The Effect of Mesenchymal Stem Cells and Aqueous Extract of *Elaeagnus Angustifolia* on the Mechanical Properties of Articular Cartilage in an Experimental Model of Rat Osteoarthritis

Mahmoud Mofid [1], Seyed Homayoon Sadraie [1*], Hossein Imani [1], Giti Torkaman [2], Gholamreza Kaka [3], Mohammad Reza Naghii [4], Gholamhossein Alishiri [5], Mohammad Hossein Asadi [1]

1. Department of Anatomical Sciences, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran.
2. Department of Physiotherapy, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
3. Neuroscience Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.
4. Department of Nutrition, Health School, Baqiyatallah University of Medical Sciences, Tehran, Iran.
5. Department of Rheumatology, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran.

Dr. Seyed Homayoon Sadraie is a professor of Department of Anatomy in Baqiyatallah University of Medical Sciences. He teaches anatomical sciences, including histology and embryology. His research fields are embryo toxicology, bone & cartilage repair.

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ABSTRACT

Introduction: Although, the effect of direct intra-articular injection of bone marrow stem cells (BMSCs) on the repair of articular cartilage and the effect of *Elaeagnus angustifolia* extract on pain relief in patients with osteoarthritis have been investigated, no studies has been conducted to compare the effects of these two therapeutic methods on the mechanical properties of articular cartilage. In the present study, the effect of these two methods on the mechanical strength of knee articular cartilage in a model of rat osteoarthritis has been studied.

Methods: In the present research, 48 mature, male Wistar rats were used. Animals were randomly divided into 6 groups of 8 as follows: control group (healthy animals), saline with mono-iodoacetate (MIA), MIA with *Elaeagnus angustifolia* extract, MIA with BMSCs, and MIA with a combination of *Elaeagnus angustifolia* extract and BMSCs. Osteoarthritis was induced by injection of 50 µL solution of MIA in rats of groups 3 to 6. About 500 mg/kg *Elaeagnus angustifolia* extract was injected intraperitoneally daily for 4 weeks and nonautologous mesenchymal stem cells were injected into the knee joint on the 14th day. Stress-relaxation test was conducted applying 0.1 mm displacement at the rate of 5 mm/min for 1000 seconds. Then, the maximum initial force, instantaneous stiffness, equilibrium force, and equilibrium stiffness were calculated.

Results: Induction of osteoarthritis model decreased instantaneous stiffness, maximum initial force, and equilibrium stiffness as compared to the healthy group (P=0.05). Using *Elaeagnus angustifolia* extract and bone marrow stem cells increased instantaneous stiffness and equilibrium stiffness compared to MIA group, although this increase was statistically significant only in the BMSCs group (P=0.04 and P=0.026, respectively). In the BMSCs group, maximum initial force also significantly increased compared to MIA group (P=0.04).

Conclusion: Apparently direct injection of BMSCs into the knee joint with osteoarthritis is more effective in increasing mechanical strength of the cartilage and improving the performance of the weight-bearing joint compared to using *Elaeagnus angustifolia* extract.

Key Words:

Osteoarthritis, Mesenchymal stem cells, Elaeagnus angustifolia, Mechanical strength of cartilage

*** Corresponding Author:**
Seyed Homayoon Sadraie, PhD
Address: *Department of Anatomical Sciences, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran. PO Box: 11365-16777*
Tel: *+98 (912) 7241170*
E-mail: *h_sadraie@yahoo.com*

1. Introduction

Osteoarthritis (OA) is one of the most common chronic diseases. It is also one of the most important causes of pain and disability among adults [1]. With increased longevity in all communities and due to the strong relationship between OA and advancing age, the prevalence of osteoarthritis is increasing. Studies have shown that approximately 15% of the world population is suffering from this inflammatory-degenerative illness. In Iran, prevalence of this disease among the urban population has been estimated to be 16.6% [1] and among the rural population approximately 20% (at least in one of the joints), which is higher than that figure in rural population of other Asian countries [2]. Although any joint with hyaline cartilage can be affected with primary osteoarthritis, this illness, especially affects the weight-bearing joints of the lower extremities [3]. There are several therapeutic methods to improve the performance of the synovial joints and repair the joints cartilage affected by OA.

Nonpharmaceutical therapeutic interventions include education, weight loss, nutrition (often prescription of antioxidants), and reinforcement of the relevant muscles. Pharmaceutical interventions include pain control (prescription of nonsteroidal anti-inflammatory drugs), intra-articular injection of steroid, hyaluronic acid, chondroitin sulfate, and glucosamine (with different results) and interventions for inhibiting the pathogenesis of the illness such as inhibiting nitric oxide, lymphokines, metalloproteinases; gene therapy; and growth stimulation and restoration of articular cartilage [4].

Nowadays, cartilage restoration methods include transplantation of osteochondral grafts, microfracturing, and autologous chondrocyte implantation (ACI) to transfer cells with or without scaffold matrix. Unfortunately, in advanced stages of osteoarthritis, which is associated with destruction of cartilage and disturbance of the mechanical function of joints, no method has yet been found to postpone full joint replacement, which is the last therapeutic strategy and in many cases, a hazardous operation. Cartilage has a poor restorative ability, and if a solution like using stem cells, postpones this operation on these patients, it can considerably help treat these individuals [5].

Stem cells derived from mature bone marrow, which have the ability to differentiate into ancestral cartilage cells, are right candidates with excellent potential for cell-based methods of restoration of articular cartilage in patients with topical and nontopical articular cartilage lesions. Using BMSCs can eliminate the need for biopsy of the cartilage, and consequently, remove the damage to the remaining healthy cartilage surface in the donation area [5]. Technically, intra-articular injection of BMSCs is the simplest therapeutic approach to osteoarthritis. Following injection, BMSCs spread across the joint space and interact with each available cell and surface [6, 7], but certainly the role of these cells in the restoration of mechanical strength of cartilage and cartilage biomechanical performance is very important and should be examined in novel studies.

On the other hand, using herbs or traditional medicine to treat diseases has a long history in medicine in the world and in Iran and since the use of herbal medicines at the conventional level has lower risks than the chemical drugs; their use is growing in the world. *Elaeagnus angustifolia* is an herb that has many uses in Iranian traditional medicine and many therapeutic properties have been attributed to its fruits, leaves, flowers, and stem [8]. Aqueous extract of *Elaeagnus angustifolia* contains the compounds of potassium, magnesium, sodium, iron, calcium, zinc, copper [9], fatty acids, flavonoids, tannins, cardiac glycosides, cytosterol, and terpenoid [10]. Its herbal treatments (using its fruits, flowers, brewed drink, and extract) have been reported to be effective on nausea, vomiting, jaundice, asthma, tympanites, and fever. It is also analgesic, anti-inflammatory for rheumatism, and muscle relaxant; eliminates osteomalacia; and heals wounds [8, 11-14].

Medicinal properties of *Elaeagnus angustifolia* are probably due to its flavonoids ingredients [15, 16]. Flavonoids can play a variety of roles in biological processes such as osteoarthritis and chondrogenesis [17, 18]. Flavonoids, as antioxidants, act as agonists and antagonists of endogenous estrogens, react with many cell receptors (15), and protect against cell death [19, 20]. Research has shown that free radicals control the synthesis of proteoglycans and collagen through chondrocyte and flavonoids, as antioxidants, may be involved in chondrogenesis [21]. However, some studies argue that prescription of *Elaeagnus angustifolia* extract inhibits chondrogenesis during the prenatal period [22]. Use of *Elaeagnus angustifolia* extract has been reported to reduce pain in patients with inflammatory joint lesions [12].

Despite reports about the effect of *Elaeagnus angustifolia* extract in reducing pain in patients with osteoarthritis, so far no studies have been conducted on the mechanical function of cartilage after using its extract. Since chondrocytes' activity and their function in the production of extracellular matrix affect the biomechanical properties of cartilage, it seems that the mechanical strength of cartilage against the mechanical forces due to daily activities will also increase. Because of the limited human studies and medical ethics considerations, the biomechanical aspects of the above

treatments must be evaluated in animal models to use its findings for the better treatment of osteoarthritis. In the present study, the effect of BMSCs and *Elaeagnus angustifolia* extract as well as the combination of these two therapeutic methods has been investigated on the biomechanical properties of the knee joint cartilage in osteoarthritis model in rats.

2. Materials and Methods

All stages of this research have been approved in the Medical Ethics Committee of Baqiyatallah University of Medical Sciences. In this research, 48 mature, male Albino-Wistar rats weighing 200-250 g, were used. Food and water was made available to them ad libitum. Rats nest temperature was kept at 22±2°C and the nest light was set as 12:12 hour light/dark cycle.

Animals were randomly divided into 6 groups of 8 rats. The control group consisted of healthy animals that underwent no intervention. A dose of 50 mL physiologic serum was injected into the right knee joint of the members of the saline group. Next one, the MIA group received 50 mL solution containing 3 mg MIA into their right knee joints. In groups 4 to 6, similarly, MIA was used. In group 4, since the 14th day, 500 mg/kg *Elaeagnus angustifolia* extract was injected intraperitoneally every day for 4 weeks. In group 5, on the 14th day, nonautologous BMSCs were injected into the right knee joints of the animals. In group 6, on the 14th day, nonautologous BMSCs were injected into the right knee joint of the animals and at the same time, *Elaeagnus angustifolia* extract 500 mg/kg was injected intraperitoneally to them every day for 4 weeks.

Method of creating osteoarthritis

First, the rats were anesthetized by using Ketamine (50 mg/kg) and Xylazine (5 mg/kg). After shaving the knee skin and bending the knee joint, a single dose of 3 mg (based on previous studies) MIA (Sigma, Aldrich) dissolved in 50 µL physiological saline was injected through infrapatellar ligament of the right knee using a 26-gauge needle. In the control group, using a 26-gauge needle, 50 µL physiological saline solution was injected into the right knee joint capsule through infrapatellar ligament.

Method of preparation of bone marrow derived stem cells

Animals were anesthetized by using a mixture of Ketamine hydrochloride (40 mg/kg) and Xylazine hydrochloride (10 mg/kg). Then, rat thigh bone marrow was aspirated with a G18 needle into a 5-mL syringe containing 1 mL Alpha-MEM (GIBCO) medium (pH=7.2) and 10% (GIBCO) fetal bovine serum (FBS). Next, it was discharged into a 25-mL flask and kept in an incubator at 37°C with 5% CO_2 in a humid condition. After 24 hours, the culture plate containing cells was replaced. The medium was changed every 2 to 3 days. Morphology and general condition of the cells was examined with invert microscope every day. After reaching over 80% density, BMSCs were detached from flask bottom using Trypsin/EDTA (SIGMA) solution and were passaged at least 4 times. In this phase, stromal nature of stem cells was confirmed through immunocytochemistry method by using antibody against fibronectin and CD44, consecutively.

Bone marrow derived stem cells transplantation method

To inject BMSCs, the cells were first removed from the culture medium and washed with PBS solution. After adding 1 mL Trypsin/EDTA and centrifuging at 2000 rpm for 4 minutes, the upper liquid was removed and 1 mL alpha-MEM culture medium was added. BMSCs cell count was performed in the suspension containing the cells with Neubauer hemocytometer. Then, the suspension was centrifuged at 2000 rpm for 4 minutes along with alpha-

Table 1. Biomechanical parameters of the cartilage in different groups.

Variable	Control (Mean±SD)	Saline (Mean±SD)	MIA (Mean±SD)	MIA+Elaeagnus angustifolia (Mean±SD)	MIA+Cell (Mean±SD)	MIA+Cell+Elaeagnus angustifolia (Mean±SD)
Maximum initial force (MPa)	0.32±0.11	0.35±0.20	0.021±0.009	0.034±0.26	0.28±0.21	0.048±0.047
Instantaneous stiffness (N/mm)	3.95±2.046	2.15±1.28	0.14±0.01	1.29±0.08	2.03±1.36	0.29±0.27
Equilibrium force (MPa)	0.16±0.17	0.08±0.04	0.004±0.005	0.04±0.029	0.068±0.05	0.031±0.022
Equilibrium stiffness (N/mm)	20.8±5.09	17.95±13.00	1.47±0.81	11.44±1.76	14.33±9.48	10.58±7.12

MEM culture medium. To count BMSCs in the suspension containing the cells with Neubauer hemocytometer, a sampler 10 µL of the suspension containing stromal cells was taken and mixed with 10 µL Trypan blue. Then, 10 µL of the obtained solution was placed under the hemocytometer for cell count. Trypan blue makes the dead cells blue and reduces the error in cell count. Cells were counted in all 4 squares of Neubauer Hemocytometer subdivided into 16 equal portions and after calculating the mean of the number of cells, the obtained value was multiplied by 20000 to determine the number of cells in the 1 mL volume of the culture medium. Afterwards, animals were anesthetized using Ketamine (50 mg/kg) and Xylazine (5 mg/kg) and after shaving the right knee joint area of the animal, the area was washed with Betadine, and then 800000 MSCs in a 50 µL volume were injected into the right knee joint of the animal through the infrapatellar ligament area over 1 minute.

Method of preparation and injection of *Elaeagnus angustifolia* extract

Elaeagnus angustifolia fruit powder was prepared in the laboratory. About 100 g of dried *Elaeagnus angustifolia* powder and 1000 mL distilled water were mixed and soaked for 24 hours at 30°C and the supernatant solution was passed through a filter. By using this method, from each 100 g *Elaeagnus angustifolia*, 20 g dry extract was obtained. This extract was then weighed and after being dissolved in physiological saline, based on previous studies, 500 mg/kg was injected intraperitoneally to animals every day (with insulin syringes and disposable needles).

Measuring the biomechanical strength of articular cartilage

At the end of the sixth week, animals were killed with a high dose of anesthetic. Then, the right knee joints of the animals were dissected and after removing the patella bone, ligaments, and meniscus without causing damage to the joint surfaces, for biomechanics test, the medial plateau of tibia was placed inside the lower jaw of the tensiometer. Stress-Relaxation test was carried out using Resistance Measuring Device (Zwick Universal Testing Machine, Zwick/Roell GmbH & Co., Ulm, Germany). Using an indenter with the diameter of 1 mm made of stainless steel, unconfined stress-relaxation test was conducted on medial plateau of tibia. In the designed holder base, it was possible to rotate the bone so that samples could be placed in the same position and the indenter be fixed on the central part of medial plateau of tibia. To fix the indenter, an initial preload of 0.01 N was applied. Displacement of 0.1 mm at the constant rate of 5 mm/min was applied and 1000 seconds was considered for reaching equilibrium. The obtained data were fed to MatLab software and after being processed, parameters of maximum initial force (in terms of MPa) at the time of reaching 0.1 mm deformation, elastic modulus (instantaneous stiffness or load-deformation curve slope at the time of reaching 0.1 mm deformation) in terms of N/mm, equilibrium force in terms of MPa, which was reaching an approximately constant force 1000 seconds after starting the test and was formulated based on the previous studies [23-27], and equilibrium stiffness (aggregate modulus or load-deformation curve slope after reaching equilibrium) in terms of N/mm were calculated.

Statistical analysis of the information

The data were analyzed using SPSS 17.0 software. Given the normal distribution of data determined by Shapiro-Wilk test, in order to compare the results among the 6 groups, the 1-way ANOVA and Tukey complementary tests were used. The level of significance in tests was considered at $P<0.05$.

3. Results

Results of cartilage biomechanics test

Table 1 presents the data obtained from cartilage biomechanics test for each group, based on the mean and standard deviation. Measurement of biomechanical parameters of cartilage indicated that using MIA for induction of OA significantly reduces the mechanical strength of cartilage compared to the control group and using cell therapy methods, *Elaeagnus angustifolia* extract. and the combined method is effective in increasing the mechanical strength of cartilage, as it will be mentioned in the following sections.

Maximum initial force of the cartilage

The results of 1-way analysis of variance showed that the maximum initial force among the study groups (Figure 1) had a significant difference (P=0.000). Tukey complementary test showed that the mean of maximum initial force in MIA (P=0.015), *Elaeagnus angustifolia* (P=0.022), and combination of cells and *Elaeagnus angustifolia* (P=0.031) significantly decreased compared to the control group. While the mean of initial force in the cell therapy group showed a significant increase compared to the above 3 groups (P=0.04, P=0.06, P=0.008, respectively). The maximum initial force in the cell therapy group did not show a significant difference with the control group.

Instantaneous stiffness of the cartilage

The results of 1-way analysis of variance showed that the instantaneous stiffness among the study groups (Figure

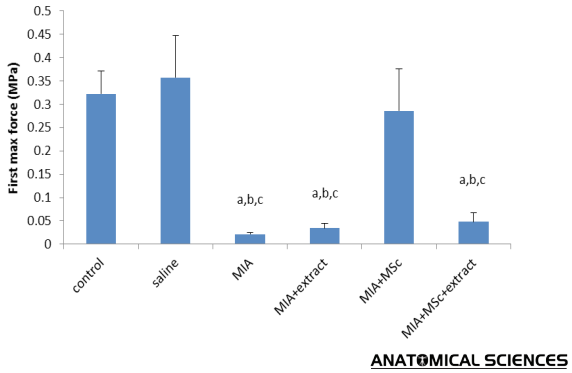

Figure 1. Initial maximum force of the cartilage in the study groups. a: significant decrease compared to the control group, b: significant decrease compared to the saline group, c: significant decrease compared to the cell therapy group.

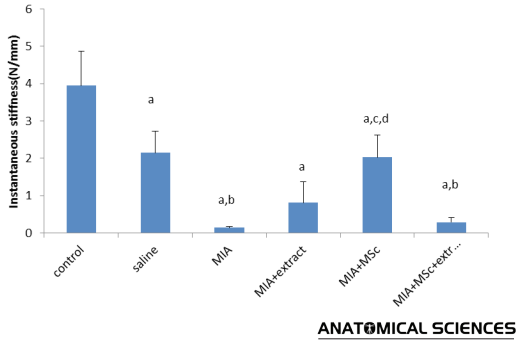

Figure 2. Instantaneous stiffness in the study groups. a: significant decrease compared to the control group, b: significant decrease compared to the saline group, c, d: significant increase compared to MIA and the combination of cells and Elaeagnus angustifolia groups.

2) had a significant difference (P=0.001). Tukey complementary test showed that the mean of instantaneous stiffness in all groups significantly decreased compared to the control group (P<0.05). While the mean of instantaneous stiffness in the MIA group (P=0.018) and combination of cells and *Elaeagnus angustifolia* (P=0.028) showed a significant decrease compared to the saline groups. The mean of instantaneous stiffness in the cell therapy group showed a significant difference with the above 3 groups (P=0.04, P=0.06, P=0.008, respectively). Instantaneous stiffness in the cell therapy group showed a significant difference with the MIA group (P=0.026) and also with the combination of cells and *Elaeagnus angustifolia* (P=0.039).

Equilibrium force of the cartilage

The results of 1-way analysis of variance showed that the difference between the initial equilibrium force in the study groups (Figure 3) was not close to the significance level (P=0.06). The mean of equilibrium force in all groups in which OA had been induced by MIA was less than the control group. The mean of equilibrium force in the cell therapy group showed a greater increase compared to the MIA, *Elaeagnus angustifolia* extract and the combination of cells and *Elaeagnus angustifolia*, although this increase was not significant.

Equilibrium stiffness of the cartilage

The results of 1-way analysis of variance showed that the difference between the equilibrium stiffness modulus in the study groups (Figure 4) was significant (P=0.021). Tukey complementary test showed that the mean of equilibrium stiffness modulus in the MIA group significantly decreased compared to the control group (P<0.018) and the saline group (P=0.08). The mean of the equilibrium stiffness modulus in the cell therapy group showed a significant increase compared to the MIA group (P=0.026). The value of this parameter in the combination of cells and *Elaeagnus angustifolia* group also showed a significant decrease compared to the control group (P=0.018).

4. Discussion

In this research, the effect of mesenchymal stem cells, *Elaeagnus angustifolia* extract and their combination on the changes in mechanical properties of cartilage in the model of rat knee joint osteoarthritis was studied. Induction of osteoarthritis model by injection of MIA reduced the mechanical strength of the cartilage. Instantaneous stiffness, maximum initial force, and equilibrium stiffness showed a significant decrease compared to the control group with healthy cartilage, which indicates articular cartilage lesions. It seems that chondrocyte apoptosis and extracellular matrix degradation, which includes glycosoaminoglycan and collagen, caused by injection of MIA [28] decreases the mechanical strength of cartilage. Injection of saline had no effect on the mechanical properties of cartilage and none of the measured parameters showed a significant difference

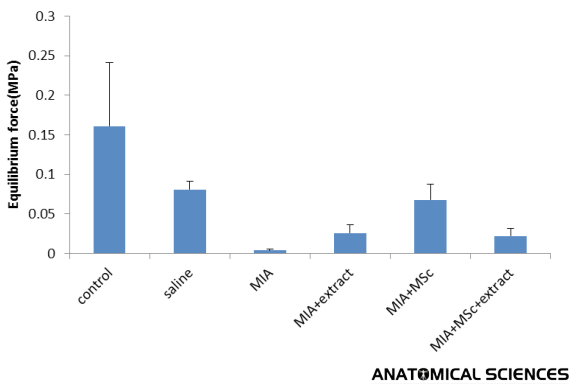

Figure 3. The amount of equilibrium force in the study groups.

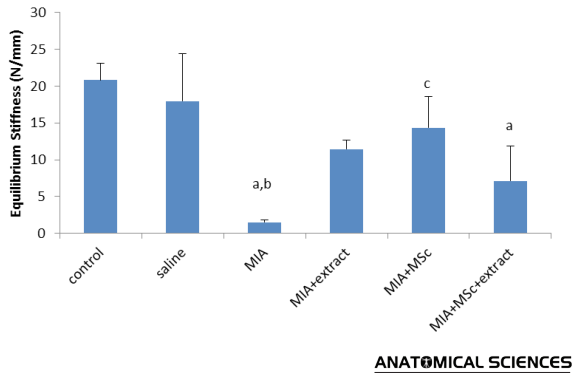

Figure 4. Equilibrium stiffness in the study groups. a: significant decrease compared to the control group, b: significant decrease compared to the saline group, c: significant increase compared to MIA group.

with the control group. In the groups that received *Elaeagnus angustifolia* extract and mesenchymal stem cells, instantaneous stiffness and equilibrium stiffness of cartilage increased compared to MIA group, but this increase was statistically significant only in the cells group.

In addition, in the cells group, the maximum initial force, which represents the initial resistance of cartilage against the pressure of indenter on the surface of the cartilage, also showed a significant increase compared to MIA group. This parameter, unlike the two groups of *Elaeagnus angustifolia* extract and the combination of cells and *Elaeagnus angustifolia*, showed no significant difference compared to the control group, i.e. the mechanical strength of cartilage in the cells group is close to normal levels in healthy cartilage. However, in case of instantaneous stiffness, both groups of *Elaeagnus angustifolia* extract and cells were close to the normal level regarding the cartilage condition and showed no significant difference with the control group. It seems that combination of the two therapeutic methods does not have a greater effect on increasing the mechanical strength of cartilage and possibly the stimulated interference mechanisms have had different effects. However, in order to determine the probable mechanisms, histological and histochemical studies must be considered in future research.

Inherent regenerative capacity of chondrocytes is very limited. Lesions with relative thickness, which do not penetrate the subchondral bone, are not usually cured spontaneously and regeneration of lesions with full thickness which reach the subchondral bone are also dependent on the conditions such as age of the patient; size, and location of the lesion. Smaller lesions may heal spontaneously by production of hyaline cartilage, but larger lesions are only restored with the production of fibrous or fibrocartilage tissue that is biomechanically different from normal hyaline cartilage, thus the cartilage will continue to have a poor mechanical performance [5].

The loss of cartilage in degenerative joint diseases, which are currently treated with analgesics and anti-inflammatory drugs, leads to pain and immobility [29-31] and in advanced cases; joint replacement (arthroplasty) is used to restore joint function [32-35]. Because of the problems associated with the long-term use of this prosthesis, its use in younger people (under 50 years) is limited. It seems that the optimal treatment of cartilage lesions in younger people is the application of tissue engineering methods for reconstruction of articular cartilage using a functional replacement tissue.

The use of tissue engineering to repair damaged weight-bearing tissues such as cartilage with its complex cell architecture, is not easily applicable. Cell source for tissue engineering is chondrocytes or mesenchymal stem cells. Disadvantages of using chondrocytes include their limited ability to replicate and the loss of phenotype in the process of replication [36]. Several studies have shown that mesenchymal stem cells derived from bone marrow and fat, have the potential to repair articular cartilage [37, 38] but most of the measured parameters in these studies have been limited to histological characteristics, measuring pain, the range of mobility of the joint, and radiologic signs. Jo et al., administered a high dose of mesenchymal stem cells (10^8 cells) in knees of patients with osteoarthritis and pain and clinical symptoms showed improvement 6 months after cell injection into the joint [7].

However, as mentioned in the introduction, study of the biomechanical properties of the cartilage, especially in weight-bearing joints is essential and must be considered in therapeutic use of mesenchymal stem cells. However, medical ethics have restrained studies on humans and in this area, so studies are limited to animals. In the study conducted by Han et al., on rabbit animal model, cartilage biomechanical parameters were evaluated by indentation test. Twelve rabbits with osteochondral knee defect were used in this study. About 2-8 mL BMSCs was obtained from the posterior iliac crest and was exposed to TGF-β3, three weeks prior to culture. Twelve weeks after culture, cartilage repair was observed in 10 out of 12 rabbits and in indentation test, Young's modulus (stiffness of cartilage) had reached 80% of the amount of normal cartilage [39].

Contrary to our results, Ravanbod et al., who used mesenchymal stem cells in experimental hemophilic arthropathy in the rabbit knees did not report a significant increase in mechanical strength of the cartilage (including initial stiffness and equilibrium stiffness). The difference in the obtained results may be due to the type of damage to the joint,

as they injected blood into the joint several times to cause arthropathy [25].

The researchers believe that therapeutic use of *Elaeagnus angustifolia*, because of its flavonoids [15, 16], may be effective in the biological process of osteogenesis and chondrogenesis [17, 18]. Flavonoids as antioxidant products, can be effective in reducing glutathione and free radicals [19] and prevent cell death [19, 20].

In previous studies, the effect of *Elaeagnus angustifolia* extract on reducing the pain of inflammatory joint lesions has been reported [12]. However, some studies argue that prescription of *Elaeagnus angustifolia* extract inhibits chondrogenesis during the prenatal period [22]. No studies have been conducted on the mechanical performance of cartilage after using *Elaeagnus angustifolia* extract. Given that the function of chondrocytes in the construction of the extracellular matrix affects cartilage biomechanical properties and increases the mechanical strength of cartilage against mechanical forces due to the daily activities, in the present research the effect of *Elaeagnus angustifolia* extract in the osteoarthritis model was studied. But despite the relative increase in equilibrium stiffness and instantaneous stiffness compared to MIA group, the obtained results were not significant.

It is likely that the frequency and doses of *Elaeagnus angustifolia* extract may affect the obtained results; something which should be examined in future studies. According to the favorable reported results of using *Elaeagnus angustifolia* extract to reduce pain and facilitate joint motion, this extract may be more effective in lubrication of the joints and reduction of joint friction. Therefore, the biomechanical function of the joint, which plays a crucial role in the performance of the joints, particularly weight-bearing joints, should be considered in future studies.

In general, it seems that direct injection of MSCs is more effective in increasing mechanical parameters of the cartilage and improving its mechanical strength, which may be due to the role of stem cells in chondrogenesis and increase in synthesis of extracellular matrix, including collagen and glycosoaminoglycan. Although *Elaeagnus angustifolia* extract increased the mechanical parameters compared to the MIA group, but in none of the cases, this increase reached a significant level, even though, just like the cell group, instantaneous stiffness of the cartilage in this group was close to the normal level and did not show a significant difference with the obtained value in the control group. Complementary studies on the mechanical performance of the joint such as joint friction measurement as well as histological and immunohistochemical studies in future research may help confirm these results.

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