Journal of Sports Physiology and Athletic Conditioning (J.S.P.A.C) 2023; 3 (8): 1-10

Research Article

The effect of endurance training and mesenchymal stem cells on ALP gene expression and osteopontin levels in rats with knee osteoarthritis

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Received: 4 January 2023
Revised: 19 February 2023
Accepted: 10 March 2023

Abstract

Background: Osteoarthritis (arthritis of the joints) is one of the most common metabolic disorders of bone tissue that reduces the process of absorption and reabsorption in bone. Exercise and stem cell injections can have beneficial effects in treating this disease. The enzymes alkaline phosphatase and osteopontin, as markers of bone formation, play an important role in diagnosing the progression or treatment of this disease. The aim of this study was to examine the effect of training, stem cells and hyaluronic acid on osteocalcin, ALP and osteopontin in the cartilage tissue of rats with osteoarthritis.

Materials and Methods: In this study, 25 rats were divided in 5 groups including: (1) healthy control, (2) patient control, (3) endurance training (3 days a week for one month), (4) recipients of mesenchymal stem cells (1 × 106 cells / Kg), and (5) simultaneous recipients of endurance training and mesenchymal stem cells. Alkaline phosphatase gene expression was assessed by RT PCR and the amount of osteopontin synthesis was measured by immunohistochemistry procedure.

Results: Training and mesenchymal stem cell injection had a significant effect on increasing alkaline phosphatase gene expression and osteopontin in patient rats compared to the patient control group ($P < 0.001$). Also, simultaneous endurance training and stem cell injection have interactive effects on increasing both factors ($P < 0.001$).

Conclusion: Based on the findings of this study, it seems that endurance training and injection of mesenchymal stem cells in the joints, either separately or simultaneously, can increase the expression of alkaline phosphatase gene and the amount of osteopontin.

Keywords:
Osteoarthritis, Mesenchymal stem cells, Endurance training, Osteopontin, Alkaline phosphatase.

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1. Introduction

Osteoarthritis is a common non-inflammatory disorder of the musculoskeletal system that presents with degenerative changes in the synovial joints, along with ossification. Symptoms of this disease are more common in the knee joints (1). The prevalence of this disease in the urban population of Iran is 16.6% and in the rural population is about 20%. About one-thirds of people over the age of 65 in the world have osteoarthritis of the knee (2). The disease can be divided into two groups: 1- Primary or idiopathic osteoarthritis in which the person has no underlying disease 2- Secondary osteoarthritis, which is a primary predisposing factor and is locally or systemically involved in the development of the disease (3). Numerous risk factors for the development or exacerbation of this disease have been identified, the most important of which are obesity, knee injury, gender, old age, muscle weakness, bone characteristics, and poor job-related status (4). In osteoarthritis, existing stem cells are depleted or their storage runs low, and on the other hand, their proliferating capacity and differentiating ability decrease; therefore, systemic or topical administration of stem cells to these individuals can lead to the repair of joint tissues (5).

Alkaline phosphatase is the most widely used biomarker of bone metabolism, which is involved in all stages of bone mineralization and is known as a specific indicator of osteoblast activity. The presence of calcium ions is essential for the activity of this enzyme (6). As alkaline phosphatase levels increase, the transfer of extracellular fluid ions to non-mineral osteoid increases and new cells are formed (7).

Osteopontin, formerly called bone sialoprotein, is an acidic glycoprotein and is an important factor in bone resorption, osteoclast calcification, cell adhesion, body chemical regulation, inflammation regulation, reproduction, and fetal growth (8). Physical activity is an important determinant of bone mass. Exercise has both a direct and indirect osteogenic effect on skeletal tissue. Aerobic exercise has been shown to help increase levels of bone detection biomarkers, including alkaline phosphatase (9,10). Reports of the effect of exercise are somewhat contradictory (11). Mesenchymal cells are pluripotent cells that have the ability to produce connective tissue such as cartilage, bone, tendons, ligaments, and stromal cords during histogenesis. In recent years, the use of mesenchymal stem cells in the treatment of cartilage damage has increased (12).

With the increasing number of surgeries for the treatment of osteoarthritis of the knee and due to the surgical risks, surgery can not be performed in all cases of osteoarthritis. In addition, few patients with osteoarthritis are willing to have surgery. On the other hand, drug interactions and side effects of these drugs make patients less inclined to undergo surgery and use anti-inflammatory and analgesic drugs, and hence, alternative therapies can become very important. For this reason, the results of this research can be a guide for many researchers in this regard.

2. Materials and Methods

Statistical sample of the study

The sample of this study included thirty 6 to 8 week-old healthy male Wistar rats with an average weight of 230 to 250 grams that were purchased from the Pasteur Institute of Iran and kept in the laboratory of Pasargad Tissue and Gene Research Center in Tehran and were then randomly divided into 5 groups (N = 6).

The standard conditions in terms of room temperature, dark-light cycle and water and food were fully observed. After one week of adaptation to the new environment and familiarity with the training protocol, the animals were divided into the following groups: 1) healthy control, 2) patient control, 3) training, 4) stem cells injection, and 5) training + stem cells injection.

The training protocol

In the first stage (preliminary stage), the rats worked on the treadmill with zero percent slope, 3 days a week, for 10 minutes at a speed of 16 meters per minute, about 60 to 70 percent of Vo_{2max}. The second stage (main training protocol) included 30 minutes of running on the treadmill without slope and at a speed of 16 meters per minute, with regard to the principle of overload progressively, so that the duration of training was 50 minutes in the eighth week.

Also, five minutes at 8 meters per minute were devoted to warming and cooling the animals before and after training. During the training protocol, the control group only stayed on the treadmill off.

Induction of osteoarthritis: In the present study, direct intervention method was used to induce osteoarthritis in rats. The rats were first anesthetized with the anesthetics ketamine and xylazine.

After shaving the right knee, a horizontal incision was made in the inside of the knee. After removing the skin, the lateral internal ligament of the knee was removed to show the internal meniscus. Then, by creating an incomplete incision, a tear and damage was created in the meniscus. Finally, the desired area was sutured with a sterile method.

Preparation and injection of mesenchymal stem cells: In order to use mesenchymal stem cells in bone marrow of adult male rats, after killing the animal under anesthesia with ketamine and xylazine, bone marrow cells were collected from the femur and tibia. After culturing these cells in DMEM culture medium, one million cells per kilogram of body weight were prepared for each rat and injected intra-articularly into the right knee joint during the recovery period.

RT PCR method for the detection of ALP: RNA related to alkaline phosphatase gene was extracted from cartilage tissue cells using RNX-Plus kit (SinaClon; RN7713C). The quantity and quality of RNA extracted was determined by ND-1000 nanodrop spectrophotometer.

CDNA synthesis was performed according to the instructions in the fermentase kit (K1622). The reverse transcription reaction was performed using the enzyme RevertAid ™ M-MuLV Reverse transcriptas. Table 1 presents the sequence of primers used in this study.

Table 1: Sequence of primers used in RT PCR related to ALP

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	AAG TTC AAC GGC ACA GTC AAG G	CAT ACT CAG CAC CAG CAT CAC C
ALP	CTTTTGGACAGCAGGGTGGG	AAGGAGGGTTGGGTTGAGGGA

Evaluation of osteopontin synthesis by immunocytochemical method: First, 4,000 cells were inserted in each cell from the 12-cell plate, then the cells in the mentioned groups were treated for 21 days. After 21 days, the cells were fixed in paraformaldehyde and after washing with PBS, the cells were incubated with Triton for 10 minutes to increase permeability.

The cells were then incubated with bovine serum albumin and PBST buffer for 30 minutes. The polyclonal antibody osteopontin ab8448 from ABCAM company was placed on the cells. Hochst's staining was then performed on the cells and finally they were photographed with a fluorescence microscope.

To analyze the obtained data in this study, independent samples t-test and three-factor ANOVA statistical procedures were used.

3. Results

The results of independent samples t-test to compare osteopontin levels between the healthy control (58.72 ± 6.64) and patient (4.24 ± 0.96) groups showed that due to the induction of osteoarthritis, the levels of this protein was significantly reduced in the patient group (P <0.001).

Also, the results of ANOVA showed that training had a significant effect on osteopontin gene expression levels (F = 3187.326, P = 0.0001, μ = 0.990). Mesenchymal stem cells injection also had a significant effect on the increase of osteopontin (F = 1117.097, P = 0.0001, μ = 0.972). Besides, the interaction of training and mesenchymal stem cells injection (F = 494.224, P = 0.0001, μ = 0.939) had a significant effect on osteopontin levels.

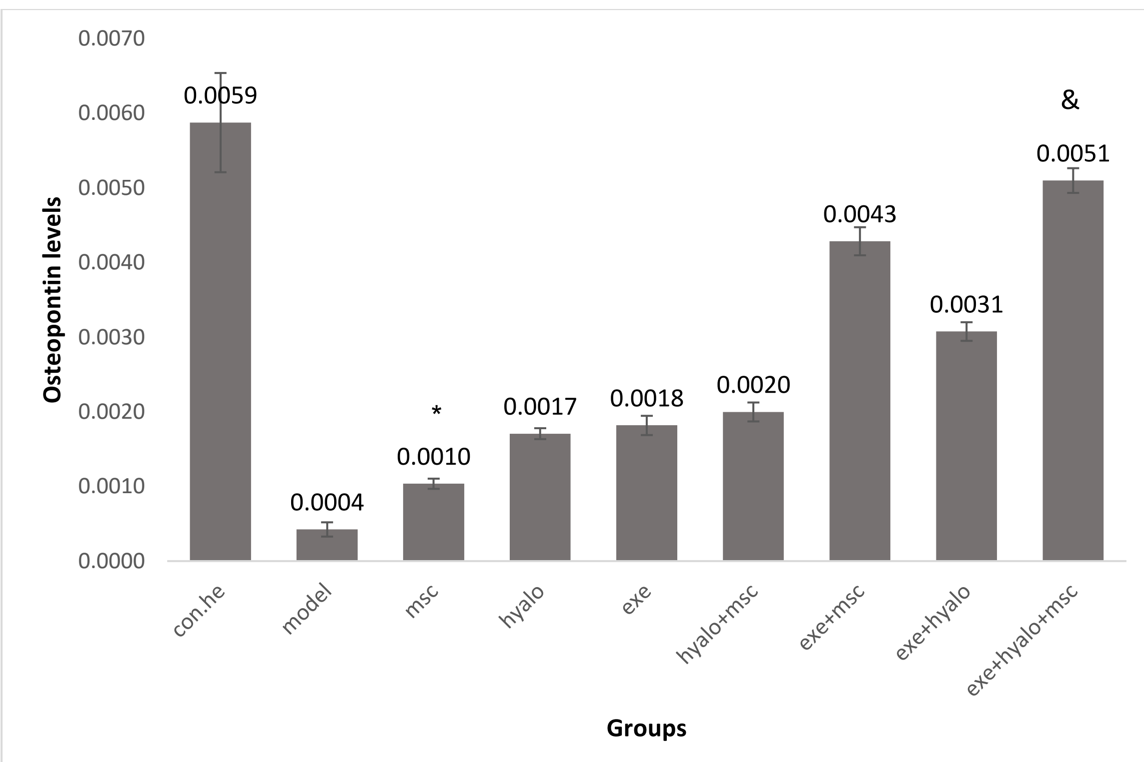

Figure 1: Osteopontin levels in the experimental groups. * Significant reduction of this protein can be seen in the patient group (model) compared to the healthy group (con.he). & Interaction of training and stem cells injection (exe + msc) had a synergistic and significant effect on osteopontin levels compared to other experimental groups.

The results of this test showed a significant difference in ALP gene expression levels between the osteoarthritis induction ($3.94 \times 10^{-4} \pm 0.8 \times 10^{-4}$) and healthy ($1.6 \times 10^{-2} \pm 0.63 \times 10^{-2}$) groups in male rats (P <0.001), so that due to the induction of osteoarthritis, the levels of this enzyme was significantly reduced in the patient group.

Based on the results of three-way analysis of variance, it was found that training had a significant effect on ALP gene expression levels (F = 97.745, P = 0.0001, μ = 0.849). Mesenchymal stem cells injection also had a significant effect on ALP gene expression levels (F = 21.697, P = 0.0001, μ = 0.753). Also, the interaction of training and stem cells injection (F = 20.976, P = 0.0001, μ = 0.001) had a significant effect on ALP gene expression levels.

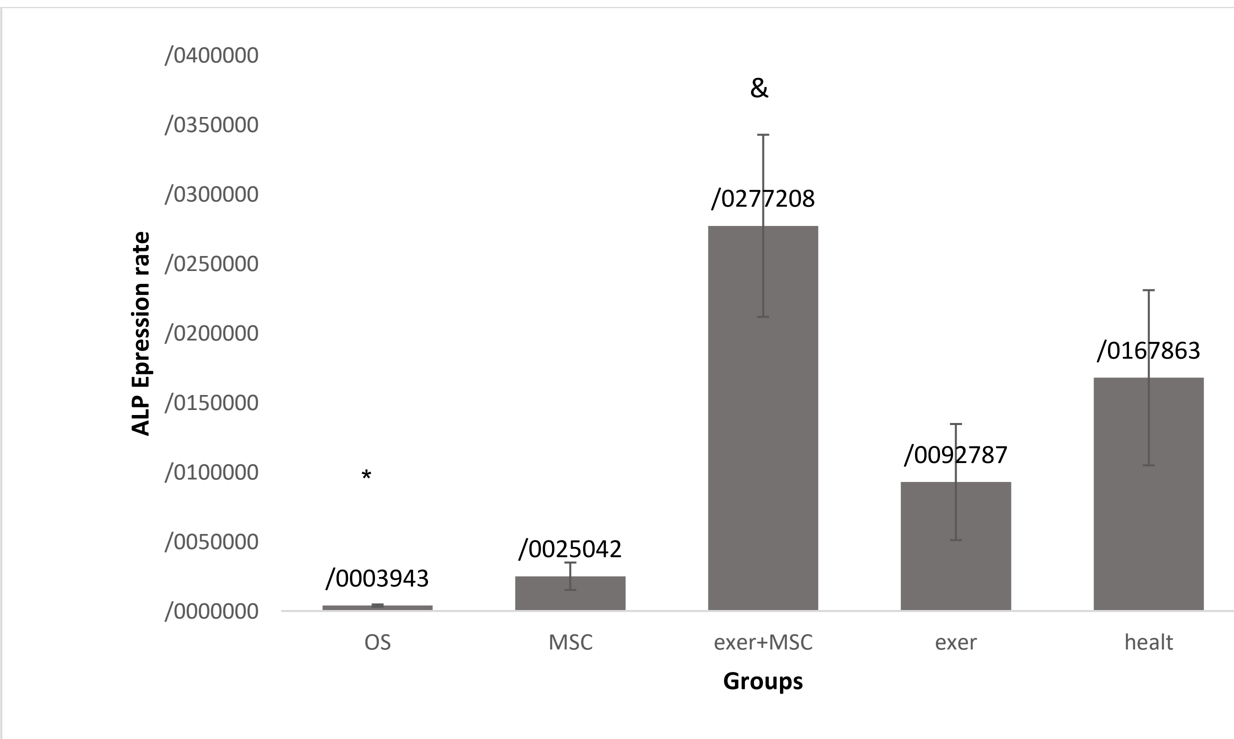

Figure 2: ALP gene expression levels in the experimental groups. * Significant reduction of this protein can be seen in the patient group (model) compared to the healthy group (con.he). & Interaction of training and stem cells injection (exe + msc) had a synergistic and significant effect on ALP gene expression levels compared to other experimental groups.

4. Discussion

The results of the present study showed that induction of osteoarthritis reduced ALP and osteopontin gene expression levels in bone tissue. Training and injection of mesenchymal stem cells alone had a significant effect on increasing ALP and osteopontin gene expression levels in rats with osteoarthritis. Still, interaction of training and stem cell injection had a significant synergistic effect on the dependent variables of this study. Exercise plays an important role in the HDAC3 / NF-KappaB signaling pathway in bone cells. According to research by Zhang et al. in 2019, exercise has a direct effect on inhibiting the transfer of the HDAC3 / NF-KappaB molecular complex into the nucleus, reducing the expression of inflammatory proteins such as MMP-13 and ADAMTS5. These proteins are a major cause of inflammatory disorders such as osteoarthritis (13).

Lubricin is produced as an anti-inflammatory agent in bone tissue cells, especially cartilage cells. Research by Blaney Davidson et al. in 2016 showed that exercise has a positive effect on gene expression of this protein. Lubricin binds to the TLR2 and TLR4 inflammatory receptors, blocking their function and ultimately reducing inflammatory processes. Increased lubricin levels can play an important role in the treatment of inflammatory diseases such as osteoarthritis. (14) It appears that inhibition of leukocyte-derived inflammatory factors, decreased inflammatory factors such as some interleukins (15), increased transcription of some bone growth and mineralization factors, and increased calcium deposition in bone tissue are some of the mechanisms of effect of exercise on bone tissue (16).

In a study conducted by Tung et al., the most important mechanisms of exercise training to increase osteoblast activity and increase calcium storage were known to be increased amounts of osteocalcin and osteopontin in the bone tissue matrix (17). The results of the present study were in line with some of the studies reported in this regard. Osteoblasts play a major role in the formation of bone tissue and originate from mesenchymal stem cells. The process of osteoblast genesis is controlled by several transcription factors. Macrophages play an important role in inducing osteoblast genesis through these transcription factors.Besides, by producing a variety of interleukins and macrophage colony-stimulating factors (M-CSF), macrophages play an important role in the process of osteoclast reabsorption and subsequent differentiation of osteoblast cells, resulting in the growth and differentiation of bone tissue (18).

In recent years, the use of mesenchymal stem cells has been considered as an anti-inflammatory factor in the treatment of osteoarthritis, because these cells are known to regulate the immune system. By acting on T-type immune cells, these stem cells transform them into regulatory type T cells; in other words, they modulate and regulate the expression of inflammatory factors in osteoarthritis (19). Research by Hanna et al. in 2018 showed that mesenchymal stem cells can increase the expression of CD105, CD90 and CD44 genes by increasing the expression and regulation of Ca^{2+} fluctuations; also, an increase in the amount of ascorbic acid can increase bone mass, the number of osteoblasts and finally, alkaline phosphatase activity in bone tissue (20).

The results of the present study on the effect of mesenchymal stem cells were completely in line with the related results reported on human and rat models.

Osteopontin produces proinflammatory cytokines and binds osteoclasts to the bone underlying substance. This protein is able to bind to CD44 and integrin receptors. On the other hand, CD44 and integrin are the main markers of mesenchymal cells.By binding to these receptors, osteopontin activates the TGF-β signaling pathway, thereby increasing the activity of proinflammatory cytokines such as IL-17, IL-6, and TNF-α. These proinflammatory cytokines destroy cartilage (21).

One of the most important findings of the present study was that the combination of endurance training with mesenchymal stem cell injection resulted in better effects on increasing bone metabolic markers. Given that endurance training has a direct effect on a wide range of signaling pathways and increasing osteopontin and alkaline phosphatase, and on the other hand, mesenchymal stem cells by reducing inflammation and increasing bone mass, play an important role in the treatment of osteoarthritis, applying these two independent variables simultaneously can have significant synergistic effects on the repair of osteoarthritis injuries in patients.

Conclusion

Based on the results obtained in this study, it was found that the use of endurance training and injection of mesenchymal stem cells, either separately or interactively, increases the levels of alkaline phosphatase and osteopontin in bone tissue and consequently repairs injuries in rats caused by osteoarthritis.

Acknowledgements

Hereby, from all the patients and people participating in the present research and their loved ones We are grateful to those who have helped us in this research.

Funding

This study did not have any funds.

Compliance with ethical standards

Conflict of interest None declared.

Ethical approval the research was conducted with regard to the ethical principles.

Informed consent Informed consent was obtained from all participants.

Author contributions

Conceptualization: M.H., M.A.A., M.P., S.A.H.; Methodology: M.H., M.A.A., M.P., S.A.H.; Software: M.H., M.A.A., M.P., S.A.H.; Validation: M.H., M.A.A., M.P.; Formal analysis: M.H., M.A.A., M.P., S.A.H.; Investigation: M.H., M.P., S.A.H.; Resources: M.H., M.A.A., M.P., S.A.H.; Data curation: M.P., S.A.H.; Writing - original draft: M.A.A., M.P., S.A.H.; Writing - review & editing: M.H., M.A.A., M.P., S.A.H.; Visualization: M.H., M.A.A., S.A.H.; Supervision: M.H., M.A.A., M.P., S.A.H.; Project administration: M.H., M.A.A., M.P., S.A.H.; Funding acquisition: M.P., S.A.H.

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