Physiology International 107 (2020) 2, 231–242
DOI: 10.1556/2060.2020.00024

AKADÉMIAI KIADÓ

Combined therapies with exercise, ozone and mesenchymal stem cells improve the expression of *HIF1* and *SOX9* in the cartilage tissue of rats with knee osteoarthritis

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Received: April 14, 2019 • Accepted: March 6, 2020
Published online: July 24, 2020

ABSTRACT

Purpose: Knee osteoarthritis (OA) is a common type of degenerative joint disease which decreases the quality of life. Sex-determining region Y box 9 (SOX9) and hypoxia-inducible factor-1 (HIF1) are considered as the key regulators of OA. We investigated the effect of combined therapies with mesenchymal stem cells (MSCs), ozone (O_3) and exercise training on *SOX9* and *HIF1* expression in the cartilage of rats with knee OA. *Methods:* Knee OA was induced by surgical method. OA rats were divided into model, MSCs, ozone, exercise, MSCs + ozone, MSCs + exercise, ozone + exercise and MSCs + ozone + exercise groups. Rats in the MSCs group received intraarticular injection of 1×10^6 cells/kg. Rats in the ozone group received O_3 at the concentration of 20 μg/mL, once weekly for 3 weeks. Rats in the exercise group were trained on rodent treadmill three times per week. 48 hours after the programs, cartilage tissues were isolated and the expression of *SOX9* and *HIF1* was determined using Real-Time PCR. *Results:* Significant differences were found in the expression of *SOX9* and *HIF1* between groups ($P < 0.0001$). Although combined therapies with exercise, MSCs and O_3 significantly increased the expression of *SOX9* and *HIF1* in the cartilage tissue of rats with knee OA, combination of exercise with O_3 was significantly more effective compared to the other combined therapies ($P < 0.001$). *Conclusions:* Combined therapy with exercise, MSCs and O_3 significantly increased the expression of *SOX9* and *HIF1* genes in the cartilage of rats with knee OA; however, exercise + O_3 was significantly more effective.

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KEYWORDS

knee osteoarthritis, O_3 therapy, MSCs therapy, exercise training, *SOX9*, *HIF1*

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by chronic pain, stiffness, and erosion in the cartilage, which subsequently decreases the quality of life in patients. Although it affects the joints in fingers, wrists, ankles, and hips, knee OA is the most common form of articular disease which affects 13% of women and 10% of men aged 60 years [1]. Recent reports demonstrated that more than 20 million people in the United States suffer from OA [2]. Genetics, obesity or overweight, life style, repetitive movements or joints injuries, inflammatory reactions and oxidative stress, as well as several metabolic disorders such as diabetes, cancer, hemochromatosis, and acromegaly are significant risk factors for increased risk of knee OA [1, 3]. Although the incidence of knee OA is high, the exact cellular and molecular mechanism of the disease development and progression is not well-understood. For this reason, unfortunately, there is no definitive medication for the prevention of knee OA, and current treatments only ameliorate signs and symptoms without a significant improvement.

Recent studies have demonstrated that various genes regulate the development and progression of OA [4–6]. Dysregulation of the genes involved can be associated with severe damages in the cartilage tissue and consequently articular diseases such as knee OA. Therefore, identification of genes and their expression patterns in patients are valuable and provide a precondition for the treatment and improvement of the disease and the quality of life in these patients. Sex-determining region Y box 9 (SOX9) is a cartilage-specific transcription factor, which plays an important role in the regulation of the expression of many extracellular matrix (ECM) proteins such as collagen type II [7]. Recent studies reported that decreased expression of SOX9 can be associated with severe cartilage damage and knee OA [8]; however, the exact mechanism of its action is unclear. In addition to SOX9, hypoxia-inducible factor-1 (HIF1) is another significant transcription factor which is highly expressed in articular chondrocytes [9]. It is a heterodimeric factor consisting of HIF-1β and HIF-1α subunits [9]. Previous studies demonstrated that mechanical stress and pro-inflammatory cytokines contribute to OA pathogenesis through the dysregulation of HIF1 [10]. These data indicate that HIF1 and SOX9 are critical factors in the development of OA and inflammatory reactions [11]. Therefore, these gene products might be targets in a possible therapy. Nevertheless, little is known about SOX9 and HIF1 expression or the effect of therapeutic methods on the expression of these genes at the early stage of OA. For this reason, in this research we evaluated *SOX9* and *HIF1* expression in the cartilage of rats with knee OA, followed by studying the effect of mesenchymal stem cells (MSCs), ozone (O_3) and exercise training on the expression of these genes.

Ozone therapy is a safe and effective method, which has frequently been used for the treatment of OA [12]. More recently, Feng and Beiping [13] revealed that O_3 therapy importantly reduces pain intensity and improves the quality of life in patients with knee OA. In addition, several lines of studies indicated that MSCs can safely improve cartilage damages and

knee OA due to their immunomodulatory and anti-inflammatory activities [14]. Although MSCs have strong immunosuppressive activity, their therapeutic advantage in OA primarily lies in stimulating the differentiation ability of chondrocytes, and hence building up new cartilage [15]. Interestingly, mild to moderate exercise training has been implicated for the treatment of knee OA by many researchers [16]. Clinical trials studies showed that regular and mild exercises can reduce pain severity and improve quality of life, walking disability, stair climbing, and sit-up speed in patients with knee OA [17]. Although these studies indicated the importance of exercise training, O_3 and MSCs therapies in the improvement of OA and quality of life, the exact mechanism of these methods is not clear. Given the critical role of *SOX9* and *HIF1* in OA, we assume that MSCs and O_3 therapy along with exercise training may be effective through the improvement of *SOX9* and *HIF1* expression. Therefore, we designed this study to compare the effect of MSCs and O_3 therapy along with exercise training on *SOX9* and *HIF1* expression and histological outcomes in rats with knee OA.

MATERIALS AND METHODS

Experimental animals

In this experimental study, 63 male Wistar rats (between 40 and 45 weeks of age with body weights of 250–300 g) were provided by the Laboratory Animal Research Center at the Islamic Azad University of Sari. Rats were housed in standard shoebox cages (42 × 26.5 × 15 cm), 3 per cage in a climate-controlled room (ambient temperature of 22 ± 2 °C, humidity 50 ± 5%, and a 12:12-h light/dark cycle). Rats were fed with standard diet and water. The study was approved by the Animal Care and Use Committee at Islamic Azad University, Sari branch.

Induction of knee OA in experimental rats

OA was induced through the surgical method according to a previously published report [18]. Before OA induction, rats were anesthetized with ketamine (30–50 mg/kg) and xylasine (3–5 mg/kg). To expose the knee joint, a 1-cm longitudinal incision was made and then the knee joint was shortly opened through lateral dislocation of the patella and the patellar ligament. A longitudinal cut was provided in the knee joint capsule through the medial parapatellar incision. An incomplete incision was made through the medial meniscotibial ligament without articular cartilage and other ligament injury. Finally, the knee joint capsule and then the skin were closed with a 6-0 absorbable suture and 6-0 silk suture, respectively.

Before starting the MSCs, exercise and O_3 therapies, these rats were fed with standard food and water for three weeks. Rats with knee OA were then randomly divided into 8 groups (7 rats in each group), including: patient or model (OA rats without any treatments), MSCs therapy, ozone therapy, exercise training, MSCs + O_3 therapy, MSCs + exercise therapy, exercise + O_3 therapy, and MSCs + O_3 + exercise groups. Rats in combined therapies were first treated with MSCs, then with O_3, and finally with exercise training. An additional group known as control group was also entered into the study. Rats in the control group were healthy and did not receive any treatments.

MSCs injection

Bone marrow-derived MSCs were purchased from the Histogenotech Company (Tehran, Iran). MSCs were extracted from healthy male Wistar rats (25–300 g). These MSCs were purchased when they reached >90% confluency at passages 3 or 4. MSCs were intraarticularly injected to the right knee at a concentration of 1×10^6 cells/kg. Rats in the MSCs group received only a single dose of injection.

Ozone therapy

Ozone was produced from medical-grade oxygen (O_2) by OZOMED 01 equipment. It was generated through a silent electric discharge, and its concentration was measured using a UV spectrophotometer at 254 nm. Ozone was injected into the knee through the tibiofemoral joint line at a concentration of 20 μg/mL, once weekly for 3 weeks, starting 3 weeks after the modeling.

Exercise program

Before exercise training, rats in this group were adapted to treadmill running for 5 days (once a week, with VO_2max 60–70%, speed of 16 m/min at 0% inclination for 10 min/day). The exercise program was started with a 30-min run on the treadmill without slope and a speed of 16 m/min in the first week, which was progressively increased to 30 minutes by the third week. Warm-up and cool-down times were done for 5 m/min at the beginning and the end of the exercise period.

Samples collection and gene expression analysis

Forty-eight hours after the end of programs, all rats were anesthetized with ketamine (30–50 mg/kg) and xylasine (3–5 mg/kg). Cartilage tissues were isolated and a piece of tissue was used for histological examination. Tissues were fixed in 10% formalin for 48 h and fragments were then dehydrated in a graded series of ethanol, embedded in paraffin and sectioned using an automatic microtome (ROTOCUT200, SCILAB Company, England) at 5 mm thickness. The sectioned tissues were stained with haematoxylin-eosin (H & E) and their morphological and histological parameters were examined by light microscopy.

lFor gene expression analysis, a fragment of cartilage tissue (∼50 mg) was homogenized in phosphate buffered saline (0.01 M; pH 7.0) at 4 °C with a homogenizer (Hielscher, UP100H). Total RNAs were then extracted using the RNX-Plus (SinaClon; RN7713C) Kit. The quantity and quality of the extracted RNAs were characterized using a Nanodrop ND-1000 spectrophotometer (Thermo Sci., Newington, NH) method. cDNA was synthesized from RNA samples using Revert Aid Reverse Transcriptase (Thermo science, Germany) at 42 °C for 1 h with random hexamer primers (Thermo science, Germany). A Rotor Gene 6,000 (Corbett Research, Australia) thermocycler was applied for amplifications. Each reaction included 5 μl master mix and 100 nm primers. Primer sequences were synthesized as follows: *SOX9*, 5′-AGGAAGTCGGTGAAGAATGGG-3′ (forward), 5′-GGTTTTGGGAGTGGTGGGT-3′ (reverse); *HIF1*, 5′-GTTGTTGTTGTTGTCTGTGGG-3′ (forward), 5′-AGTGAAAATGAAG-GAGGAAGGG-3′ (reverse), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-AAGTTCAACGGCACAGTCAAGG-3′ (forward); 5′-CATACTCAGCACCAGCATCACC-3′ (reverse), as a reference gene. The mRNA levels of *HIF1* and *SOX9* were normalized relative to the amount of *GAPDH* mRNA. Delta Ct (ΔCT) was calculated using the following formula

according to the method described by Livak and Schmittgen [19]: [ΔCT = CT (target) – CT]. The expression profile of each gene was determined in triplicates. We determined the mean of Ct of each gene and then calculated delta-Ct and, on that basis, fold change by calculating $2^{-\Delta Ct}$ [19].

Statistical analysis

The mean expression of *HIF1* and *SOX9* between all groups was compared using the One-Way ANOVA: Post Hoc-Tukey test. SPSS software (IBM, version 19, USA) was applied for analysis of data. A $P < 0.05$ was considered as significant.

RESULTS

Chondroblasts and chondrocytes were arranged regularly inside the cavities in the normal group (Fig. 1). The space between each cartilage cell and ECM showed as a colored aura around the cell, suggesting the existence of glycoproteins around the chondrocyte cavities. Bone tissue was

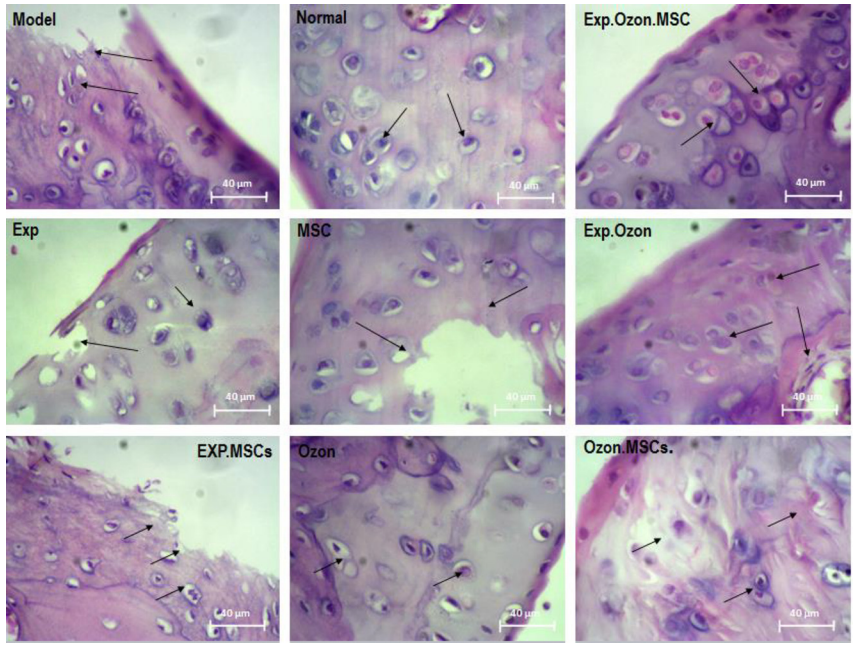

Fig. 1. Histological examination of cartilage in each study group by H&E staining. Chondroblasts and chondrocytes were arranged regularly inside the cavities in the normal group. In the model group, chondrocyte cells were destroyed. Moderate abnormalities are observed in the O_3, MSCs exercise training groups. The proliferating chondrocyte cells were observed in combined therapies with MSC, ozone and exercise, and injured cartilaginous tissues were completely replaced with normal tissues. EXP: exercise training

normal in structure and had cavities consisting of bone marrow tissue and regular arrangement of osteocyte cells. Synovial membranes, lateral ligaments and retaining muscles around the joint and articular capsules had normal structure (Fig. 1).

In the model group, chondrocyte cells were destroyed, which was observed in the synovial membrane, lateral ligaments and retaining muscles. Articular joint and collateral ligament injuries with apoptotic chondrocytes were also observed. A small number of isogenous groups were found, which indicates mild proliferation of cartilage cells in this group (Fig. 1). Although treatment with O_3, MSCs exercise training alone slightly improved these abnormalities in the cartilage and bone tissues, moderate abnormalities can be seen in these groups (Fig. 1). Combined therapies with MSC, ozone and exercise improved these abnormalities more than in the other groups. Proliferating chondrocyte cells were observed within the lacunae as isogenic groups, in even higher numbers than in the healthy group. Injured cartilaginous tissues were completely replaced with normal tissues. The articular capsule of the knee joint and the medial ligament were well-repaired (Fig. 1).

Comparison of *HIF1* expression in all groups is shown in Fig. 2. The ANOVA test analysis showed significant differences in the expression of *HIF1* between groups ($P < 0.0001$). The control group had significantly higher expression of *HIF1* compared to the model (2,529.42-fold; $P < 0.001$), MSCs (17.32-fold; $P < 0.001$), exercise (20.02-fold; $P < 0.001$), ozone (75.19-fold; $P < 0.001$), MSCs + O_3 (1.85-fold; $P < 0.001$), MSCs + exercise (6.28-fold; $P < 0.001$), MSCs + O_3 + exercise (1.92-fold; $P < 0.001$) groups (Table 1). Although exercise training, MSCs and O_3 therapies increased the expression of HIF1 in the cartilage tissue of OA rats, this improvement was not statically significant (Fig. 2). Although combined therapies with exercise, MSCs and O_3 significantly increased the expression of *HIF1*, combination of exercise with O_3 was significantly more effective compared to the other combined therapies (Fig. 2).

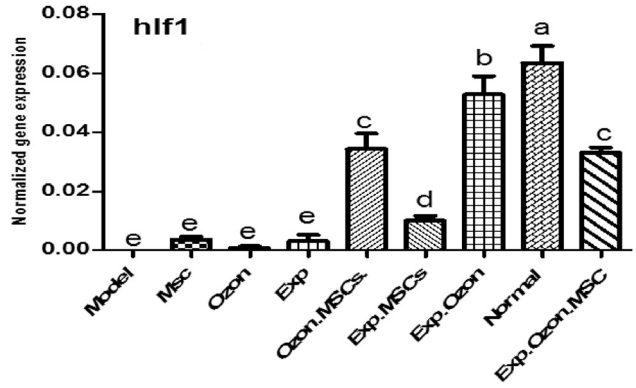

Fig. 2. Comparison of the mean mRNA levels of *HIF*. Gene expression was detected by Real-Time PCR. There was no significant difference in the mRNA levels of *HIF* between groups with similar MSC: mesenchymal stem cell; Exp: exercise training symbols (a–e). The order of the mean mRNA levels of *HIF* was a > b > c > d > e. One-Way ANOVA: Post Hoc-Tukey test was applied to compare the mean values of *HIF* expression pattern between all groups.

Table 1. Fold change ratio of *HIF1* expression in each group

	Fold-change ratio	Up-/down-regulation	*P*-value
Control vs. Model	2,529.42	Up-regulated	<0.001
MSCs vs. Model	14.67	Up-regulated	0.46
O_3 vs. Model	3.64	Up-regulated	0.86
Exp vs. Model	12.63	Up-regulated	0.52
MSCs + O_3 vs. Model	1,369.48	Up-regulated	<0.001
MSCs + Exp vs. Model	403.06	Up-regulated	0.048
Exp + O_3 vs. Model	2,102.61	Up-regulated	<0.001
MSCs + O_3 + Exp vs. Model	1,319.89	Up-regulated	<0.001
Control vs. MSCs	17.32	Up-regulated	<0.001
Control vs. O_3	75.19	Up-regulated	<0.001
Control vs. Exp	20.02	Up-regulated	<0.001
Control vs. MSCs + O_3	1.85	Up-regulated	<0.001
Control vs. MSCs + Exp	6.28	Up-regulated	<0.001
Control vs. Exp + O_3	1.20	Up-regulated	0.036
Control vs. MSCs + O_3 + Exp	1.92	Up-regulated	0.000
MSCs vs. O_3	4.34	Up-regulated	0.57
MSCs vs. Exp	1.16	Up-regulated	0.92
Exp vs. O_3	3.76	Up-regulated	0.63
O_3 + Exp vs. MSCs	14.39	Up-regulated	<0.001
O_3 + Exp vs. Exp	16.64	Up-regulated	<0.001
O_3 + Exp vs. O_3	62.50	Up-regulated	<0.001
O_3 + Exp vs. MSCs + O_3	1.54	Up-regulated	<0.001
O_3 + Exp vs. MSCs + Exp	5.22	Up-regulated	<0.001
O_3 + Exp vs. MSCs + O_3 +Exp	1.59	Up-regulated	<0.001
MSCs + O_3 vs. MSCs	9.38	Up-regulated	<0.001
MSCs + O_3 vs. Exp	10.84	Up-regulated	<0.001
MSCs + O_3 vs. O_3	40.71	Up-regulated	<0.001
MSCs + O_3 vs. MSCs + Exp	3.40	Up-regulated	<0.001
MSCs + O_3 vs. MSCs + O_3 + Exp	1.04	Up-regulated	0.8
MSCs + Exp vs. Exp	3.19	Up-regulated	0.16
MSCs + Exp vs. O_3	11.98	Up-regulated	0.068
MSCs + Exp vs. MSCs	2.76	Up-regulated	0.19
MSCs + Exp vs. MSCs + O_3 +Exp	3.27	Down-regulated	<0.001
MSCs + O_3 + Exp vs. O_3	39.24	Up-regulated	<0.001
MSCs + O_3 + Exp vs. Exp	10.44	Up-regulated	<0.001
MSCs + O_3 + Exp vs. MSCs	9.04	Up-regulated	<0.001

*$P < 0.05$ is considered as significant; One-Way ANOVA: Post Hoc-Tukey test was applied to compare the mean value of *HIF1* expression pattern between all groups.

Comparison of *SOX9* expression is shown in Fig. 3. Significant differences were found in the expression of *SOX9* between groups ($P < 0.0001$). The control group had significantly higher expression of *SOX9* compared to the model (52.65-fold; $P = 0.000$), MSCs (7.14-fold; $P = 0.001$), ozone (3.37-fold; $P = 0.000$), exercise (11.51-fold; $P = 0.000$), MSCs + exercise (8.81-fold; $P = 0.000$), MSCs + O_3 (1.83-fold; $P = 0.000$), and MSCs + O_3 + exercise (1.4-fold; $P =$

Table 2. Fold change ratio of *SOX9* expression in each group

	Fold-change ratio	Up-/down-regulation	*P*-value
Control vs. Model	52.65	Up-regulated	<0.001
MSCs vs. Model	7.37	Up-regulated	0.15
O_3 vs. Model	15.62	Up-regulated	0.002
Exp vs. Model	4.58	Up-regulated	0.42
MSCs + O_3 vs. Model	28.77	Up-regulated	<0.001
MSCs + Exp vs. Model	5.98	Up-regulated	0.26
Exp + O_3 vs. Model	45.63	Up-regulated	<0.001
MSCs + O_3 + Exp vs. Model	37.64	Up-regulated	<0.001
Control vs. MSCs	7.14	Up-regulated	<0.001
Control vs. O_3	3.37	Up-regulated	<0.001
Control vs. Exp	11.51	Up-regulated	<0.001
Control vs. MSCs + O_3	1.83	Up-regulated	<0.001
Control vs. MSCs + Exp	8.81	Up-regulated	<0.001
Control vs. Exp + O_3	1.15	Up-regulated	0.12
Control vs. MSCs + O_3 + Exp	1.40	Up-regulated	0.002
MSCs vs. O_3	2.12	Down-regulated	0.07
MSCs vs. Exp	1.61	Up-regulated	0.53
Exp vs. O_3	3.41	Down-regulated	0.017
O_3 + Exp vs. MSCs	6.19	Up-regulated	<0.001
O_3 + Exp vs. Exp	9.97	Up-regulated	<0.001
O_3 + Exp vs. O_3	2.92	Up-regulated	<0.001
O_3 + Exp vs. MSCs + O_3	1.59	Up-regulated	0.001
O_3 + Exp vs. MSCs + Exp	7.64	Up-regulated	<0.001
O_3 + Exp vs. MSCs + O_3 +Exp	1.21	Up-regulated	0.079
MSCs + O_3 vs. MSCs	3.90	Up-regulated	<0.001
MSCs + O_3 vs. Exp	6.29	Up-regulated	<0.001
MSCs + O_3 vs. O_3	1.84	Up-regulated	0.005
MSCs + O_3 vs. MSCs + Exp	4.82	Up-regulated	<0.001
MSCs + O_3 vs. MSCs + O_3 + Exp	1.31	Down-regulated	0.053
MSCs + Exp vs. Exp	1.31	Up-regulated	0.75
MSCs + Exp vs. O_3	2.61	Down-regulated	0.036
MSCs + Exp vs. MSCs	1.23	Down-regulated	0.75
MSCs + Exp vs. MSCs + O_3 +Exp	6.30	Down-regulated	<0.001
MSCs + O_3 + Exp vs. O_3	2.41	Up-regulated	<0.001
MSCs + O_3 + Exp vs. Exp	8.22	Up-regulated	<0.001
MSCs + O_3 + Exp vs. MSCs	5.10	Up-regulated	<0.001

*$P < 0.05$ is considered as significant; One-Way ANOVA: Post Hoc-Tukey test was applied to compare the mean value of *SOX9* expression pattern between all groups.

0.002) groups (Table 2). There was no significant difference in *SOX9* expression between the control and exercise + O_3 groups ($P = 0.12$). Although exercise, MSCs and ozone therapies increased the expression of SOX9 in the cartilage tissue of OA rats, their combined therapy, especially exercise + O_3, was significantly more effective (Fig. 3).

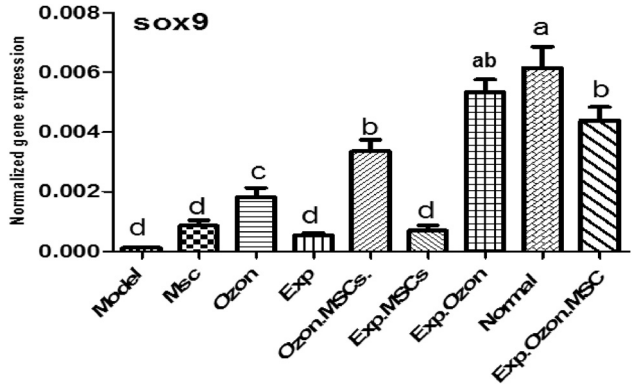

Fig. 3. Comparison of the mean mRNA levels of *SOX9*. Gene expression was detected by Real-Time PCR. There was no significant difference in the mRNA levels of *SOX9* between groups with similar symbols (a–d). The order of the mean mRNA levels of *SOX9* was a > b > c > d. One-Way ANOVA: Post Hoc-Tukey test was applied to compare the mean values of *SOX9* expression pattern between all groups. MSC: mesenchymal stem cell; Exp: exercise training

DISCUSSION

Here, we compared the effect of exercise training, ozone and MSCs therapies and their combination on the expression of *SOX9* and *HIF1* in the cartilage tissue of rats with knee OA. On histological examination, arthritic rats showed chondrocyte cells destruction possibly due to apoptosis, as well as articular joint and collateral ligament injuries. Our findings also show that knee OA is associated with a significant decrease in the expression of *SOX9* and *HIF1* in the cartilage tissue of arthritic rats. This is in agreement with previous research that showed downregulation of *SOX9* and *HIF1* mRNA and proteins in osteoarthritic subjects [20–22]. HIF has an important function in the control of energy production, matrix synthesis and cell survival by articular and growth-plate chondrocytes [23]. It is also crucial for chondrogenesis and regulates the maintenance of articular cartilage via the induction of anabolic factors and the repression of catabolic factors [24]. SOX9 is the other important factor which is critical for cartilage ECM formation and maintenance of the chondrocyte phenotype. Many studies reported decreased level and expression of SOX9 in OA subjects [25, 26]. Therefore, these data suggest that downregulation of SOX9 and HIF may be a main reason for the loss of cartilage ECM and increased risk of OA. Furthermore, these factors are necessary for the maintenance of articular cartilage, and the dysregulation of these genes can cause cartilage degeneration and increased risk of OA development and progression.

According to these findings and the pathogenesis of OA, *SOX9* and *HIF* can be considered as targets for treatment of the disease. To study the pathogenesis or therapeutic options for knee OA, we compared the effect of exercise training, MSCs and ozone therapies on *SOX9* and *HIF* expression in the cartilage tissue of rats with knee OA. We found that combined therapies with exercise training, MSCs and O_3 significantly increased the expression of *SOX9* and *HIF* in the cartilage of osteoarthritic rats; however, combination of exercise with O_3 was more effective. Furthermore, this combined therapy was associated with an increased number of proliferating

chondrocyte cells within the lacunae and also with repair of injured cartilaginous tissues. Therefore, these data indicate the importance of these combined therapies in the treatment of knee OA disease, which is probably mediated through the increase in *SOX9* and *HIF* expression. In support of this theory, Lindholm et al. [27] showed that exercise training increases *HIF1* expression in skeletal muscle. Similarly, Mason and Johnson [28] demonstrated that endurance training causes overexpression of HIF1 in the skeletal muscle of mice.

To our knowledge, the effects of MSCs and ozone therapies on the expression of *HIF1* and *SOX9* in the cartilage of OA subjects have not been investigated yet. Here, we showed for the first time the effectiveness of MSCs and O_3 to enhance the expression of these genes. However, several studies have reported the importance of exercise training, MSCs and ozone therapies in the treatment of knee OA. For instance, Helmark et al. [29] demonstrated that exercise training significantly increases the expression of anti-inflammatory cytokines in the articular cartilage of patients with knee OA. de Jesus et al. [30] reported that O_3 therapy is effective for pain relief and improvement of quality of life in patients with knee OA [30]. A more recent study has demonstrated that O_3 therapy is significantly associated with an improvement in hind-paw diameter, arthritis severity and inflammation in Wistar rats [31]. Some studies have indicated that O_3 therapy may improve OA through the improvement of the antioxidant defense system and the prevention of inflammatory cytokines such as IL-6, TNF-α and IL-8 [32, 33]. Our results reveal that increased expression of *HIF1* and *SOX9* may be another significant mechanism by which O_3 therapy causes OA improvement.

Based on this study and accomplished studies, we hypothesized that combined therapy with O_3, MSCs and exercise training, especially O_3 + exercise, is effective to mitigate clinical and histopathological outcomes of arthritis by increasing the expression of the *HIF1* and *SOX9* genes in the cartilage tissue of rats with knee OA. However, histochemical analysis is essential to study the expression of these genes at the protein level and also to determine its relevance on quality of life in human subjects.

In conclusion, the findings of the current study revealed that knee OA is strongly associated with decreased expression of *HIF1* and *SOX9* in the cartilage tissues. Combined therapies with O_3, MSCs, and exercise training, especially O_3 + exercise training, are effective for the improvement of knee OA. However, we recommend another study at the protein level, as we only examined these factors at the mRNA level.

Funding: This study was supported by a grant received from the Islamic Azad University, Sari Branch, Iran.

ACKNOWLEDGMENT

This work was supported by the Exercise Physiology, Islamic Azad University, Sari Branch, Iran. We would also like to thank the staffs of the Exercise Physiology Centers of the Islamic Azad University, Sari, Iran.

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