Original Article

Effect of exercise, ozone and mesenchymal stem cells therapies on the expression of IL-10 and TNF-α in the cartilage tissue of rats with knee osteoarthritis

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Abstract

Background: Knee osteoarthritis (OA) is a common type of articular disorder worldwide. Interleukin 10 (IL-10) and tumor necrosis factor-α (TNF-α) are considered as an essential regulator contributing to inflammation and knee OA pathogenesis. In this study, the effects of mesenchymal stem cells (MSCs), ozone (O3) and exercise training were considered on IL-10 and TNF-α expression in rats with knee OA.

Methods: In this experimental study, knee OA was induced by surgical method in rats. OA rats were randomly divided into the patient, MSCs, ozone, and exercise groups. Rats in the MSCs group received an intra articular injection of 1×10^6 cells/kg. Rats in the ozone group received O3 at the concentration of 20μg/ml, once weekly for 3 weeks. Rats in the exercise group were trained on rodent treadmill for three times per week. 48 hours after the programs, cartilage tissues were isolated and expression of IL-10 and TNF-α was considered using Real-Time PCR (RT-PCR).

Results: Ozone therapy significantly increased the expression of *IL-10* compared to the patient (3.12 fold; P=0.031), MSCs (2.78 fold; P=0.042) and exercise (4.64 fold; P=0.034) groups. The patient group had significantly higher expression of *TNF-α* compared to the control (32.27 fold; P<0.001), MSCs (1.58 fold; P=0.001) and ozone (3.02 fold; P<0.001) groups. MSCs and ozone therapies significantly decreased *TNF-α* expression compared to the patients (P=0.001 and P<0.001, respectively) and exercise (P=0.042 and P<0.001, respectively) groups; however, ozone therapy was significantly more effective than MSC therapy (P=0.007).

Conclusion: Ozone therapy was significantly more effective than exercise and MSC therapy to improve knee OA in rats.

Keywords: Interleukin-10; Osteoarthritis; Mesenchymal Stromal Cells; Ozone; Knee Joint; Cartilage; Inflammation

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Introduction

Osteoarthritis (OA) is a common form of arthritis which is associated with cartilage erosion, chronic joint pain and stiffness. It affects more than 20 million individuals in the United States (1). Knee OA is more important because of its high prevalence compared to the other kinds of arthritis (2). A recent report has revealed

that the incidence of knee OA has been doubled since the mid-20th century (3). Overweight, aging, genetics, diabetes, moderate intensity activity, and walking disability are important risk factors for knee OA (4, 5). Despite the high incidence rate, there are approved medications for knee OA and current treatments ameliorate symptoms and chronic inflammation. For this reason, a great number of studies have been focused on cellular and molecular mechanisms of OA pathogenesis and its therapeutic pathways.

Recent pieces of evidence have shown that inflammation plays a critical role in OA development and progression (6). A large number of studies demonstrated the important role of pro- and anti-inflammatory factors in the development and progression of OA in both human and animal models. Interleukin 10 (IL-10) and tumor necrosis factor-α (TNF-α) are important factors contributing to knee OA development and progression. IL-10 is a potent anti-inflammatory cytokine which serves as a regulator in preventing inflammatory and autoimmune pathologies (7). Many studies showed that deficiency or decreased expression of IL-10 enhances inflammatory response and leads to develop a number of autoimmune diseases (8). Decreased expression of IL-10 has been reported in different pathologies such as osteoarthritis and rheumatoid arthritis (9). TNF-α is considered as a central mediator of a broad range of biological activities (10). It is a key regulator of the inflammatory response in different pathologies such as rheumatoid arthritis (11). Several studies showed increased expression of TNF-α in osteoarthritis and rheumatoid arthritis diseases (12). Therefore, these data indicated that impaired expression of IL-10 and TNF-α can result in exacerbated immunopathology and tissue damage in different diseases.

Given the regulatory function of TNF-α and IL-10 in the development of OA, they can be considered as targets for therapeutic strategies to treat osteoarthritis. Recent investigations have suggested that ozone (O3) therapy can be used safely in the treatment of OA (13). For instance, Feng et al. showed that O3 therapy significantly improves the pain intensity in patients with OA (14). Several studies have indicated that mesenchymal stem cells (MSCs) are effective for cartilage repair and treatment of knee osteoarthritis due to their immunomodulatory properties (15). Moderate exercise training is another significant strategy which has been suggested for the treatment of knee OA (16). Several clinical trials revealed that exercise improves the pain severity, walking disability, stair climbing, and sit up speed in patients with knee OA (17). Although some studies considered the effectiveness of O3 therapy, MSC therapy and exercise training, the mechanism of action of these therapies in patients with knee OA is not compared and fully understood. Given the critical role of *IL-10* and *TNF-α* in OA development and inflammatory reaction, we assume that MSCs and O3 therapy along with exercise training may be effective in knee OA through the improvement of *IL-10* and *TNF-α* expression. Thus, we designed this study to compare the effects of MSCs and O3 therapy along with exercise training on *IL-10* and *TNF-α* expression in rats with knee OA.

Methods

Experimental animals

In this experimental study, 35 male Wistar rats (age range 8-12 weeks and body weight of 250-300 g) were selected from laboratory animal research center at Islamic Azad University of Sari. This study was approved by the animal care and use committee at Islamic Azad University, Sari branch (Ethical code: NO.19.33.2018). Rats were housed 3 per cage (42×26.5×15 cm) in a climate controlled room (ambient temperature of 22±2°C, humidity 50±5, and a 12:12 light/dark cycle). During the study, rats were fed with a standard diet and water.

Induction of osteoarthritis

Osteoarthritis was induced by the surgical method as previously described by Zhao et al. (18). Briefly, rats were anesthetized with ketamine (30-50 mg/kg) and xylazine (3-5 mg/kg). After shaving the right knees, a 1 cm longitudinal incision was made to expose knee joint. The knee joint was immediately opened through lateral dislocation of the patella and patellar ligament. A longitudinally cut was provided in knee joint capsule through the medial parapatellar incision. Lateral dislocation of the patella and patellar ligament was performed with forceps and then an incomplete incision was made through the medial meniscotibial ligament without articular cartilage and other ligaments injury. Eventually, the knee joint capsule was closed with a 6-0 absorbable suture. The skin was closed with 6-0 silk suture. Rats were fed with standard food and water for three weeks.

Experimental groups

The osteoarthritic rats were randomized into 4 groups (7 rats in each group) including patient (osteoarthritic rats), MSCs, ozone, and exercise groups. Rats in control group had normal knee joints and didn't receive any further treatments.

MSC therapy

Bone marrow-derived MSCs were purchased from the Histogenotech Company (Tehran, Iran). The purchased MSCs were extracted from healthy male Wistar rats (25-300 g). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 20% fetal bovine serum (FBS), incubated overnight to select adherent cells. The culture medium in the flasks was changed every 3 days and MSCs were passaged 3-4 times. Rats were selected for injection when the confluency reached >90% confluency at passages 3 or 4. Rats in MSCs groups received a single intra articular injection of 1×10^6 cells/kg. MSCs were injected into the right knee joint.

Ozone therapy

Ozone (O_3) was generated from medical-grade oxygen (O_2) by OZOMED 01 equipment. Ozone was produced through a silent electric discharge, and its concentration was measured using UV spectrophotometer at 254 nm. Ozone was injected into the knee through the tibiofemoral joint line at the concentration of 20μg/ml, once weekly for 3 weeks starting at 21 days after the modeling.

Exercise training

Before exercise program, rats were adapted with a rodent treadmill for 5 days (one time per week, VO_2 max 60-70%, the speed of 16 m/min at 0% inclination for 10 min/day). Briefly, the exercise program was initiated with a 30-minute run on a treadmill without slope and a speed of 16 m/min in the first week which was gradually reached to 50 minutes in the third week. Warm-up and cool-down time were done for 5m/min at the beginning and end of the exercise.

Samples collection and gene expression analysis

Forty and eight hours after the interventions, rats in each group were anesthetized with ketamine (30-50 mg/kg) and xylazine (3-5 mg/kg). Cartilage tissues were isolated and homogenized in phosphate buffer saline (0.01 M; pH 7.0) at 4 °C with a homogenizer (Hielscher, UP100H). RNA was extracted from cartilage tissues using the RNX-Plus (SinaClon; RN7713C) Kit. The quantity and quality of extracted RNAs were considered using Nanodrop ND-1000 spectrophotometer (Thermo Sci., Newington, NH) method. A complementary DNA (cDNA) was synthesized from RNA samples using RevertAid Reverse Transcriptase (Thermo science, Germany) at 42° C for one hour and random hexamer primers (Thermo science, Germ any). A Rotor-Gene 6000 (Corbett Research, Australia) thermocycler and Real Q-PCR 29 Master Mix Kit (Amplicon, Denmark) in 40 cycles were applied for amplification. Each reaction included 5 μl master mix and 100 nm primers.

The holding stage for RT-PCR was 95.0°C 10:00 minutes. Cycle stages were as the following: 40 cycles; 95.0 °C for 15 seconds; 60.0 °C for one minute. Primer sequences were synthesized as follows: *IL-10*, 5′-CTGTCATTGATTTCTCCCCT-3′ (forward), 5′-TCTATTTATGTCCTGCTGTCC-3′ (reverse); *TNF-α*, 5′-GAGATGTGGAAATGGCAGAGGA -3′ (forward), 5′-GAGAAGATGATGTGAGTGTGAGG -3′ (reverse), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-AAGTTCAACGGCACAGTCAAGG-3′ (forward); 5′-CATACTCAGCACCAGCATCACC-3′ (reverse), as a reference gene. The mRNA levels of *IL-10* and *TNF-α* were normalized relative to the amount of *GAPDH* mRNA. Delta Ct (ΔCT) was calculated using the following formula:

[ΔCT= CT (target) - CT].

Gene expression level was determined by $2^{-\Delta Ct}$ method.

Statistical analysis

Comparison of the mean expression of *IL-10* and *TNF-α* between all groups were performed using one-way ANOVA with tukey post hoc tests. IBM SPSS Statistics for Windows, Version 19.0. was used for data analysis. P value of lower than 0.05 was considered statistically significant.

Table 1. Fold change ratio of IL-10 expression in each group

	Fold change ratio	Up-/down-regulation	P^*
Patient vs Control	12.869	Down-regulated	<0.001
MSCs vs Patient	1.124	Up-regulated	0.91
Ozone vs Patient	3.126	Up-regulated	0.03
Exercise vs Patient	0.673	Down-regulated	0.76
Control vs MSCs	11.452	Up-regulated	<0.001
Control vs Ozone	4.117	Up-regulated	<0.001
Control vs Exercise	19.114	Up-regulated	<0.001
Ozone vs MSCs	2.781	Up-regulated	0.04
MSCs vs Exercise	1.669	Up-regulated	0.68
Ozone vs Exercise	4.643	Up-regulated	0.03

* One-Way ANOVA

Table 2. Fold change ratio of TNF-α expression in each group

	Fold change ratio	Up-/down-regulation	P^*
Patient vs Control	39.279	Up-regulated	<0.001
MSCs vs Patient	1.587	Down-regulated	0.001
Ozone vs Patient	3.026	Down-regulated	<0.001
Exercise vs Patient	1.182	Down-regulated	0.13
Control vs MSCs	24.755	Down-regulated	<0.001
Control vs Ozone	12.982	Down-regulated	0.006
Control vs Exercise	33.227	Down-regulated	<0.001
Ozone vs MSCs	1.907	Down-regulated	0.007
MSCs vs Exercise	1.342	Down-regulated	0.04
Ozone vs Exercise	2.560	Down-regulated	<0.001

* One-Way ANOVA

Results

Comparison of the IL-10 expression in all groups is shown in Figure 1. The ANOVA test analysis showed a significant difference in the expression of IL-10 between groups (P<0.001). Control group had significantly higher expression of IL-10 compared to the patient (12.86 fold; P<0.001), MSCs (11.45 fold; P<0.001), exercise (19.11 fold; P<0.001) and ozone (4.11 fold; P<0.001) groups (Table 1). Ozone therapy significantly increased the expression of IL-10 compared to the patient (3.12 folds; P=0.031), MSCs (2.78 fold; P=0.042) and exercise (4.64 fold; P=0.034) groups (Table 1). There was no significant difference in the expression of IL-10 between the patient, MSCs and exercise groups (Figure 1).

Comparison of the TNF-α expression is shown in Figure 2. The patient group had a significantly higher expression of TNF-α compared to the control (32.27 fold; P<0.001), MSCs (1.58 fold; P=0.001) and ozone (3.02 fold; P<0.001) groups (Table 2). There was no significant difference in TNF-α expression between patient and exercise group. MSCs and ozone therapies significantly decreased TNF-α expression compared to the patients (P=0.001 and P<0.001, respectively) and exercise (P=0.042 and P<0.001, respectively) groups; however, ozone therapy was significantly more effective than MSC therapy (P=0.007).

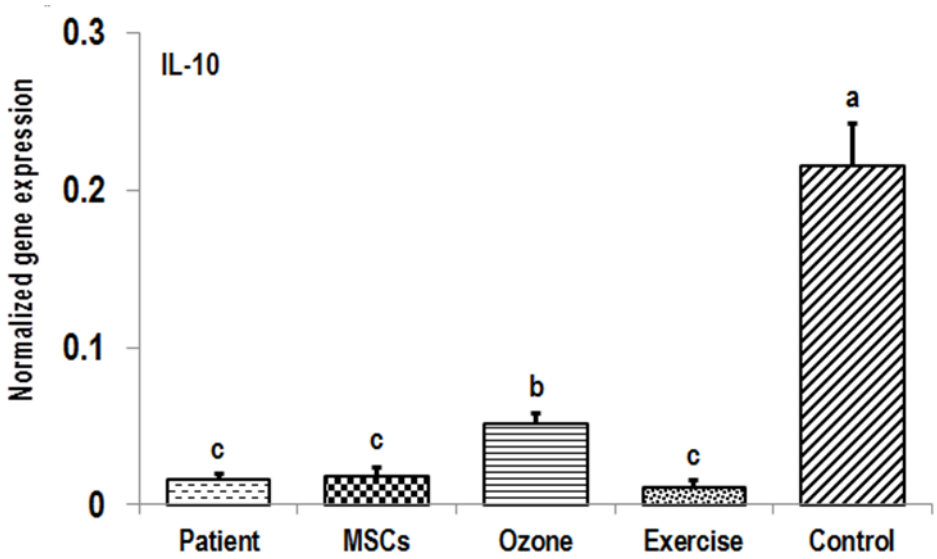

Figure 1. Comparison of the mean mRNA levels of IL-10. Gene expression was detected by RT-PCR. There was no significant difference in the mRNA levels of IL-10 between groups with similar symbols (a-c). The mean mRNA level of IL-10 was in order a>b>c. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of IL-10 expression pattern between all groups.
MSC: mesenchymal stem cell

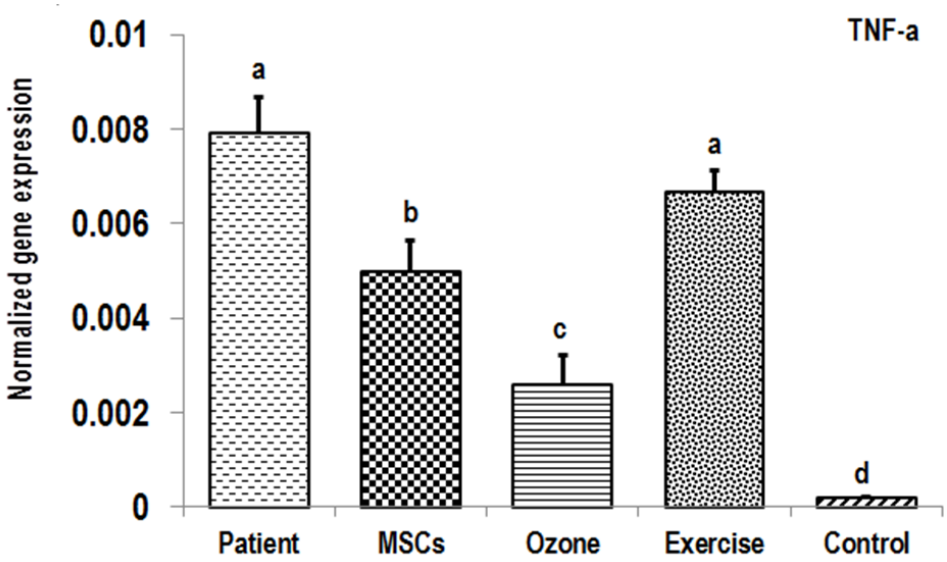

Figure 2. Comparison of the mean mRNA levels of TNF-α. Gene expression was detected by RT-PCR. There was no significant difference in the mRNA levels of TNF-α between groups with similar symbols (a-d). The mean mRNA level of TNF-α was in order a>b>c>d. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of TNF-α expression pattern between all groups.
MSC: mesenchymal stem cell

Discussion

Our findings have revealed that knee OA was associated with a significant decrease in the expression of IL-10, while TNF-α was significantly increased in the cartilage tissue of arthritic rats. Our finding was in agreement with Rojas-Ortega et al. which reported decreased expression of IL-10 in the articular cartilage of rats with knee OA (19). A previous study found that patients with early psoriatic arthritis and early rheumatoid arthritis had higher IL-10 contents compared to patients in later stages of the disease (20). On the other hand, Han et al., demonstrated that polymorphisms or mutations in TNF-α increase individual susceptibility to OA in the Korean population (21). Therefore, these data suggest that there is a tight link between TNF-α and IL-10 expression and increased risk of knee OA. Furthermore, they are necessary for the maintenance of articular cartilage homeostasis and the dysregulation of these genes can cause cartilage degeneration and increased risk of osteoarthritis development and progression.

According to our findings and pathogenesis of osteoarthritis, TNF-α and IL-10 genes can be considered as a target for the disease treatment. We found that MSCs and ozone therapies significantly decreased TNF-α expression in the cartilage of osteoarthritic rats; however, ozone was more effective. Additionally, ozone therapy significantly increased the expression of IL-10 compared to MSC therapy and exercise training. MSC therapy and exercise training didn't affect IL-10 expression. These data indicate that ozone therapy is more effective to regulate inflammatory mediators in OA disease.

Several lines of studies considered the effect of exercise training, MSCs and ozone therapies on inflammatory biomarkers and OA treatment. In a clinical trial study, Helmark et al. demonstrated that exercise training significantly increases the expression of IL-10 in the articular cartilage of patients with knee OA (22). Similarly, Rojas-Ortega et al. showed that exercise training improves the expression of IL-10 in the articular cartilage of rats with knee OA (19).

Increased expression and production of IL-10 during exercise may possibly restrict the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8 and subsequently improve disease treatment (22). On the other hand, several studies did not find a significant change in IL-10 concentration after exercise (23). Similarly, we couldn't find a relationship between exercise training and the expression of IL-10 and TNF-α. However, it seems that multiple factors such as the type of exercise, disease severity and exercise duration may be involved in disease improvement and should be further considered.

Studies have reported the effectiveness of ozone therapy on arthritis. In a clinical trial study, de Jesus et al. showed that O3 therapy is associated with pain relief, functional improvement, and quality of life in patients with knee osteoarthritis (24). More recently, Bozbaş et al. have revealed that O3 therapy significantly improves hind-paw diameter, arthritis severity, and histopathological findings of inflammation in Wistar rats (25). Furthermore, several experimental studies showed that O3 therapy improves osteoarthritis by improving antioxidant status and preventing oxidative damages and inflammatory mediators such as IL-6 and IL-8 (26, 27). Our findings have indicated that increased expression of IL-10 and decreased expression of *TNF-α* may be another significant mechanism by which O3 therapy causes arthritis improvement. In support of this fimding, de Souza et al., showed that O3 therapy modulates the inflammatory response by increasing IL-10 and decreasing TNF-α in rats with acute lung injury (28). A previous study found that O3 therapy decreases TNF-α production and on the other hand it improves antioxidant-prooxidant balance by increasing of endogenous antioxidant systems (29). More recently, Wei et al. showed that O3 therapy ameliorates inflammation and endometrial injury in rats with pelvic inflammatory disease by regulating the expression of inflammatory factors, including IL-6, TNF-α and IL-2 (30).

We also found that MSC therapy decreases the expression of TNF-α, while it didn't affect IL-10 expression. Salazar et al. demonstrated that mesenchymal stem cells produce TGF-β, which in turn induces the proliferation and expression of procollagen in lung fibroblasts (31).

In conclusion, the findings of the current study revealed that knee OA is strongly associated with reduced expression of IL-10 and overexpression of TNF-α in the cartilage tissues. Ozone therapy was more effective compared to MSC therapy and exercise training. However, we recommend another study at the protein level as we only considered these factors at mRNAs levels.

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Conflict of interest

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