EXPERIMENTAL STUDY

Acta Orthop Traumatol Turc 2014;48(2):187-195
doi: 10.3944/AOTT.2014.2985

Mesenchymal stem cell application improves tendon healing via anti-apoptotic effect (Animal study)

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Objective: The aim of this study was to determine the effects of mesenchymal stem cell (MSC) application and the possible pathways of MSC's effects on tendon strength and healing after tendon repair.

Methods: The study included 40 Wistar albino rats. Mesenchymal stem cells were obtained from the femurs and tibias of 6 rats. Achilles tendons of the remaining 34 rats were cut and repaired with open surgical procedures. Rats were divided into 2 groups. Percutaneous MSCs were applied to the study group (n=17) and physiological serum only was applied to the control group (n=17) at the 4th week. Rats were sacrificed using the cervical dislocation method under ether anesthesia at the 12th week and samples were analyzed by histological and immunohistochemical methods. For biomechanical analysis, a traction force was applied at 10 mm/min and load to failure was recorded for each specimen in Newtons.

Results: Histologically, there was no significant difference between groups ($p>0.05$). In the immunohistochemical studies, MSCs were located more intensively at the repair zone. Apoptosis was minimally present in the study group and was clearly increased in the control group. Increase in tendon strength was significantly higher in the study group than in the control group at the 12th week ($p<0.05$).

Conclusion: The application of MSCs to decrease re-ruptures has a positive effect on tendon strength, probably due to their anti-apoptotic effects. Mesenchymal stem cell application can be used percutaneously and is effective in clinical practice in the late stages of tendon healing.

Key words: Apoptosis; mesenchymal stem cell; tendon healing.

Mesenchymal stem cell (MSC) application studies have shown a considerable increase in tendon strength. However, no histopathologic differences have been demonstrated and the mechanism of strength increase has not yet been fully explained.[1]

Tendon healing involves three phases; inflammation, repair and remodeling. At the second phase of tendon healing, the number of healing tendon fibroblasts in the wound increases to carry out matrix deposition and new collagen fibers are formed. At the late healing phase of

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Submitted: July 21, 2012 **Accepted:** October 09, 2013

Available online at
www.aott.org.tr
doi: 10.3944/AOTT.2014.2985
QR (Quick Response) Code

normal tendon healing in particular, the increased number of fibroblasts in the healing mass must decrease in order to restore normal cell-to-matrix ratio. This process occurs through apoptosis of healing tendon fibroblasts which increase in number.[2] It has been shown in rat patellar tendon injury models that maximum apoptosis occurs at the 28th day of healing.[2] In addition, Steiner reported that during the tendon healing process, tendon strength is 50% of normal at the 3rd week and 25% at the 4th week of healing.[3]

The aim of this experimental study was to investigate the possible effects of MSC application in the late stages of tendon healing in terms of histopathologic and biomechanical properties. We hypothesized that MSC application may reduce apoptosis during tendon healing. This process may be one of the pathways of MSC's effects on tendon healing.

Materials and methods

This study included 40 female, non-pregnant Wistar albino rats weighing 200 to 300 grams. In the first step of the study, 6 rats were sacrificed and their femurs and tibias excised in order to obtain rat bone marrow-derived mesenchymal stem cells (rBM-MSCs). The remaining rats were divided into two groups. The study group (n=17) was given rBM-MSCs and the control group (n=17) physiological serum. Rats were sacrificed using the cervical dislocation method under ether anesthesia at the 12th week of the study. The experimental design and all procedures were approved by the University Animal Study Ethics Committee.

The isolation and culture of rBM-MSCs were performed as previously described by Karaoz et al.[4]

To confirm that rBM-MSCs maintained their phenotypic characteristics after growth in culture, undifferentiated stem cells were subjected to flow cytometry analysis. Three surface markers of rBM-MSCs at Passage 3 were assayed as previously described by Karaoz et al and Kiliç et al.[4,5]

rBM-MSCs were plated at a density of $1x10^6$ cells into T75 flasks and incubated for 2 days until they reached 50% confluence. The medium was exchanged with the same medium containing 10 mM of BrdU (Sigma, St. Louis, MO, USA). After 48 hours of incubation at 37°C and 5% CO2, the BrdU-labeled MSCs were trypsinized and evaluated for viability.[6]

Rats were anesthetized intraperitoneally with 50 mg/kg ketamine HCl (Ketalar®; Pfizer) combined with 5 mg/kg xylazine HCl (Rompun®; Bayer). All rats were operated under sterile conditions by the same surgeon. A 2 cm longitudinal incision was made in the midline of the right cruris, exposing the Achilles and plantaris tendons. A transverse incision was made in Achilles tendons at 0.5 cm proximal to their calcaneal insertion. The plantaris tendons were left intact to serve as internal splints. The Achilles tendons were then repaired with 3/0 Ethibond sutures using a modified Kessler-type suture technique and the skin was then sutured with 4/0 monofilament nylon.[7,8] Postoperatively, rats were placed in plastic cages and monitored without immobilization.

At the 4th week after the surgical procedure, rBM-MSCs (0.5 mL, $1.0x10^6$ cells) were injected percutaneously around the repair site peritendinously in the study group and physiological serum in the control group. This procedure was done under ether anesthesia in both groups.

Of the 34 rats included in this study, 1 rat in the control group died at the 3rd week and 1 rat in the study group was excluded due to an incorrect sampling of the Achilles tendon. Randomly, 14 rats were selected for biomechanical testing (study group n=7, control group n=7) and the remaining 18 rats were selected for immunohistochemical examination (study group n=9, control group n=9). All specimens are examined in a blinded manner. There were no infection signs in any rats.

For histological and immunohistochemical assessments, 9 tissue samples were obtained from each group. Tissues were fixed in 10% formalin for approximately 24 hours and then embedded in paraffin.[6] Four longitudinal serial sections, each 5 μm thick, were taken from each paraffin embedded tendon sample. Hematoxylin and eosin (H&E) and Alcian blue staining were performed. Intensity of fibrosis, density of blood capillaries in tendon, distribution of inflammatory cells and cartilaginous metaplasia were evaluated. Inflammation, fibrosis and vascularization was graded as 0 (no), 1 (minimal), 2 (moderate) or 3 (severe); cartilaginous metaplasia was graded according to its presence: 0 (no) or 1 (present).[7]

To perform cell tracing after injection of the BrdU-labeled rBM-MSCs, the immunofluorescence double-staining protocol previously described by Adas et al. was used.[6]

Tendon samples were embedded in paraffin to prepare 5 longitudinal sections at a thickness of 5 μm. TUNEL and Caspase-3 immunohistochemical staining were used to evaluate apoptosis and anti-BrdU immunohistochemical staining to mark BrdU-positive cells. Fibronectin and desmin monoclonal antibodies were used as second staining for samples that were stained with BrdU and Caspase-3. The number of TUNEL-

positive cells in samples were determined by counting the stained cells per area (294 mm^2 each) in 3 randomly selected sections on slide and normalized to the total area. The total number of cells was determined with a light microscope (Leica DMI 4000B; Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany).

For mechanical testing, the harvested Achilles tendons with the calcaneus on the distal end and a portion of muscle on the other end were placed into a container with 0.9% saline-soaked gauze and biomechanical measurements were carried out in 3 hours. The Achilles tendons were mounted with special clamping jaws on the testing device. A traction force was applied at 10 mm/min and load to failure was recorded for each specimen in Newtons.

Data were analyzed using SPSS for Windows v13.0 (SPSS Inc., Chicago, IL, USA) software. Statistical analysis of the data obtained from the histological evaluation of the 18 specimens was performed using the chi-square test. The non-parametric Mann-Whitney U test was used for the analysis of biomechanical data of 14 specimens. A p value of less than 0.05 was considered statistically significant.

Results

Mesenchymal stem cells attached to the culture flasks sparsely and displayed a fibroblast-like, spindle-shaped morphology during the initial days of incubation. After 3 to 4 days of incubation, proliferation began and the cells gradually grew into small colonies. By Day 6 to 8, colonies with different sizes increased in number (Figs. 1a and b). As growth continued, adjacent colonies interconnected with each other and a monolayer confluence was obtained after 12 to 15 days and the viability was higher than 95%.[6]

rBM-MSCs expressed CD29 and CD90 antibodies, but not CD45 and maintained their phenotype throughout passages (Fig. 1e).

While fibrosis and increased vascularity were seen more prominent in the control group, the difference was not statistically significant (p>0.05). There were no differences in inflammatory cells and cartilage metaplasia between groups (p>0.05).

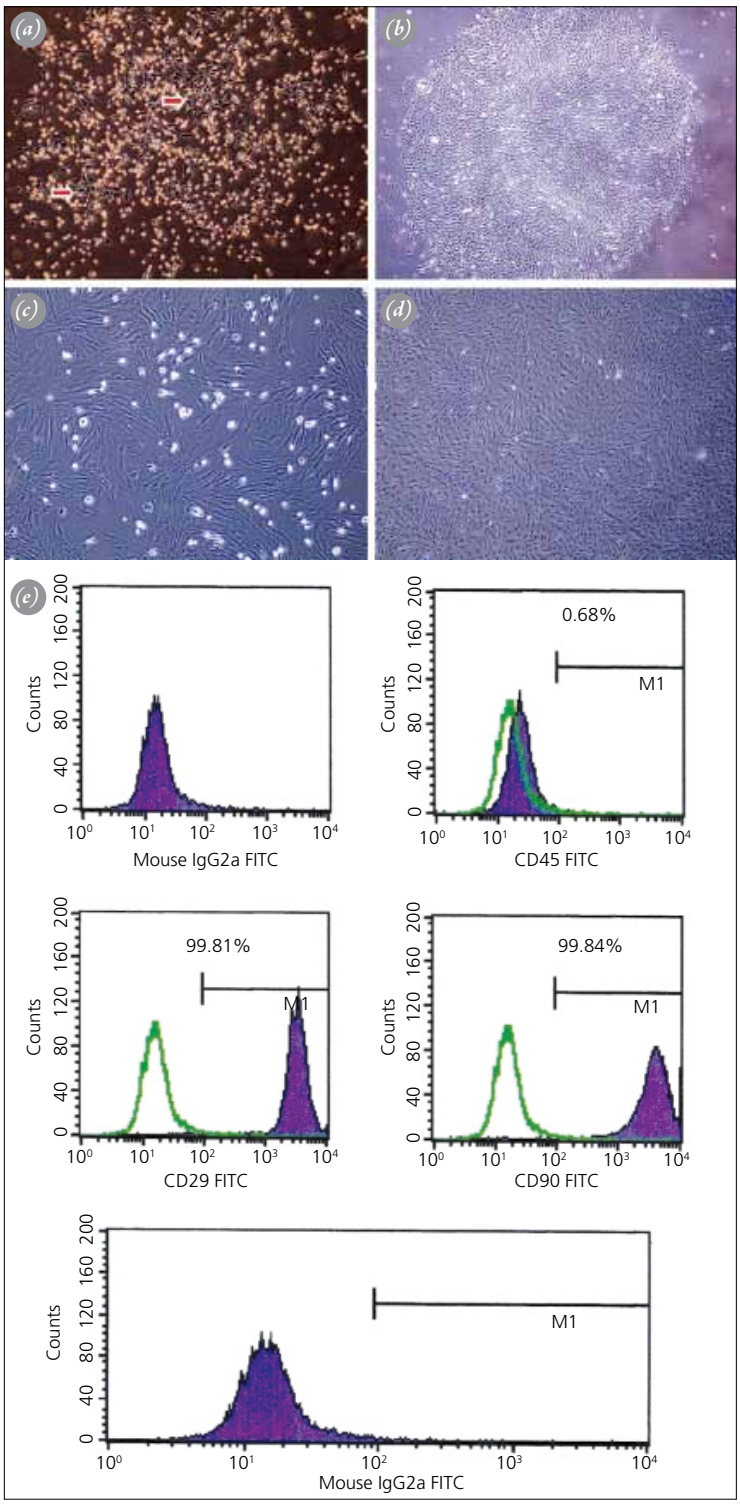

Fig. 1. **(a, b)** Fields showing rBM-MSC morphologies for primary culture and **(c, d)** different passages. **(a)** During the onset of culture (P0-7th day), the isolated cells from rBM formed single-cell-derived colonies (shown by arrows). **(b)** At P0-9th day, single cell colony is larger. Later passages, (**c**: P1-5th day, **d**: P4-9th day) most of these MSCs exhibited large, flattened or fibroblast-like morphology (original magnifications: **(a-c)**: x40, **d**: x100). **(e)** Flow cytometry analysis of cell-surface markers in rBM-MSCs at Passage 3. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Collagen fibers lost their organized structures. Young connective tissue, especially around the repair site, was observed to be less organized, mixoid and rich in cellularity. In the histological evaluation, there were no tissue differences indicating any immune response and rejection.

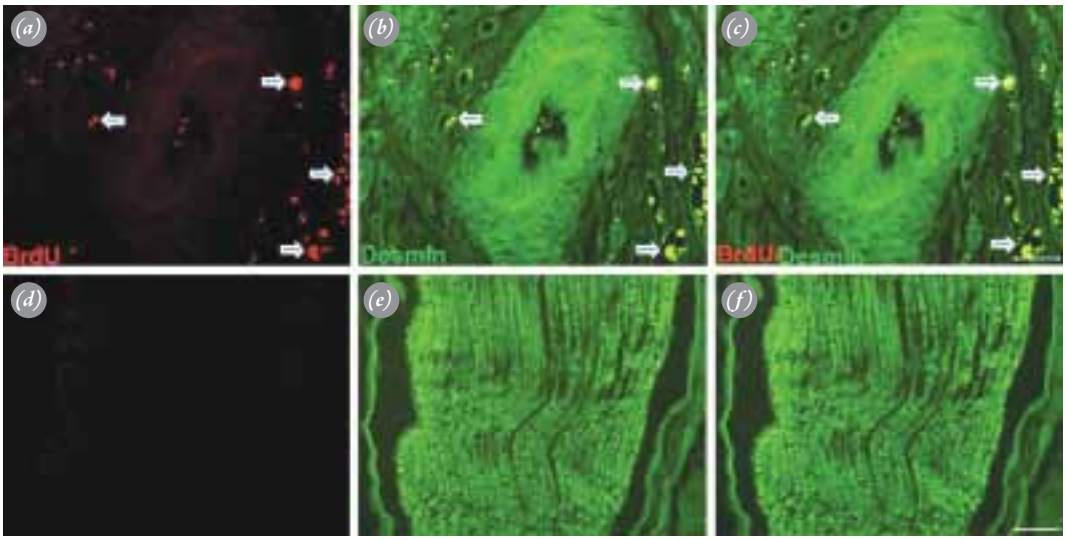

Fig. 2. **(a-f)** Cross-sections belonging to control group tendons after immune staining for BrdU and desmin. No positive staining is observed for BrdU except autofluorescent intravascular erythrocytes (shown by arrows) (Scale bars: 50 μm). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

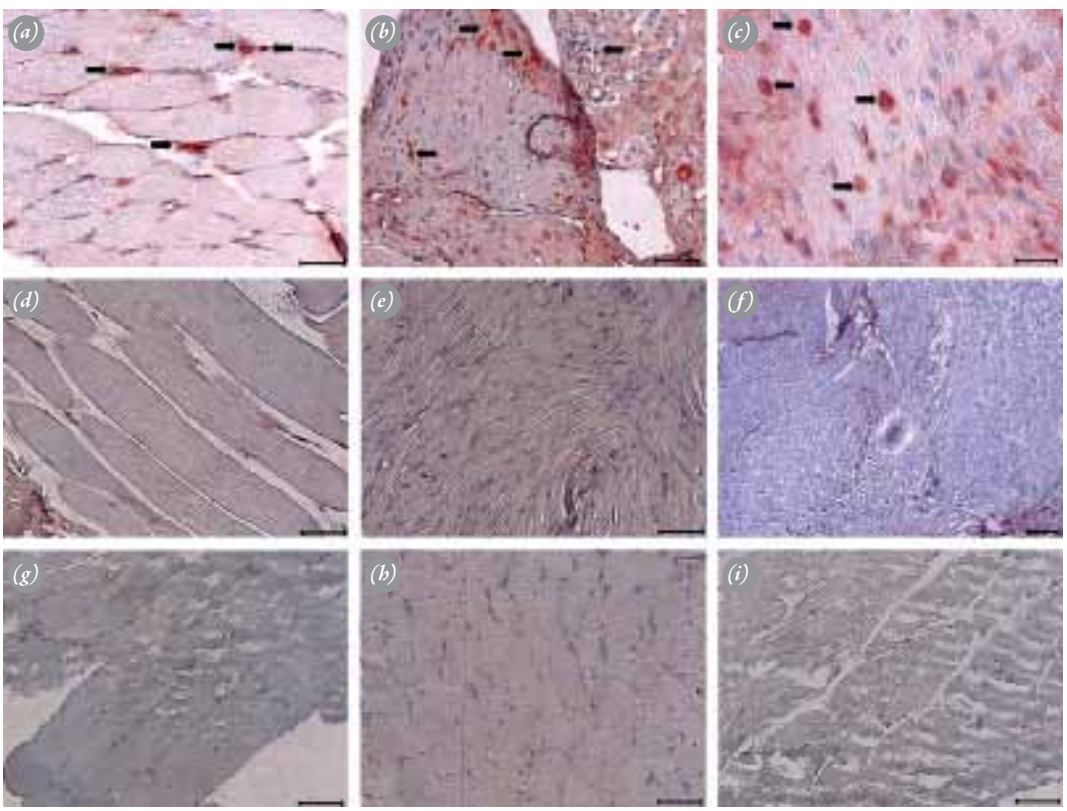

Fig. 3. Results of immunohistochemical staining for BrdU on tendons. **(a-c)** Study group tendons, **(d-f)** control group tendons, and **(g-i)** normal tendons. In the group with stem cell application, BrdU-positive cells are observed (shown by arrows) whereas no such positivity is observed in the other groups (Scale bars: a-c, d, e, g-i: 50 μm, F: 70 μm). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

In the control group, no positive staining was observed under immunofluorescence microscope in addition to the intravascular autofluorescence erythrocytes after immunostaining with anti-BrdU antibodies (Fig. 2). Normal tendon samples were also taken in order to evaluate BrdU immunohistochemical staining results (Figs. 3g-i). As a result, BrdU-positive cells were not seen in tendon specimens of the control group in either microscopic examination (fluorescence and light microscopy).

After rBM-MSC application at the 4th week, BrdU-positive cells were observed more intensively at the repair sites of samples taken at the 12th week. BrdU-positive cells were seen in all of the samples taken from different tendon sites, revealing that applied rBM-MSCs are not located only at the injury site (Figs. 3a-c and Fig. 4).

Although diffuse apoptosis was seen in all samples in the control group (mean cell number ratio: 28±3.75), it was significantly lower in the study group (mean cell number ratio: 12±4.30) with TUNEL and Caspase-3 immunostaining at the 12th week ($p<0.05$) (Figs. 5 and 6).

The average load to failure of tendons was 38.3 N in the study group (n=7) and 23.7 N in the control group (n=7) (Table 1). The difference between groups was statistically significant ($p<0.05$). The rupture point was calcaneal insertion in 10 rats, proximal to repair site in 2 rats and repair site in 2. Although there was no rupture

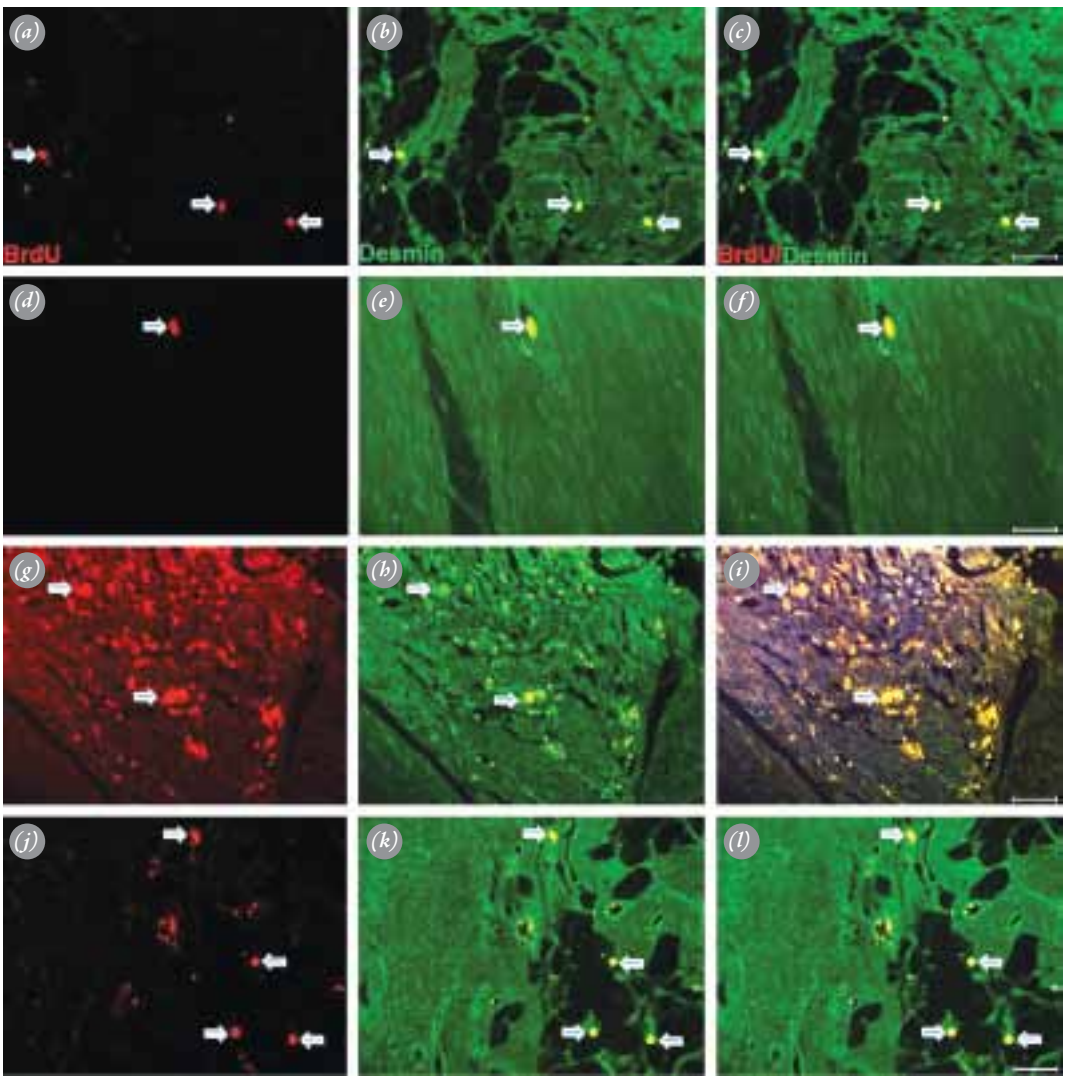

Fig. 4. **(a-l)** Microscopic views of cross-sections obtained from different rats belonging to study group (group with rBM-MSC application) after immunofluorescent staining. Nuclei of the BrdU-labeled stem cells are observed in red (shown by arrows). Cell components reacting positively to desmin (green) represent tenocytes or fibroblasts (Scale bars: 50 μm). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

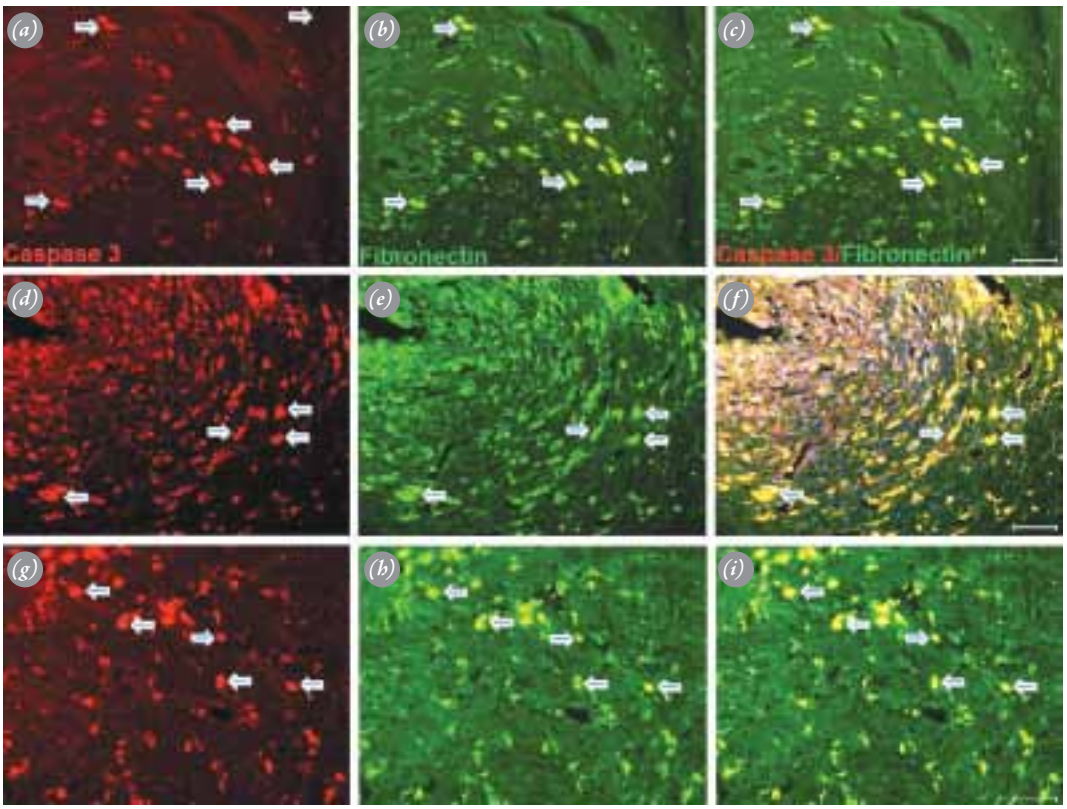

Fig. 5. **(a-i)** Cross-sections belonging to the control group after immune staining for Caspase-3 (TR-red) and fibronectin (FITC-green). In the cross-sections obtained from different rats, apoptotic cells reacting positively to Caspase-3 (nuclear) are observed in abundance (shown by arrows) (Scale bars: 50 μm). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

of the repair site in the study group, 2 tendons of the control group ruptured from repair site ($p>0.05$).

Discussion

Tendon healing involves inflammation, cell migration and cell maturation in the early stages. The number of fibroblasts in the wound increases rapidly to carry out matrix deposition and remodeling. During inflammation, growth factors and cytokines stimulate fibroblasts to maturate and secrete extracellular matrix. New collagen fibers are formed and cell-to-matrix ratio is restored. In the late stages of tendon healing, the number of cells decreases in order to restore the cell-to-matrix ratio. Although the exact mechanism is unknown, Lui et al.[2] has attributed it to apoptosis. He demonstrated that fibroblast-like cells increase in number at Day 4 before decreasing in the following days and that maximum apoptosis occurs at Day 28. Increased incidence of re-rupture at late stages of tendon healing may be due to the increase in apoptosis to restore cell-to-matrix ratio.

The therapeutic effects of MSC are believed to occur not only by direct differentiation into injured tissue but also by production of paracrine and autocrine factors. Mesenchymal stem cells at the injury site can promote the secretion of a variety of cytokines and growth factors that have both paracrine and autocrine activities.[9,10] Several studies have been performed on this topic. In examining the mechanism of treating effects of MSCs, several researchers observed that when MSC is transplanted into a heart muscle with infarction, the local hypoxia

Table 1. Load to failure of tendons in the two groups.

	Load to failure (N)				
	n	**Minimum**	**Maximum**	**Mean**	**Standard deviation**
Study group	7	22.1	67.1	38.28	14.777
Control group	7	15.3	40.5	23.68	9.07

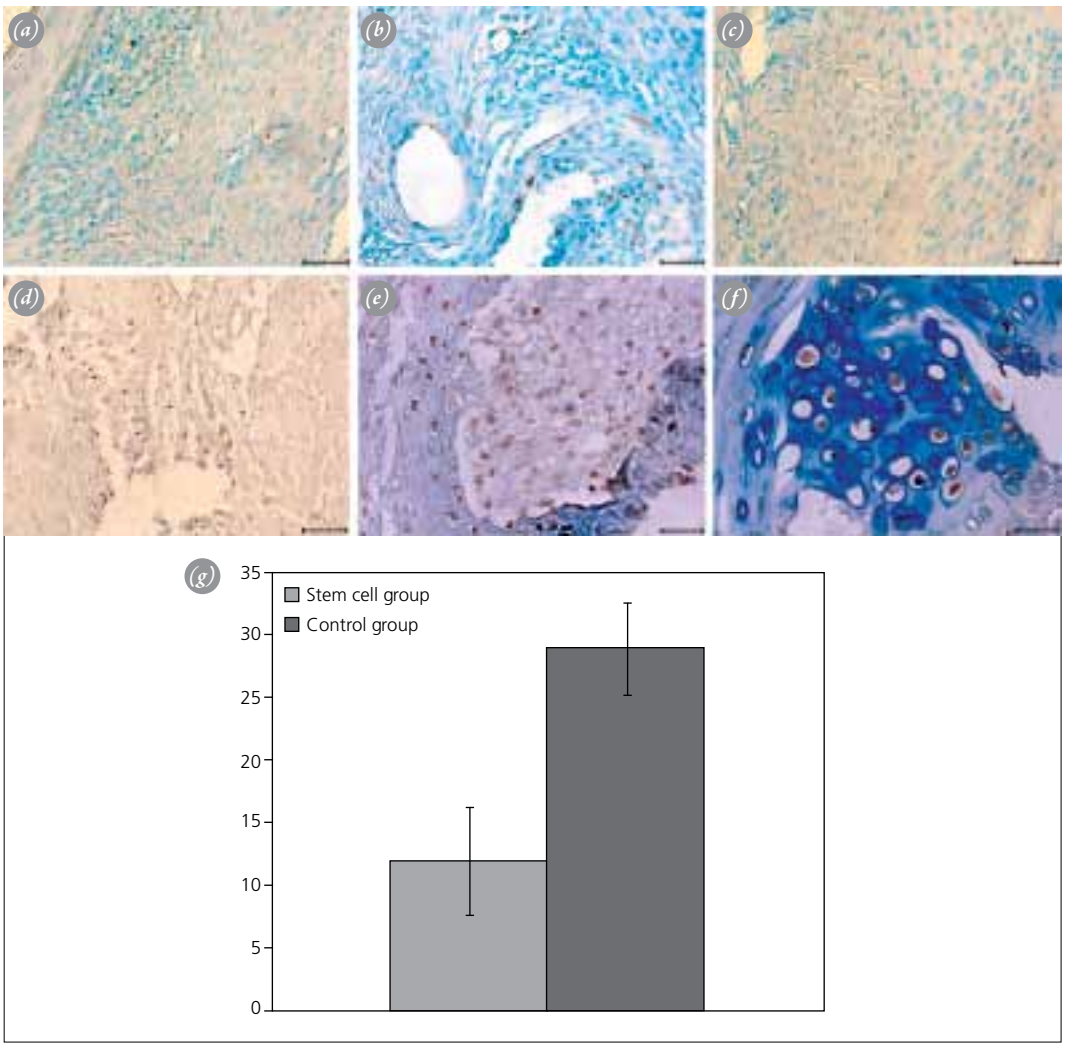

Fig. 6. Microscopic views of the paraffin-embedded sample sections after immunostaining with TUNEL for **(a-c)** the study group with rBM-MSC application and **(d-f)** control group without rBM-MSC application. Apoptotic cells with nuclei observed in brown are considerably fewer in the study group compared to the control group. **(e, f)** Apoptosis in fibrocartilaginous tissue cells is particularly remarkable. **(g)** In the group with stem cell application, number of TUNEL-positive cells was significantly lower ($p<0.05$) compared to the control group (Scale bars: 50 μm). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

promotes a greater secretion of cardioprotective growth factors than in in vitro normoxic conditions.[11] Co-culturing with rBM-MSCs might have significant potential in protecting streptozotocin (STZ)-induced injured pancreatic islets through paracrine actions (cytoprotective, anti-inflammatory and anti-apoptotic effects).[12] Research has suggested that the underlying mechanisms modulating pancreatic islet viability can be attributed to paracrine mediators, IL6, TGF-β1, osteopontin and fibronectin secreted by MSCs.

In a study on rat patellar tendon allografts, Mae et al. reported that allograft was weakest at Week 4 (20% of regular strength).[13] Steiner[3] reported that tendon strength was 50% at Week 3 and 25% at Week 4 in a biomechanical study on rat Achilles tendons. This change at the 3rd week was attributed to early fibrodysplasia and the weakening at the 4th week to the replacement of fibrodysplasia by larger fibers replacing and the effort of achieving longitudinal order between fibers. In light of studies by Steiner, Mae et al., and Lui et al. on apoptosis as well as other studies showing the presence of re-rupture at late stages, in the present study we chose to apply MSCs at the 4th week after tendon repair, i.e. at the time when the tendon is the weakest.[14-18]

While significant results in biomechanical properties between MSC application and control groups have been obtained, no statistically significant histological differences have been found between these groups in most studies. In a study using MSCs and rabbit patellar tendons, Awad et al. observed no histological differences

between groups.[19] In a study on rabbit Achilles tendons, Chong et al. reported that among all histological criteria, differences in the group with MSC application were found only in the early stage (Week 3) and only in the nuclear aspect ratio and nuclear orientation angle.[20] Young et al. observed a larger cross-sectional area and better organized collagen fibers in the MSCs in the collagen gel group than in the control group and that evident fibrosis and inflammatory cells common around the suture were spotted in every group, especially in the early stages of examination.[21] In our study, although there was less fibrosis and vascularization in the group with MSC application, no statistically significant difference was established. In particular, inflammatory cells spread around the suture material were present in both groups. A nuclei of tendon cells growing and becoming rounder in evident cytoplasm is interpreted as cartilage metaplasia although the mechanism of this phenomenon has not been fully explained.[22] While cartilage metaplasia was observed in some sections regardless of groups, no statistically significant difference could be ascertained in our study.

Labeled MSCs, when applied at the same time in the tendon defects model formed, settle and remain alive at the defect site.[20] However, no studies have been performed examining the effects of late stage MSC application to a previously formed tendon defect. In the current study, BrdU-labeled MSCs were applied percutaneously in the second stage of healing after tendon repair, i.e. in the fibroblast (repair) stage. Transplanted MSCs were found to be present, both in differentiated and undifferentiated states, at the site of healing in the 12th week. In the first stage of healing (inflammation stage), chemotactic factors enabling the migration of MSCs to the site of healing are secreted by the inflammatory cells. The settling of MSCs at the site of healing when applied percutaneously in the late stage in our study shows that secretion of chemotactic factors continues in the latter stages of healing.

In our study, a very small amount of apoptosis was observed at the 12th week in the study group with late stage MSC application. However, in the control group, extensive apoptosis was present. Increased apoptotic activity at the repair site may be one of the factors that enable MSCs to settle more specifically at this site, despite the application taking place in the late stage of healing. We hypothesize that MSCs decrease apoptosis due to the anti-apoptotic genes they express and lead to the increase in tendon strength. Various theories have been proposed for the action mechanisms of MSCs. One is that MSCs differentiate into tenocytes and act at the site of healing and the stage of remodeling. Another theory assumes that MSCs act as 'growth factor pumps', rather than terminal differentiation taking place.[23] We suggest that MSCs transplanted to the site of injury have an anti-apoptotic function through the paracrine action mechanism.

Rats were used in this study due to their high reproducibility, ease of maintenance, lower position in the phylogenetic scale, low infection risk, and the availability of more data in stem cell studies. On the other end, the shortness of their Achilles tendons is a disadvantage for this type of study. At the time of MSC injection at the 4th week of our study, the visibility of suture color under the skin enabled percutaneous injection. However, when the histological results were examined, inflammatory cells and fibrosis were observed intensively around the suture material. In view of histological examination, models resulting in tendon defects are appropriate rather than tendon repair. In this study, MSC application was employed once at the 4th week after surgery and the results at the 12th week were assessed. However, it was observed histologically that tendon repair was not complete even at the 12th week. In future studies, repeated MSC applications and longer follow-up periods may lead to better histological results.

In conclusion, MSCs give successful results in various experimental or clinical applications and they are believed to be a future solution to many diseases. Nevertheless, despite these successful results, their action mechanism has not yet been fully understood. We suggest that application of MSCs to reduce the incidence of late stage re-ruptures increases the strength of the tendon owing to the possible anti-apoptotic effects of these cells.

Conflicts of Interest: No conflicts declared.

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