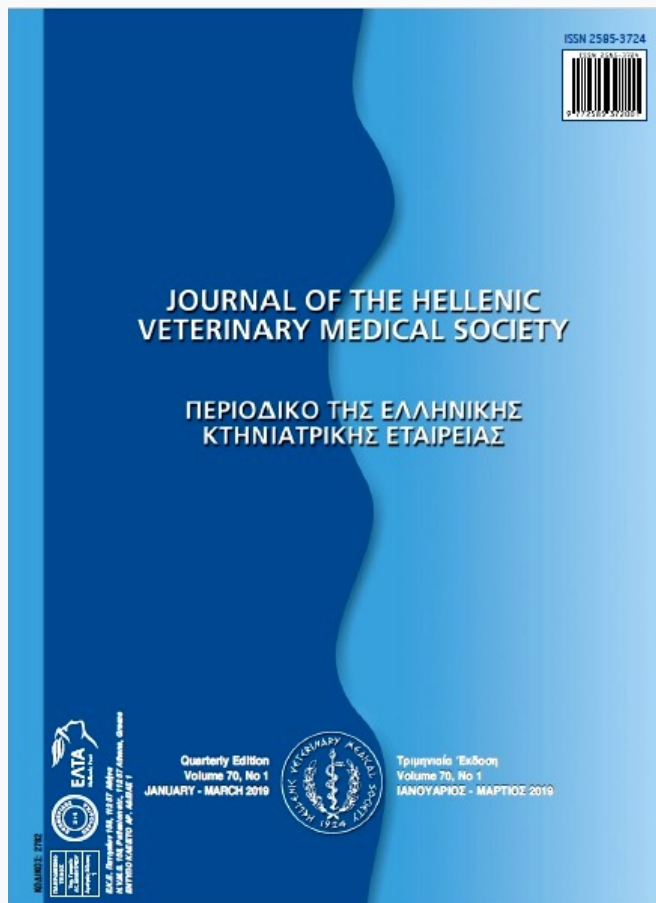


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The healing effect of bone marrow-derived stem cells and aquatic activity in Achilles tendon injury

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ABSTRACT. The common treatment recommended for Achilles tendon rupture as the most common tendon injury during exercise is surgical intervention, while it eventually causes various clinical problems. This study assessed the healing effect of bone marrow-derived stem cells (BMSCs) and aquatic activities in Achilles tendon injury. Forty rats were randomly divided into 5 equal groups. Group 1 underwent aquatic activity, 72 h after a crush lesion formed on Achilles tendon, group 2 received 1×10^6 intra-articular BMSCs post-tendon injury, group 3 had aquatic activity together with BMSCs transplantation after tendon damage, group 4 just experienced tendon injury without any treatment intervention and group 5 was considered as the control group while did not undergo any tendon injury and did not receive any treatment measure. After 8 weeks, the animals were sacrificed and the tendons were transferred in 10% formalin for histological evaluation. There was a significant increase in fibroblast number in group 3 in comparison to other groups. However, there was a significant increase in collagen deposition in groups 2, 3 and 5 in comparison to group 1 and 4. A significant decrease was noted for cellularity in group 2 when compared to groups 1 and 4. Regarding tendon diameter in group 3; a significant healing was observed when compared to groups 2, 4 and 5. It was shown that aquatic activity together with cell transplantation was an effective therapeutic measure enhancing the healing in tendon injuries. These findings can open a window in sport medicine in treatment of tendon injuries.

Keywords: Aquatic activity, Bone marrow, Mesenchymal stem cells, Healing, Tendon.

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INTRODUCTION

The common treatment recommended for Achilles tendon rupture as the most common tendon injury during exercise is surgical intervention, while it eventually causes various clinical problems (Rodeo et al., 2010). During the recent decade, it was shown that biological factors may accelerate tendon healing in damaged tendons (Rajabi et al., 2015). Although many studies have indicated that exercising and physical activity is among effective factors in increasing tendon strength, the question whether physical activity after the surgery could accelerate the recovery of tendons biomechanical properties and lead to fundamental changes in healing process have still remained controversial (Rajabi et al., 2015).

Several researchers have mentioned the significant role of mesenchymal stem cells (MSCs) in tendon healing (McGoldrick et al., 2017; Romero et al., 2017). MSCs have been isolated from different tissues including bone marrow (BM) (Aliborzi et al., 2015), adipose tissue (Mehrabani et al., 2015a), menstrual blood (Famarzi et al., 2016), dental pulp (Mehrabani et al., 2017), and endometrium (Shamosi et al., 2017). They have osteogenic (Shaterzadeh Yazdi et al., 2015), adipogenic (Mehrabani et al., 2015b) and neurogenic (Razeghian Jahromi et al., 2015) differentiation properties with similar features such as favorable proliferative capability, self-renewal, and differentiation potential (Mehrabani et al., 2013). Bone marrow-derived stem cells BMSCs) have opened a new window in regenerative medicine and cell transplantation purposes getting help from tissue engineering too (Hosseinkhani et al., 2014).

The beneficial effects of MSCs in healing of Achilles tendon injuries were previously reported demonstrating that use of MSCs could improve the biological and mechanical parameters of the tissue (Chong et al., 2007). Therefore, this study was conducted to determine the healing effect of a combination of BMSCs and aquatic activities in Achilles tendon injuries in experimental rat model.

MATERIAL AND METHODS

Forty male Sprague-Dawley rats weighing 200 ± 20 g and aging 3 months were provided from Laboratory Animal Center of Shiraz University of Medical

Sciences, Shiraz, Iran and housed in groups of 4 in transparent polycarbonate cages with dimensions of $54\times 18\times 18$ cm at an environment of $21\pm 2^\circ\text{C}$ with the lighting of 12:12 light-to-dark ratio; light at 7:30 AM in 50 ± 5 percent humidity. All procedures were done based on laws of Animal Care by Iran Veterinary Organization. The rats were randomly divided into 5 equal groups.

Group 1 underwent aquatic activity, 72 h after a crush lesion formed on Achilles tendon using a mosquito hemostat (Akinbo et al., 2008), group 2 received 1×10^6 intra-articular BMSCs post-tendon injury, group 3 had aquatic activity together with BMSCs transplantation after tendon damage, group 4 just experienced tendon injury without any treatment intervention and group 5 was considered as the control group while did not undergo any tendon injury and did not receive any treatment measure.

In order to prepare BMSCs, 5 rats were euthanized and under sterile conditions, femoral and tibial bones were removed while, the muscular and connective tissues were later detached. Under sterile conditions, both ends of the bones were cut and the BM was flushed out using a 10 ml syringe filled with Dulbecco's Modified Eagle Medium (DMEM; Biovet, Bulgaria) and 1% penicillin streptomycin (Sigma, USA). BM was then transferred on ice to the stem cell laboratory, Shiraz University of Medical Sciences, Shiraz, Iran.

The BM was diluted in an equal volume of DMEM, and centrifugation was undertaken at 1200 rpm for 7 minutes. After removal of the supernatant, the precipitate was transferred in 25 cm² flasks containing DMEM supplemented with 10% fetal bovine serum (FBS; Biovet, Bulgaria), 1% L-glutamine (Sigma, USA) and 1% penicillin and streptomycin and the culture flasks were placed in CO₂ incubator at 37°C with 5% CO₂ and saturated humidity while the medium was replaced every 3 days. The adherent cells were subcultured at 80% confluency, by washing twice with PBS (Gibco, USA) and adding 0.25% trypsin (Gibco, USA) for 3 minutes. To inactivate the trypsin activity, equal volume of DMEM was added. Cell passage was continued until passage 5 and at each passage, the cells were counted too. Cell morphology was assessed by inverted microscope (Olympus, USA) as described by Gashmardi et al. (2017).

To assess the osteogenic differentiation property, BMSCs from passage 5 were seeded into 6 well plates and at 80% confluency, the cells were cultured for 21 days with low glucose DMEM containing 100 nM dexamethasone (Sigma, USA), 0.051 M ascorbate-2-phosphate (Wako Chemicals, USA), 10 mM b-glycerophosphate (Sigma, USA), 1% penicillin/streptomycin and 10% FBS. The medium was changed every 3 days. After 3 weeks, osteogenic differentiation was evaluated with Alizarin Red (Sigma, USA) staining (Gashmardi et al., 2017).

RT-PCR was done to determine the expression of MSC markers. So after extraction of the total RNA using the column RNA isolation kit (Denazist-Asia, Iran) based on manufacturer's instructions, it was evaluated by spectrophotometry. The complementary DNA (cDNA) was provided by AccuPower Cycle Script (RT PreMix Kit Bioneer, Korea) upon manufacturer's instruction. For each reaction; 15 µL of total RNA was used to reach a volume of 20 µL with the DEPC water. Twelve thermal cycles were conducted including 30 s at 20°C for primer annealing, 4 min at 42°C for cDNA synthesis, and 30 s at 55°C for melting secondary structure and cDNA synthesis and 5 min at 95°C for inactivation (Rahmanifar et al., 2016).

Then, 1 µL of template cDNA and PCR buffer, H₂O, dNTPs, MgCl₂, Taq DNA polymerase, and forward and reverse primers were mixed. The microtubules containing 20 µL of the mentioned mixture were transferred to a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany) and 30 amplification cycles were performed (30 s denaturation at 95°C, 30 s annealing at 64°C, 62°C, and 61°C and 30 s extension at 72°C with the 5 min at 95°C for primary denaturation and 5 min at 72°C for final extension). PCR products were assessed for defined bands by gel electrophoresis by DNA safe stain in 1.5% agarose gel medium. The bands were seen by UV radiation and a gel documentation system (UVtec, Cambridge, UK) and photography was done.

Aquatic activity was undertaken for 8 weeks (five sessions per week). For adaptation, rats were put into a glass aquarium with the dimensions of 50×40×50 cm filled with 34°C water for 5 minutes for 3 days. Then the aquatic activity was extended for 8 weeks

5 days per week (5-30 minutes each session, Table 1) as described by Hart et al. (2001) and McVeigh et al. (2010). After 8 weeks, the animals were euthanized and sacrificed and the Achilles tendon was removed and

Table 1. Eight-week incremental aquatic activity program of studied rats.

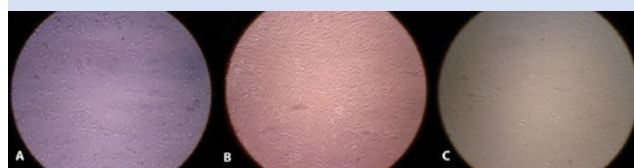
Week	Stage
	Introduction stage: In this stage, to have optional activity, rats were put into a glass aquarium
1	With the dimensions of 50×40×50 cm filled
2	with 34°C water for 5 minutes for 3 days.
3	Aquatic activity: 5 min: Overload Stage:
4	days 1 to 5
5	days 1 to 5: Aquatic activity: 10 min
6	days 1 to 5: Aquatic activity: 15 min
7	days 1 to 5: Aquatic activity: 20 min
8	days 1 to 5: Aquatic activity: 25 min
	days 1 to 5: Aquatic activity: 25 min
	days 1 to 5: Aquatic activity: 30 min
	days 1 to 5: Aquatic activity: 30 min

transferred into 10% formalin buffer for histological evaluation using hematoxylin and eosin (H&E) staining. The studied factors in histological evaluation were the number of fibroblasts, collagen deposition, cellularity and tendon diameter. For statistical analysis, SPSS software (version 16, Chicago, IL, USA) was used applying Kolmogorov Smirnov, one-way ANOVA and Tukey post hoc tests. The significance level was considered a p value less than 0.05.

RESULTS

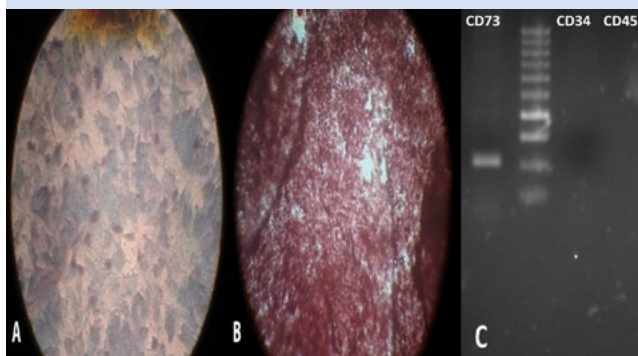
BMSCs were plastic adherent and fibroblast-like throughout all passages (Figure 1A-C). Culture of BMSCs in osteogenic media resulted into osteogenic differentiation of these cells due to presence

Figure 1. BMSCs were fibroblast-like throughout all passages. A: Passage 1, B: Passage 2 and C: Passage 3.



of calcium deposits after three weeks verified by Alizarin Red staining (Figure 2A, 2B). They showed

Figure 2. The osteogenic differentiation of BMSCs by Alizarin Red staining. A: Control, B: Osteogenic induction). C: The positive expression of CD73 marker confirming the mesenchymal stem cell property and negative expression of CD45 for markers of hematopoietic stem cells.



positive expression of CD73 marker confirming the mesenchymal stem cells and absence of CD34 and CD45 as markers for hematopoietic stem cells (Figure 2C). Table 2 shows the histological findings denoting to a significant increase in the number of fibroblast in group 3 in comparison to other groups (Figure 3A). A significant increase was visible in collagen deposition in groups 2, 3 and 5 in comparison to group 1 ($p=0.001$) and 4 ($p=0.001$,

Figure 3. The mean and standard deviation of Fibroblast numbers (A), Collagen deposition (B), Cellularity (C) and Tendon diameter (D) were shown in different groups. * $P<0.05$.

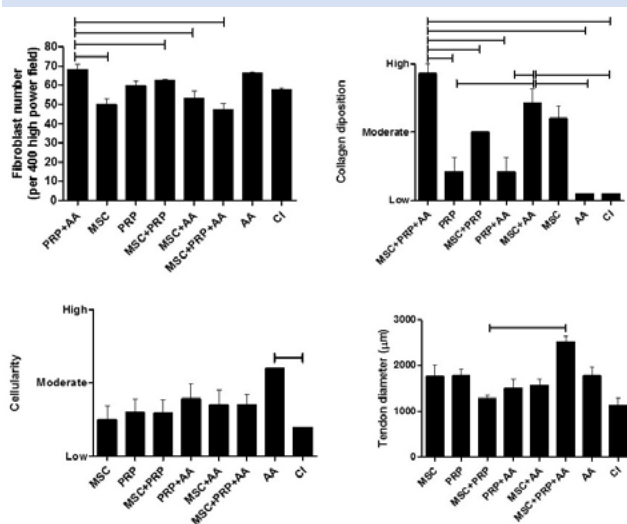
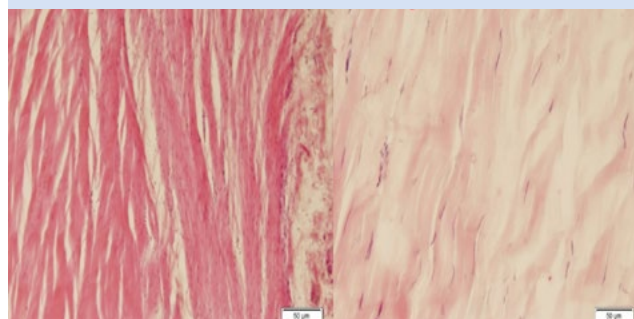


Figure 3B). There was a significant decrease in cellularity in group 2 compared to groups 1 ($p=0.009$) and 4 ($p=0.04$, Figure 3C).

Regarding tendon diameter revealing healing process, in the group receiving BMSCs and undergoing aquatic activity, an increase in tendon diameter was seen compared to the group just underwent cell transplantation ($p=0.04$) and the group with tendon injury and any treatment measure ($p=0.01$) and the control group with no tendon injury ($p=0.04$, Figure 3D). Figure 4 shows a moderate healing in Achilles tendon with collagen deposition in group 3.

Figure 4. Moderate healing in Achilles tendon with collagen deposition.



It shows that BMSCs together with aquatic activity could increase the collagen deposition compared to other groups while the difference was statistically significant too.

DISCUSSION

Our findings suggested that transplantation of BMSCs along with aquatic activities could be effective in repair of Achilles tendon injuries. The histological factors in assessment of healing of Achilles tendon injuries showed that the number of fibroblasts in transplantation of BMSCs along with aquatic activities had a statistically significant higher healing property in comparison to other groups. In response to tendon damage, fibroblasts migrated to the inflamed wound area together with type III collagen which later was substituted by type I collagen (Lisa and Zena, 2002). It was shown that the mechanical pressures due to the physical activities affected the collagens homeostasis that increased in the injured tissues, such as tendons and ligaments (Heinemeier, 2007). Injection of BMSCs along

Table 2. Mean and standard deviation of effective factors related to the healing of Achilles tendon in studied groups.

Variables	Collagen deposition				
Group	Fibroblasts	Cellularity	Tendon diameter	Total	
1: Aquatic activity	51.25±10.8	1±0.0	2±0.0	1704.78±412.1	1759.03±408.6
2: BMSC transplant	48.75±7.9	2.5±0.75	1.2±0.46	1589.9±307.5	1642.49±308.5
3: BMSC transplant +Aquatic activity	65.25±2.9	2.5±0.53	1.6±0.51	2187.32±689.9	2256.7±690.39
4: Tendon injury without treatment	50.75±6.3	1±0.0	1.87±0.35	1485.97±307.08	1539.6±310.5
5: Control with no tendon injury	51.62±5.2	2.87±0.35	1.5±0.53	1582.33±128.37	1638.33±12.13

with aquatic activities or injection of BMSCs alone was demonstrated to increase collagens deposition significantly in comparison to injury group. Physical activity was shown to increase the number of fibroblasts and as a result, the collagen deposition.

Tendons are considered as facilitators of force transfer and the arrangement of tendon fibers is important in passive forces and pressures absorbing, as tendon strength is dependent on orientation and length of collagen fibers and consistency of transplanted tendons. The collagen and transplanted tissue metabolism was shown to be influenced by the amount of physical activity. Physical activity was demonstrated to increase the differentiation and change of the transplanted tissue in the tendons denoting to the physiological adaptations and repair of the damages on the extracellular matrix based on physical activities (Kjaer, 2004). In our study, injection of cells alone lead to an increase in collagen deposition, which substituted various cellular sources for tendon repair. Similarly, the role of MSCs in tendon repair was shown before, due to their high proliferation capacity and differentiation properties (Harris et al., 2004).

We reported a significant increase in cellularity in

the group undergoing aquatic activity, compared to the control group that underwent just injury. Platelet rich plasma (PRP) was identically used to increase the performance and cellularity of the tendons and the amount of collagens and cellularity that were due to the increase in the metabolic activity and its positive impact on tissue repair (Lane et al., 2013). Our findings are also in accordance with the aforementioned study on the increase in cellularity.

We showed that the tendon diameter in the group undergoing aquatic activity together with cell transplantation revealed a significant increase. Similarly, it was also demonstrated that physical activity on treadmill for a week could increase the size of collagen fibers and tendon diameter in rats (Michna and Hartmann, 1989). In another study, an intense endurance exercise was found to increase in collagen deposition in Achilles tendon (Curwin et al., 1988). The physical exercise was noted to increase several growth factors in Achilles tendon which could stimulate the collagen synthesis and cell proliferation (Wang, 2006).

Rajabi et al. (2015) in their study on the healing effects of aquatic activities and allogenic injection of PRP on injuries of Achilles tendon based on

the number of fibroblast, cellular density, collagen deposition, and tendon diameter, showed that aquatic activity together with PRP injection was the therapeutic measure of choice enhances healing in tendon injuries. Godwin et al. in their study on implantation of BMSCs in horse with overstrain injury of the superficial digital flexor tendon showed that cell implantation was safe and appeared to reduce the re-injury rate (Godwin et al., 2012). Lacitignola and colleagues (2008) in cell therapy for tendinitis in horses by BMSCs together with exercise program stated that re-injury rate reduced and good to excellent outcome in term of athletic success were noted.

He et al. (2015) by applying BMSCs revealed attenuated adhesions in the early time point following flexor tendon repair and healing in rabbit flexor tendon. Human adipose stem cells were also shown as another cell source as a potential approach for tendon tissue repair (Vuornos et al., 2016). Transplantation of tendon-derived stem cells pre-treated with connective tissue growth factor and ascorbic acid as a different source of cells was found to promote better tendon repair in a patellar tendon window injury rat model (Lui et al., 2016). Umbilical cord blood-derived mesenchymal stem cells in a rabbit model with full-thickness rotator cuff tendon tear demonstrated the effectiveness of local injection of MSCs into the rotator cuff tendon to be a useful

conservative treatment for full-thickness rotator cuff tendon tear repair (Park et al., 2015).

CONCLUSION

Identical to many studies that did not report pain for experimental animals, this limitation existed in our study too. We showed that injection of MSCs together with the aquatic activity could promote healing process that may be due to accumulation of monocytes, proliferation and differentiation of stem cells, migration of fibroblasts and angiogenesis. So based on our findings on the number of fibroblast, cellularity, collagen deposition, and tendon diameter, it was shown that aquatic activity together with MSC injection was a therapeutic of choice enhancing the healing of tendon injuries that can open a window in treatment of damages to tendons.

Disclosure of potential conflicts: All authors declare no financial or other conflicts of interest.

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CONFLICT OF INTEREST STATEMENT

No potential conflict of interest relevant to this article was reported. ■

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