported [6]. OA is characterized by progressive cartilage degeneration, osteophyte formation, synovial inflammation (synovitis), and extracellular matrix (ECM) degradation. Synovitis in OA is associated with the phagocytosis of cartilage debris resulting from ECM degradation, which contributes to joint pain [7]. Severe synovitis correlates with increased pain severity, as demonstrated by magnetic resonance imaging (MRI) [8]. Consequently, synovitis is a key target for pain management in knee OA. Histological analysis of joint tissue in OA reveals hyperplasia caused by an imbalance between ECM synthesis and degradation and this imbalance triggers pathological catabolic metabolism, leading to tissue remodeling and subsequent damage [9].

ECM degradation occurs due to the increased activity of matrix metalloproteinase (MMP) enzymes [10]. Disruption of the ECM is driven by impaired chondrocyte function, which can be triggered by aging, trauma, metabolic diseases, and other factors [11]. MMPs are a group of zinc-dependent endopeptidases that modulate the ECM by cleaving internal peptides, thereby degrading specific components such as proteoglycans, collagen, fibronectin, and laminin [12]. Collagenase (MMP-13) plays a key role in ECM degradation and is stimulated by pro-inflammatory cytokines produced by chondrocytes synthesis. MMP-13 specifically degrades type II collagen, further contributing to joint damage. Both proteolytic enzymes and pro-inflammatory cytokines produced by synoviocytes mediate the progression of OA and its associated pain [13].

The general management of OA includes pharmacotherapy with analgesic drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) [14]. However, long-term use of NSAIDs has been reported to cause significant side effects, including gastric ulcers and impaired renal function, particularly in the elderly [15]. Moreover, pain reduction achieved through OA pharmacotherapy is not accompanied by ECM repair [16]. Therefore, alternative therapies are needed to mitigate the long-term side effects of NSAIDs [17]. A study suggested that regular walking exercise before the onset of OA could reduce joint pain by increasing the expression of anti-inflammatory cytokine mRNAs, such as IL-4 and IL-10, in cartilage and synovial fluid while simultaneously suppressing IL-13 expression [18]. Therefore, exercise has been proposed as a potential approach to preventing musculoskeletal pain [19]. However, the effects of regular walking exercise vary among OA patients [20]. There are limited studies investigating the optimal intensity of regular walking exercise for reducing pain symptoms and modulating cytokine expression in OA. Therefore, the aim of this study was to analyze the effects of regular walking exercise at varying intensities (light, medium, and high) on levels of IL-1, TNF-α and IL-4 was well as MMP-13 expression in cartilage and pain threshold in OA animal models.

Methods

Study design and setting

An animal experimental study was conducted to determine the effects of walking exercise intensity on the levels of IL-1, TNF-α, and IL-4, and MMP-13 and pain threshold in OA animal models. Thirty healthy adult male *Rattus norvegicus* (Wistar strain), aged 6–8 weeks and weighing 200–250 grams, were housed under standard laboratory conditions. The rats were kept in pairs in standard cages under a 12:12-hour dark/light cycle at an ambient temperature of 25°C. Food and water were provided ad libitum. The rats were divided into five groups (i.e., six animal each group): (1) control; (2) OA group; (3) OA with light-intensity regular walking (OA1); (4) OA with medium-intensity regular walking (OA2); and (5) OA with high-intensity regular walking

(OA3). Radiographic imaging to confirmed OA were conducted at 1st, 2nd and 3rd week. Animals were subsequently tested for pain threshold before exercise and after six weeks of exercise. Biomarker analysis from knee joint tissue was conducted only at the six weeks after the exercise Enzyme-linked immunosorbent assay (ELISA) was used to measure IL-1, TNF-α, and IL-4 levels, while immunohistochemistry was performed to assess MMP-13 expression.

Osteoarthritis (OA) induction

Femorotibial joint OA was induced using monosodium iodoacetate (MIA) (Sigma, St. Louis, MO, USA). A solution of 2 mg of MIA in 25 µL was injected into the right femorotibial joint, and the progression of OA was assessed on days 7, 14, and 21 [19]. Control group rats received an intra-articular injection of 25 µL sterile saline. The successful establishment of the OA model was confirmed through radiographic examination (X-ray) on day 7. The severity of OA was classified using the Kellgren and Lawrence grading scale as grade 0, grade I, grade II, grade III, and grade IV [10].

Radiographic imaging

The Agfa 24/30 cassette (Agfa, Mortsel, Belgium) was divided into six equal zones and placed on the table of a Shimadzu conventional X-ray device (RF Co., Kyoto, Japan). After disinfection, the rat's knee was secured with tape, and the X-ray beam was directed vertically toward the cassette. The lens was focused on the rats' knee with an appropriate focal length and the exposure time was set at 5 min to ensure clear images. Specific parameters, including constant kilovoltage (kV) and weight were maintained throughout the procedure. Radiographic images of the joint were classified according to the grading system as previously described [10,19].

Regular walking exercise treatment using a treadmill

After successful confirmation of OA in the 1st–3rd weeks, the rats were adapted for three days. Each treatment group (OA1, OA2 and OA3) was then subjected to a different intensity of regular walking exercise. The walking protocol was as follows: light intensity (10 meters/minute), medium intensity (15 meters/minute), and high intensity (20 meters/minute) on an animal treadmill (Columbus Instruments Exer-3R treadmill, Columbus, OH, USA). This treatment was administered once per day in the morning, five days per week, for a total of six weeks as recommended previously [21]. The control group received no any exercise and were kept sedentary. Rats were evaluated after completing the treadmill exercise program.

Pressure pain threshold measurement

The pressure pain threshold (PPT) was assessed one week after MIA injection (baseline) and in the final week after treatment. An analgesymeter device (Ugo Basile, Varese, Italy) was used to determine the pain threshold of the right femorotibial joint. Briefly, a constant increasing pressure (48 g/s) was applied through a blunt transducer probe to the lateral side of the joint. The pressure was gradually increased until the pain threshold was reached, as indicated by the withdrawal flexion reflex and vocalization [19].

Tissue collection and preparation

After the last training session in the 6th weeks, the animals were anesthetized using ketamine 75 mg/kg and xylazine 10 mg/kg. The rats in a deep state of anesthesia were then killed by guillotine decapitation dissected for right femorotibial joint tissue collection. The joint tissue was divided into two portions, with one half used for ELISA and the other for immunohistochemistry. For ELISA, approximately 100 mg of joint tissue was mechanically homogenized and extracted using PRO-PREPTM Protein Extraction Solution for Tissues (iNtRON Biotechnology, Gyeonggi-do, South Korea). The homogenate was then centrifuged at low speed (3000 rpm for 10–15 minutes) to obtain the protein supernatant [22].

Enzyme-linked immunosorbent assay

The levels of IL-1, TNF-α, and IL-4 in cartilage (protein supernatant) were determined using Rat Interleukin 1 (IL-1) ELISA Kit (cat. E0107Ra), Rat Tumor Necrosis Factor Alpha (TNF-α) ELISA Kit (cat. E0764Ra) and Rat Interleukin 4 (IL-4) ELISA Kit (E0133Ra), respectively (all from BT-

LAB, Sanghai, China). All procedures were conducted following the manufacturer's instructions. The optical density of was measured using a microplate ELISA reader (Diatek DR-200Bc, Wuxi Hiwell-Diatek Instriments, Jiangsu, China) with a 450±10 nm wavelength following the manufacturer's instructions.

Histopathological preparation and immunohistochemistry

The preparation of histopathological sections of cartilage was carried out according to the standard protocol [9]. Briefly, the joint tissue was first fixed in a 10% formaldehyde solution for 24 hours, followed by dehydration using a graded series of alcohol solutions from low to high concentrations. The tissue was then cleared in xylene to enhance transparency. After clearing, the tissue underwent impregnation, where it was placed in a tissue cassette and immersed in liquid paraffin. The tissue was then embedded in paraffin using a paraffin block molding tool and sectioned using a microtome to a thickness of 4–5 µm.

The expression of MMP-13 in tissue was determined using immunohistochemistry. After deparaffinization and rehydration of the tissue sections, immunostaining was performed using a two-step method following the kit manufacturer's instructions. The sections were incubated overnight at 4°C with a rabbit polyclonal anti-MMP-13 antibody (bs-10581R; Bioss Antibodies, MA, USA). The slides were then washed three times with PBS, followed by a 20-minute incubation at 37°C. After incubation, the sections were mounted with Entellan and covered with a cover glass. The preparations were allowed to dry before examination. Imaging was performed using a BX53 microscope (Olympus, Tokyo, Japan). Scoring for each preparation began at 10× magnification. The slide was then divided into four quadrants, and each quadrant was assessed at 40× magnification. The number of brown-stained chondrocytes (indicating MMP-13 expression) was counted in each quadrant, and the average MMP-13 expression was calculated [22].

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 10 (Dotmatics, Boston, Massachusetts, USA). Differences in IL-1, TNF-α, and IL-4 levels, MMP-13 expression, and pain threshold among groups were analyzed using one-way ANOVA followed by Tukey's HSD post hoc test. A significance level of $p<0.05$ was considered statistically significant.

Results

Radiographic imaging

Our preliminary study showed that the radiographic images of the tibiofemoral knee joint demonstrated a time-dependent progression of MIA-induced joint pathology at weekly intervals. Joint pathology exhibited changes from weeks 1 to 3. Based on the Kallgren and Lawrence grading classification [10], the healthy control group (**Figure 1A**) had no osteophytes, narrowing of the joint gap, or sclerosis.

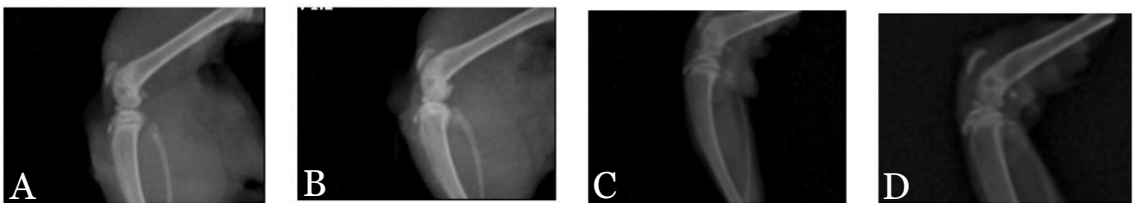

Figure 1. Radiographic representation of the femorotibial joint in monosodium iodoacetate (MIA)-induced osteoarthritis (OA) rats. The severity of OA increased over time: (A) healthy animal, showing grade 0 based on the Kellgren and Lawrence scale; (B) one-week post-MIA induction, showing grade 1 OA; (C) two weeks post-MIA induction, showing grade 2; and (D) three weeks post-MIA induction, showing grade 3.

The MIA injection group had grade 1 in the first week with mild signs of inflammation, joint space changes, or early bone and cartilage changes (**Figure 1B**). Grade 2 in the second week of OA development characterized by joint space narrowing, thickening of the subchondral bone, and

possible appearance of small osteophytes can be seen more clearly (**Figure 1C**). Grade 3 severe OA was observed in week 3 with more signs of advanced OA, with larger osteophytes, changes in the joint structure, and possible erosion of the cartilage (**Figure 1D**). Radiographic examination of the OA model animals showed significant progression between the 1st, 2nd and 3rd weeks, with more animals showing obvious OA symptoms over time. Referring to these results it was determined that the treatment (walking exercise) would be given on the week 1 (day 7) after MIA injection.

Effect of regular walking exercise on pain threshold

The pain threshold measured one week after MIA injection (baseline) was 13.2±1.2 g in the control group, 5.16±1.3 g in the OA group, 5.3±0.7 g in the OA1 group, 6.3±0.8 g in the OA2 group, and 5.6±0.6 g in the OA3 group. After six weeks, no significant difference in pain threshold was observed in the OA group (5.16±1.3 vs 5.4±0.7 g; p=0.626). However, light-intensity regular walking exercise significantly increased the pain threshold (5.3±0.7 vs 10.9±0.6 g; p=0.001), as did moderate-intensity (6.3±0.8 vs 11.6±1.5 g; p=0.007) and high-intensity exercise (5.6±0.6 vs 9.9±1.9 g; p=0.043). All treatment groups (light, moderate, and high intensity) showed a similar increase in pain threshold following the intervention (**Figure 2**).

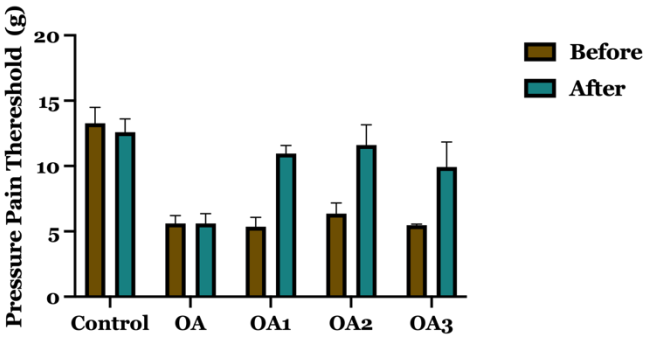

Figure 2. Measurement of pain threshold before and after treatment. The control group served as the negative control and was induced with saline, while the OA group was the positive control, induced with monosodium iodoacetate (MIA) to model osteoarthritis (OA). The OA1 group consisted of OA animals treated with light-intensity regular walking exercise (10 m/min), the OA2 group received moderate-intensity regular walking exercise (15 m/min), and the OA3 group underwent high-intensity regular walking exercise (20 m/min).

Effect of regular walking exercise on joint tissue cytokine levels

Our data indicated that the induction of OA increase the levels of IL-1- and six-week treatments reduced the IL-1 levels (**Figure 3A**). Light-intensity walking exercise (OA1) significantly reduced IL-1 levels compared to the OA group (12.01±0.87 pg/mL vs 9.84±0.25 pg/mL; p=0.007). Similarly, IL-1 levels were lower in the other treatment groups than in the OA group (10.41±0.33 pg/mL; p=0.007 for OA2, and 8.72±0.51 pg/mL; p<0.001 for OA3) (**Figure 3A**). Similar patterns also observed for TNF-α level. The TNF-α level in the OA group was 54.33±6.13 ng/mL and the reduced levels of TNF-α were observed in OA1 (32.35±2.37 ng/mL; p<0.001), OA2 (42.84±1.72 ng/mL; p<0.001), and OA3 (5.42±0.42 ng/mL; p<0.001) (**Figure 3B**). Regular walking exercise however increased the IL-4 levels in OA rats. The IL-4 level in the OA group was 8,824±1,089 ng/L, whereas significant increases were observed in OA1 (14,357±1,010 ng/L; p<0.001), OA2 (18,458±1,183 ng/L; p<0.001), and OA3 (15,416±0.416 ng/L; p<0.001) (**Figure 3C**).

Effect of regular walking exercise on joint tissue MMP-13 expression

The effect of regular walking exercise on joint tissue MMP-13 expression was measured after six weeks of treatment (**Figure 4**). Immunohistochemistry analysis demonstrated a reduction in MMP-13 expression following regular walking exercise treatment. OA rats exhibited MMP-13 expression of 18.07±5.97%. The MMP-13 expression was significantly reduced in OA1 (18.07±5.97% vs 8.80±3.93%; p<0.001), OA2 (18.07±5.97% vs 9.60±2.69%; p<0.001), and OA3 (18.07±5.97% vs 9.40±2.25%; p<0.001) (**Figure 4B**). A decrease in MMP-13 expression was

observed in the OA1, OA2, and OA3 groups compared to the OA group. However, no significant difference in MMP-3 expression was detected between the OA1, OA2, and OA3 groups (**Figure 4B**).

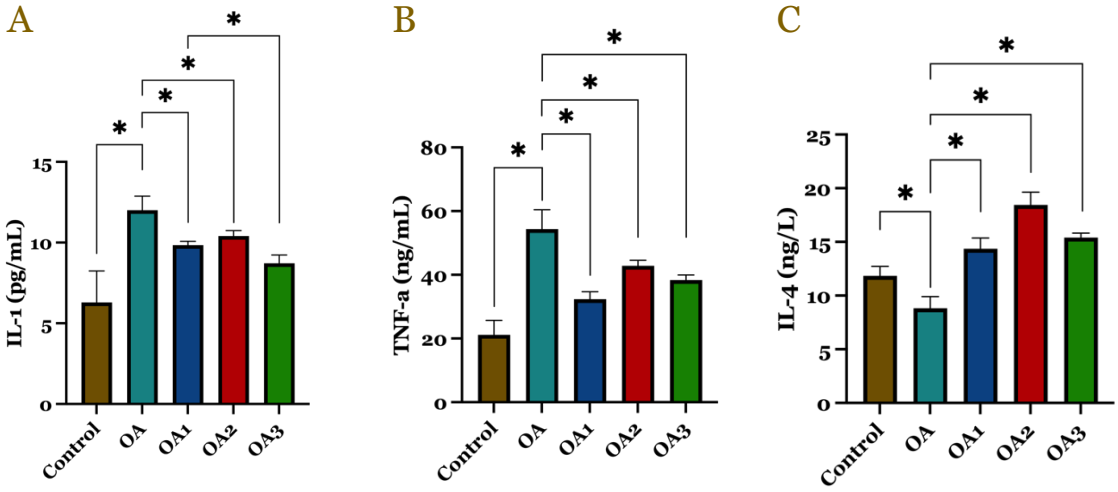

Figure 3. Comparisons of levels of (A) IL-1, (B) TNF-α, and (C) IL-4 post six weeks of regular walking exercise with different intensity in model osteoarthritis (OA) animal models. The control group served as the negative control and was induced with saline, while the OA group was the positive control, induced with monosodium iodoacetate (MIA) to model OA. The OA1 group consisted of OA animals treated with light-intensity regular walking exercise (10 m/min), the OA2 group received moderate-intensity regular walking exercise (15 m/min), and the OA3 group underwent high-intensity regular walking exercise (20 m/min). The data are presented as a mean, where the symbol (*) indicating a significant difference at $p<0.05$.

Discussion

This study found that the MIA-induced OA model led to the development of tissue osteophytes, joint gap narrowing, and sclerosis. All treatment groups exhibited significantly lower levels of pro-inflammatory cytokines and MMP-13 expression, as well as higher levels of anti-inflammatory cytokines, compared to the OA group. No significant differences were observed in cytokine levels, MMP-13 expression, or pain threshold among the three treatment groups, indicating that light-intensity regular walking exercise significantly increased pain threshold values by reducing inflammation.

These findings are consistent with a previous study, which reported that walking exercise at an intensity of 10 meters per minute reduced IL-4 and IL-10 levels [19]. Physical activity (walking at an intensity of 10 meters per minute) inhibited cartilage degradation by reducing OA-related biomarkers such as IL-1β, TNF-α, and MMP-13 while simultaneously increasing chondroprotective biomarkers, including IL-4, IL-10, and lubricin [23]. Similar findings were reported in a study comparing the effectiveness of treadmill exercise and swimming in OA animal models [24]. Treadmill exercise (10 meters per minute) was more effective in suppressing pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, and IL-6) and enhancing anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β compared to swimming [24]. Another study also demonstrated that exercise therapy reduced cytokine levels and cytokine-related gene expression in synovial fluid, thereby inhibiting cartilage degradation mediated by inflammatory factors in OA patients [25].

Physical activity is widely used as a non-pharmacological therapy for OA. Biomechanical stimuli and cell-cell interactions generate intracellular signals that can either trigger or suppress pro-inflammatory cytokines in chondrocytes during exercise [21]. As key pro-inflammatory cytokines, IL-1 and TNF-α contribute to the suppression of COL2A1 expression, which plays a crucial role in cartilage maintenance, strengthening, and regeneration [7]. In this study, all treadmill exercise frequencies reduced IL-1 and TNF-α expression, aligning with previous studies emphasizing the importance of modulating IL-1 to slow the progression of structural changes in OA [19].

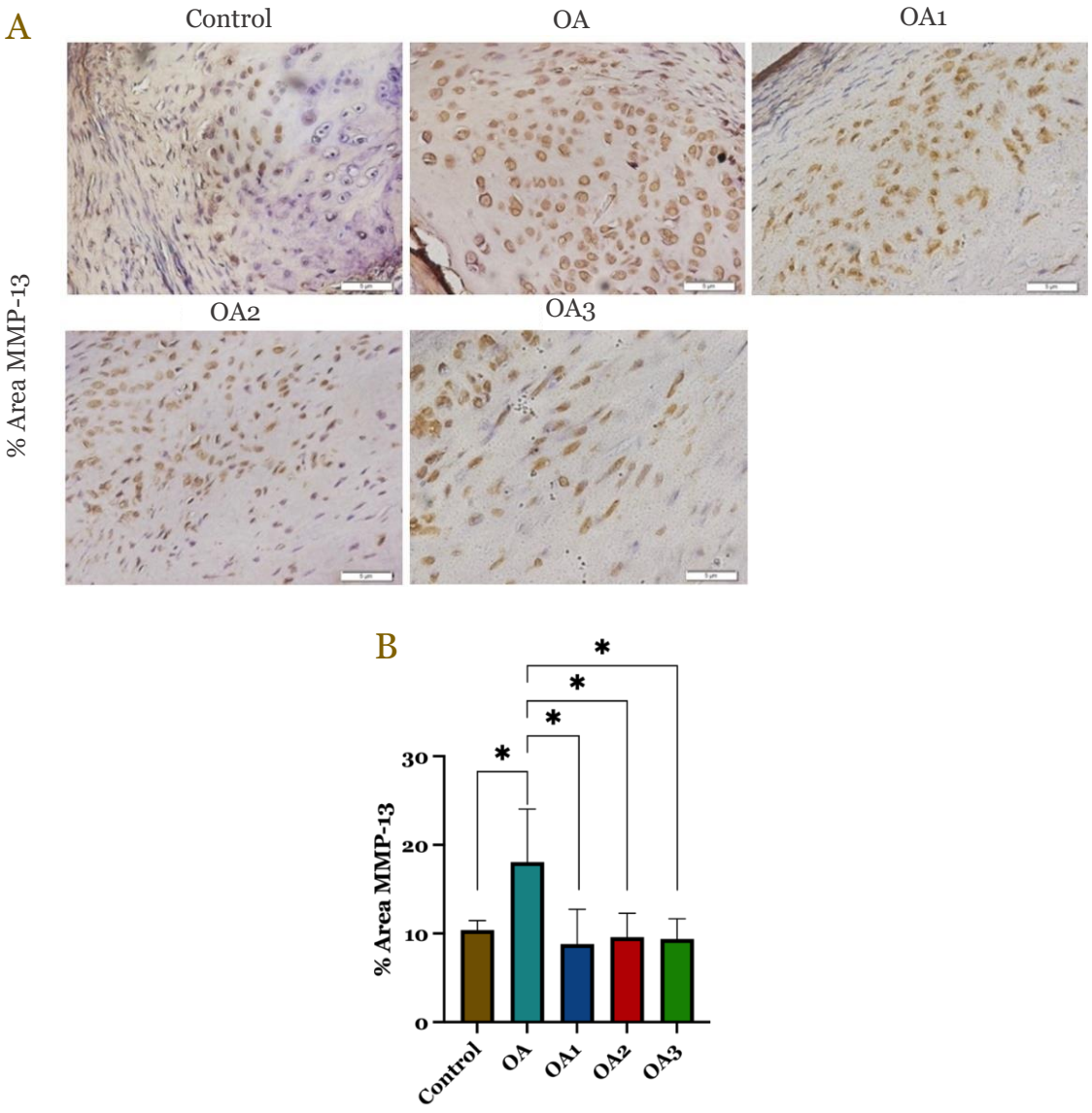

Figure 4. Effect of regular walking exercise on joint tissue MMP-13 expression. (A) Immunohistochemical examination of knee joint tissue (cartilage) showing the intensity of MMP-13 immunohistochemical staining of each group. The brown color indicates the percentage of cells that express MMP-13. (B) Different expression of MMP-13 among groups. The control group served as the negative control and was induced with saline, while the osteoarthritis (OA) group was the positive control, induced with monosodium iodoacetate (MIA) to model OA. The OA1 group consisted of OA animals treated with light-intensity regular walking exercise (10 m/min), the OA2 group received moderate-intensity regular walking exercise (15 m/min), and the OA3 group underwent high-intensity regular walking exercise (20 m/min). The data are presented as a mean, where the symbol (*) indicating a significant difference at $p<0.05$.

The progression of OA is characterized by catabolic changes in the joint, marked by increased collagenase and aggrecanase activity, which drive cartilage ECM degradation. Several biomarkers, including MMPs, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), cathepsins, and inflammatory mediators such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and prostaglandin E2 (PGE2), are induced by pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [14]. Among these, MMP-13 plays a pivotal role in OA pathogenesis by actively degrading type II collagen, the main structural component of cartilage [21].

Anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 regulate inflammatory processes through their effects on target cells [26]. An in vitro study demonstrated that IL-4 suppresses TNF-α and IL-1β synthesis via a mechanism similar to that of low-dose dexamethasone [7].

IL-10 inhibits IL-1 and TNF-α synthesis and has been proposed as a potential therapeutic target for OA [13]. Another study suggested that regular walking exercise increases IL-4 and IL-10 expression in the knee joint [19]. IL-4 induces the polarization of resident macrophages and M1 macrophages into M2 macrophages [7], a crucial response in resolving inflammation [19].

Most cytokines are produced by chondrocytes, synoviocytes, and macrophages. Rather than entering systemic circulation, cytokines secreted by chondrocytes remain in interstitial tissue fluid and synovial fluid [27]. A study indicates that cytokines contribute to pain symptoms through sequential mechanisms [18]. First, cytokines stimulate the production of classical pain mediators such as prostaglandins, which activate and/or sensitize nociceptive neurons. Second, cytokines act directly on nociceptive sensory neurons, many of which express receptors for TNF-α, IL-1β, and other cytokines [27]. A previous study found a positive correlation between TNF-α concentration in synovial fluid and pain intensity in knee OA patients (Kellgren-Lawrence grades 1–4) [16]. However, no significant correlation was observed between TNF-α concentration and radiographic grade, with TNF-α levels tending to be lower in advanced-stage OA than in early-stage OA [16].

This study has some limitations that need to be discussed. First, the use of an MIA-induced OA rat model may not fully replicate the complexity of human OA, as differences in biomechanics, immune responses, and disease progression could affect the translation of these findings to clinical applications. Second, the study duration was limited to six weeks, which may not be sufficient to determine the long-term effects of walking exercise on OA progression and cartilage degeneration. Third, although pain threshold and cytokine levels were assessed, direct structural evaluations using imaging techniques such as MRI or histological analysis were not performed to confirm cartilage integrity. Additionally, the study only examined three specific walking intensities, without exploring variations in duration, frequency, or alternative exercise modalities such as resistance training or aquatic therapy. Furthermore, while inflammatory and anti-inflammatory cytokines were measured, deeper molecular investigations, such as gene expression profiling or signaling pathway analyses, were not conducted to clarify the mechanisms underlying the observed effects.

Conclusion

This study demonstrated that regular walking exercise at varying intensities significantly increased the pain threshold and reduced pro-inflammatory cytokine levels in MIA-induced OA rats. All exercise groups showed a significant decrease in IL-1 and TNF-α levels, along with an increase in anti-inflammatory cytokines such as IL-4. Additionally, MMP-13 expression was significantly reduced in all treatment groups, indicating a protective effect against cartilage degradation. These findings suggest that regular walking exercise, even at light intensity, can effectively alleviate OA symptoms by modulating inflammatory responses. Nevertheless, further studies should explore the long-term effects of different intensities of walking exercise on OA progression, particularly in relation to cartilage regeneration and joint structural changes. Investigating the molecular mechanisms underlying the anti-inflammatory effects of exercise, including its impact on chondrocyte metabolism and macrophage polarization, would provide deeper insights into its therapeutic potential.

Ethics approval

All animal procedures were previously approved by Research Ethic Committee, Universitas Brawijaya, Malang, Indonesia (No. 061-KEP-UB-2024).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request. We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, ormanuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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