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ORIGINAL ARTICLE

Adipose derived stromal vascular fraction improves early tendon healing: an experimental study in rabbits

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Abstract

Tendon never restores the complete biological and mechanical properties after healing. Bone marrow and recently adipose tissue have been used as the sources of mesenchymal stem cells, which have been proven to enhance tendon healing. Stromal vascular fraction (SVF), derived from adipose tissue by an enzymatic digestion, represents an alternative source of multipotent cells, which undergo differentiation into multiple lineages to be used in regenerative medicine. In the present study, we investigated potentials of this source on tendon healing. Twenty rabbits were divided into control and treatment groups. Five rabbits were used as donors of adipose tissue. The injury model was unilateral complete transection through the middle one third of deep digital flexor tendon. Immediately after suture repair, either fresh stromal vascular fraction from enzymatic digestion of adipose tissue or placebo was intratendinously injected into the suture site in treatments and controls, respectively. Cast immobilization was continued for two weeks after surgery. Animals were sacrificed at the third week and tendons underwent histological, immunohistochemical, and mechanical evaluations. By histology, improved fibrillar organization and remodeling of neotendon were observed in treatment group. Immunohistochemistry revealed an insignificant increase in collagen type III and I expression in treatments over controls. Mechanical testing showed significant increase in maximum load and energy absorption in SVF treated tendons. The present study showed that intratendinous injection of uncultured adipose derived stromal vascular fraction improved structural and mechanical properties of repaired tendon and it could be an effective modality for treating tendon laceration.

Introduction

Tendon connects muscle to bone and acts as a flexible force-transmitting element, resulting in joint movement. Tendon injuries such as lacerations, ruptures, or inflammation cause marked morbidity and can have a major impact on work, recreational activities, and daily needs. Surgical treatment of tendon ruptures and lacerations is currently the most common therapeutic modality.[1] However, its outcome is often unsatisfactory and repair of ruptured, lacerated and surgically transected tendons remains a tremendous challenge for orthopaedic surgeons.[2]

Therefore, offering an optimal combination of maximal efficacy and high potency for successful treatment is critical. A recently advocated new approach is cell-based therapy which encompasses a growing list of treatment modalities with promising potential for treating tendon injuries.[3] Bone marrow mesenchymal stem cells (BM-MSCs)[4,5] and adipose-derived stem cells (ASCs)[6,7] have been investigated as important sources of undifferentiated cells in the field of regenerative medicine for tendon injuries.

Stromal vascular fraction (SVF) is an enzymatically digested cell population derived from adipose tissue.[8] The freshly isolated SVF actually contains a heterogeneous mixture of cells, including endothelial cells, smooth muscle cells, pericytes, fibroblasts, mast cells, pre-adipocytes, and a rich source of ASCs.[9] It has been successfully used in orthopaedic surgeries.[10-12] However, the potentials of SVF to enhance tendon structural characteristics as well as its mechanical properties have been poorly investigated. Owing to the great concentration of ASCs normally present in the adipose tissue harvest, it is possible to instantaneously obtain cell doses, with no need for further expansion in

culture.[13] In the present study; the effects of fresh adipose derived SVF on the healing of a surgical model of acute tendon injury in rabbit were investigated. It was hypothesized that a single intratendinous injection of SVF would promote structural and mechanical properties of experimentally induced tendon injury.

Material and methods

All procedures were carried out in accordance with the guidelines of the Ethics Committee and the University Research Council approved all experiments.

Twenty-five healthy adult male New Zealand White rabbits weighing 2.5-3.0 kg were subjected to the study. Animals were randomized into donor of adipose tissue (n = 5), control (n = 10), and treatment (n = 10) groups.

Collection of adipose tissue and preparation of stromal vascular fraction. Donor rabbits were anaesthetized by injection of xylazine HCl (5 mg kg^{-1}, IM, Alfasan, The Netherlands) and ketamine HCl (40 mg kg^{-1}, IM, Alfasan, The Netherlands). Under aseptic condition, a midline suprapubic skin incision was made to access the bilateral inguinal fat pad and approximately 6 to 8 g of subcutaneous adipose tissue was obtained from each donor. Then, stromal vascular fraction was isolated using a method as described in a previous study.[14] Briefly, adipose tissue was finely minced and washed with phosphate-buffered saline (PBS) and centrifuged at 1200g for 2 min to remove erythrocytes and cellular debris. Samples were then digested in a water bath for 60 min at 37 °C by 0.1% collagenase type II (C6885, Sigma-Aldrich, USA) in PBS. After digestion, the collagenase was neutralized by adding an equal volume of Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, USA). The digestate was centrifuged for 10 min at 1200g at room temperature to separate the SVF from adipocytes, cellular debris and undigested tissue. After removal of the supernatant, containing mature adipocytes, the cell suspension was filtered through a sterile 100 µm nylon cell strainer into a new tube and centrifuged again. The resulting pellet was re-suspended in PBS and freshly transferred to the operating room for transplantation procedure.

Surgical procedure. The model animals were anesthetized using the same anesthetic protocol (see above). One hind limb of each rabbit was randomly prepared for aseptic surgical operation. Skin was incised longitudinally on the plantar aspect of middle third of the metatarsus over the flexor tendons. The subcutaneous tissues were dissected and the deep digital flexor tendon was exposed. The injury model was a sharp complete transection through the central one third of the tendon. Subsequently, the tendon stumps were sutured with 3/0 monofilament nylon (Ethilon, Ethicon, Inc., USA) in modified Kessler pattern. Then, 0.2 mL PBS solution containing 4×10^6 nucleated cells of freshly isolated SVF was injected intratendinously at the suture site in treatment group. Control rabbits underwent the identical procedures to those of treatment group except that they just received the same volume of PBS solution (Fig. 1). The skin was closed with simple interrupted 3/0 nylon suture. A below stifle plaster cast (Iran Ortho Co., Tehran, Iran) was applied after surgery and immobilization was continued for two weeks. No antibiotics were used during study period.

Three weeks after surgery, all rabbits were sacrificed with thiopental sodium overdose (50 mg kg^{-1}, IV, Sandoz, Austria) and surgical incision reopened. Tendons were harvested by proximal and distal transverse incisions approximately 2 cm away from the repaired site. Operated tendons were harvested from all animals and processed for histological and immunohistochemical studies. For mechanical evaluations, the tendons from both hind limbs of the animals were harvested, wrapped in PBS soaked gauze and immediately stored at -20 °C.

Histological evaluations. Paraffin-embedded longitudinal sections (5 µm in thickness) were stained with hematoxylin and eosin (H&E) and were evaluated under a light microscope, equipped with a vernier scale to the nearest 0.1 mm, by the following criteria:

Fibrillar linearity, fibrillar continuity, angiogenesis in neotendon and epitenon, and epitenon thickness.

Repaired areas including longitudinally oriented collagen fibres were histologically assessed. In this regard, total area of neotendon between the severed ends of tendons was measured at 40× magnification and the area

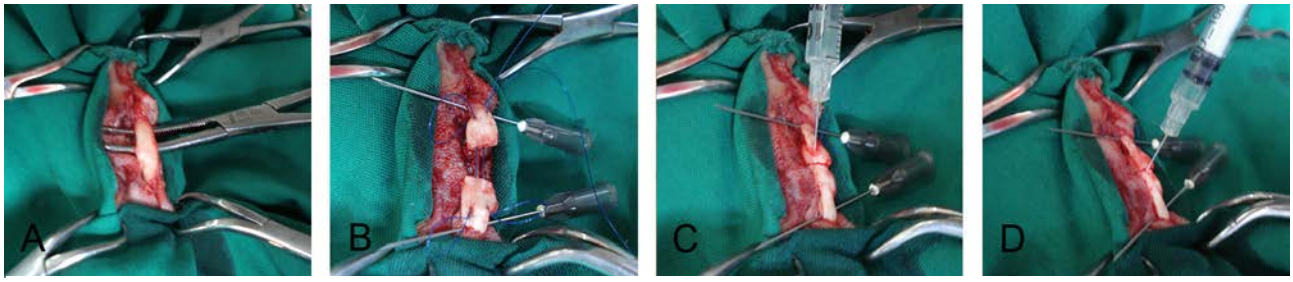

Fig 1. Intraoperative photographs **(A)** illustrates exposed the deep digital flexor tendon, **(B)** modified Kessler suture placement after complete transection of the tendon, **(C)** intratendinous injection of either SVF or PBS in tendon stumps and **(D)** at the repair site.

consisting longitudinal pattern of collagen fibres was calculated. The ratio of the values was defined as the percentage of fibrillar linearity for each tendon. The width of the widest part of neotendon including fibres following the direction of those in native tendon was measured at 40× magnification in both tendon-neotendon junctions and the ratio of their mean to the mean width of junctions was defined as the percentage of fibrillar continuity for each specimen. To examine the rate of angiogenesis within the neotendon and newly formed epitenon, the number of blood capillaries was counted at 100× magnification. For this evaluation, five randomly selected fields were examined per each tendon sample. The number of capillaries was averaged and reported for each specimen. The thickness of the epitenon was measured at 100× magnification in three randomly selected points of epitenon on both sides of neotendon and the records were averaged for each specimen.

Immunohistochemical evaluations. To evaluate the type of collagen synthesized in neotendons, immunohistochemical staining of paraffin embedded sections was used. Mouse monoclonal antibody to collagen I (Col I) (ab6308, Abcam, UK) and mouse monoclonal antibody to collagen III (Col III) (ab6310, Abcam, UK) were used. The expression of Col I and III was measured according to the immunohistochemistry kit instructions.

Immunohistochemical evaluations were made for intensity and localisation of the immunostaining of Col I and III at the repaired site of specimens, at 100× magnification. A score of 0 (absence of antigen expression), 1 (weak and spotted antigen expression), 2 (weak but diffuse antigen expression throughout the entire repaired site), and 3 (diffuse and strong antigen expression) was assigned to the semi quantitative evaluation of the immunohistochemical reaction to each antigen employed.[15]

Mechanical testing. Before testing, tendons were removed from the freezer, thawed for 2 hours at room temperature. Suture material was removed from the repair site in both groups. All tests were performed at room temperature. Ten tendons (five operated and five unoperated from contralateral limb) from each group were submitted to the mechanical test of traction using H10KS (Hounsfield, Salfords, UK) testing machine. The two ends of the tendons were clamped in the serrated jaws of the machine. The distance between the clamping jaws was set to 30 mm. The upper clamp was attached to a 500 N load cell and its displacement was controlled with the aid of a computer, endowed with QMat software (Ver. 2.22, Hounsfield, Salford, UK), responsible for commanding the equipment and for plotting the force-elongation curve. The dynamic testing took place under axial tension with a constant speed of 50 mm min^{-1}. The mechanical testing consisted of a single-cycle load-to-failure. The force and elongation of the tendon were continuously recorded until the tendon failed.

For each test the force-elongation curve was plotted and the following mechanical parameters were obtained: ultimate load (N), energy absorption (N.mm) and stiffness ($N.mm^{-1}$). The ultimate load was defined as the maximum force measured in the tendon during the failure test. Energy absorption values were measured by calculating the area under the force-elongation curve up to the point of maximum force. Stiffness was determined as the maximum gradient in the linear region of the force-elongation curve. The parameters were calculated as percentage of the values of the healing tendon as compared to the unoperated contralateral limb of the same animal rather than using absolute testing values, in order to minimize the biological variation of tensile stress among individuals.

Statistical Analysis. Statistical analyses of quantitative data were carried out using PASW Statistics (Release 18, SPSS Inc., Chicago, Illinois, USA). The residuals were tested for normality by Shapiro-Wilk's test and normality plots (histograms and quantile-quantile plots) and for homogeneity of variation by Levene's test and examining residual plot. Normality and/or homogeneity of variance assumptions for other variables were not satisfied and prior to statistical analysis these variables were logarithmically transformed to fulfil model assumptions. Statistical analysis of data was assessed using one-way analysis of variance (ANOVA). Data are presented as mean ± standard deviation. The level of significance was set at $P < 0.05$. Comparison of immunohistochemical scores across groups was performed with Kruskal-Wallis one-way analysis of variance by ranks, followed by Mann-Whitney U-test for pairwise comparison. The results are expressed as median and interquartile range (IQR 25 and IQR 75).

Results

The mean number of nucleated cells isolated from one gram of adipose tissue was approximately $2 \pm 0.5 \times 10^6$. Cell viability assessed by trypan blue exclusion assay was higher than 95%.

In macroscopic gross evaluation of tendons, the tenotomy site had healed well in all animals. No evidence of faulty union and local or systemic complications was observed. Dehiscence of the suture with gap formation between the tendon stumps was not observed in any of the tendons. There was no visible adhesion formation between the tendons and its surrounding tissues in all groups. In addition, failure mode was not influenced by treatment as it was ruptured at the repaired site in all operated tendons.

The histological and immunohistochemical results of study are presented in Tables 1 and 2, respectively. Histological analysis showed that SVF treated tendons exhibited more organised neotendon characterised by parallel and linear orientation of collagen fibres (fibrillar linearity) ($P = 0.003$). Furthermore, the rate of fibrillar

continuity was higher at the repaired site in treatment group compared to that of control group (P = 0.001). In contrast, the control specimens demonstrated remarkable distortion of the architecture, showing disruption of the normal linear orientation of collagen bundles and characterised by displaying fibrils in an irregular pattern (Fig. 2A versus 2B).

The number of blood capillaries in neotendon was also significantly higher than that in controls (P = 0.016) (Fig. 2C versus 2D). Epitenon thickness and its neovascularisation were also significantly increased in treatment group (P = 0.003 and P = 0.001, respectively).

According to the immunohistochemistry-based findings, the increase in expression of Col III (Fig. 3A versus 3B) and Col I (Fig. 3C versus 3D) observed in SVF treated tendons at third week was not statistically significant (P = 0.056 and P = 0.222, respectively).

Mechanical properties of tendons are presented in Table 3. Tensile strength parameters including ultimate load and energy absorption were significantly higher in SVF injected tendons compared to control group that had been given no cells (P = 0.002 and P = 0.001, respectively). However, no significant increase was observed in terms of stiffness between the groups (P = 0.119).

Discussion

The results of the present study showed that a single intratendinous injection of SVF could improve structural and mechanical properties of experimentally induced tendon injury in rabbit. Histopathological and mechanical indexes of this study suggested that immediate SVF transplantation would be an effective intervention in repair of tendon lacerations. In the present study, uniform neotendon formation and increased collagen type III production at the repaired site in treatment group compared to control group in the histological and immunohistochemical assays indicated positive effects of local SVF transplantation on improvement of tendon healing since increasing of collagen synthesis is the main consequence of proliferative phase of tendon healing.[16]

Furthermore, an intratendinous injection of SVF cells provoked a better angiogenesis within the neotendon in treatment group compared to control group.

Because, angiogenesis is most important for the restoration of blood flow, and delivering of oxygen and nutrients to the healing site.[17]

In the present study, beside the increased angiogenesis in neotendon, the higher rate of angiogenesis within epitenon and also increasing of epitenon thickness in treatment group in comparison with control group could suggest the positive effects of SVF intratendinous injection. Epitenon has an important role on tendon healing and probably SVF might stimulate epitenon to more participation in the regeneration. Tendon healing can occur intrinsically, by proliferation of tenocytes from epitenon and endotenon, or extrinsically, by invasion of cells from the surrounding sheath and synovium.[1] Epitenon tenoblasts initiate the repair process through proliferation and migration.[18] In addition, healing of severed tendons can be achieved by cells from the epitenon alone, without reliance on adhesions for vascularity or cellular support.[19]

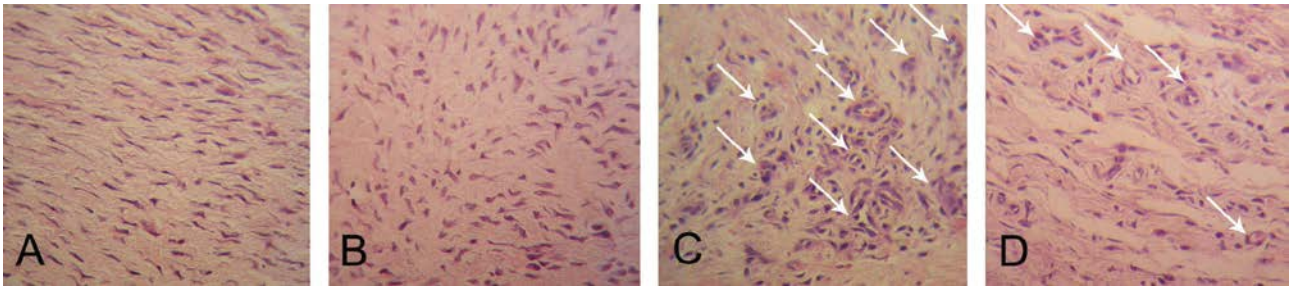

Fig 2. Photomicrographs demonstrating the differences in the histological appearances between the groups. Parallel orientation of collagen fibers in treatments indicating superior remodeling of neotendon **(A)** compared to controls **(B).** Arrows show the blood capillaries in neotendon which were significantly increased in treatments **(C)** over controls **(D)** (H&E, 100×).

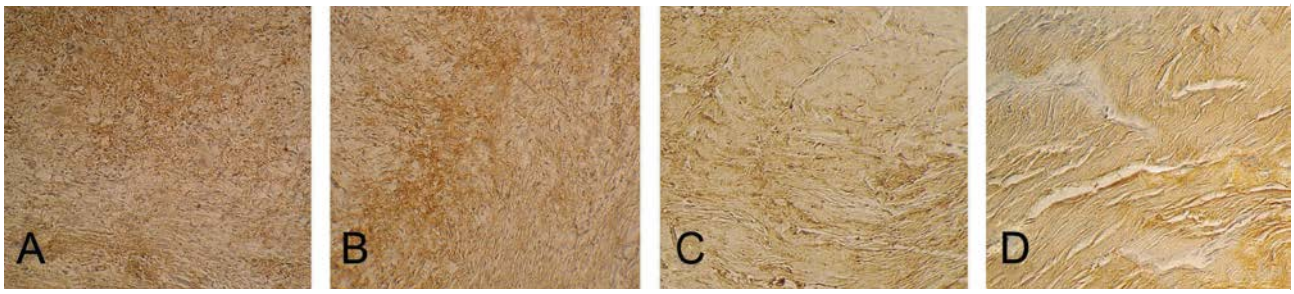

Fig 3. Photomicrographs demonstrating the differences in the immunohistochemical appearances between the groups. Immunohistochemistry demonstrated no change in expression of Col III between SVF treated tendons **(A)** and controls **(B)**. The expression of Col I in treatment group **(C)** was increased over controls **(D)**, however, the difference was not statistically significant (Brown DAB, 100×).

Actually, the first response in tendon healing is thickening of the epitenon and it is believed that initially, collagen is produced by epitenon cells.[20]

During tendon healing, the injured area is replaced by neotendon, which is composed mainly of collagen fibres with non-arrangement random directions. By maturity, the collagen fibres become more obviously oriented in line with local stresses[21] which takes a long period of time, around six weeks, in normal conditions,[19] whereas in the present study SVF intratendinous injection accelerated the commencement of this event in treatment group compared to control at third week after surgery as was found by significant increased linearity. In addition, the intrinsic mechanism is responsible for the reorganization of the collagen fibres and maintenance of fibrillar continuity.[1] In this study, SVF transplantation resulted in significant increase in fibrillar continuity in the treatment group.

In mechanical testing of tendon, ultimate load reflects the ultimate tensile strength of the specimen[22], and to store and release high loads without damage, tendons require a great energy-absorbing capacity. Therefore, increasing the energy capacity of tendons must be one of the key points in the prevention and treatment of tendon injuries.[23]

Results of the mechanical testing in the present study demonstrated significant increase in the ultimate load and energy absorption of SVF treated tendons. The increased tensile strength could be resulted from improvement in remodelling of collagen fibres in the treatment group. Since the better remodelling phase occurs, the more repair site strength appears.[24] However, insignificant difference in tendon stiffness in the present study between two groups could be due to low quantity of collagen cross-links.[25] Reportedly, the final maturation stage occurs after ten weeks, and during this time there is an increase in cross-linking of the collagen fibrils, which causes the tissue to become stiffer.[26] Therefore, at the time of sampling (three weeks after surgery), collagen fibres in all repaired tendons did not reach the time for proper cross-linking.

In this study, expression of col I in treatment group might be attributed to the presence of growth factors in SVF.[27] It is believed that high expression of col I is essential to achieve faster healing of injured tendons.[28] Reportedly, SVF contains growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor beta (TGF-β).[29] Several growth factors like TGF-β have been used therapeutically to improve tendon regeneration.[30]

In our study, remodelling of neotendon begun at the end of the third week in treatment group, while under normal circumstances remodelling of neotendon begins 6-8 weeks after injury which is characterized by increase in type I collagen synthesis[31], it was concluded that SVF could have an acceleratory effect on healing process of injured tendons. This finding is in agreement with those of James *et al.* (2008) stating delivery of required dosages of necessary factors over the repair phase is critical to a successful treatment.[1] We used histomorphometrical and mechanical indexes for tendon repair assessment. The golden standard for efficacy assessment of any technique for the evaluation of tendon regeneration is still the mechanical testing of the tensile strength.[16] In a clinical trial using allograft horse adipose derived SVF on collagenase induced tendinitis, the mechanical properties of the repaired tendons were not assessed[12], thereby our findings could support the idea of encouraged use of SVF in clinical cases.

In conclusion, the ease of preparation, elimination of lengthy cell culturing processes providing rapid single step-procedure feasibility beside remarkable results observed in this trial study suggest that SVF could be a promising option for future clinical use in tendon regenerative medicine.

Acknowledgments

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Table 1. Results of histological studies (mean ± SD) of repaired tendons at three weeks after surgery.

	No.	Percentage of fibrillar linearity	Percentage of fibrillar continuity	Number of capillaries in neotendon	Number of capillaries in epitenon	Epitenon Thickness (μm)
Controls	5	15.42 ± 4.76*	24.49 ± 3.97*	28.08 ± 0.32*	9.32 ± 1.24*	42.24 ± 11.93*
Treatments	5	30.87 ± 7.8*	52.42 ± 7.10*	47.79 ± 12.69*	19.26 ± 4.98*	64.99 ± 6.85*

* Values with same symbol in each column are significantly different ($P < 0.05$).

Table 2. Results of immunohistochemical evaluation [median and IQR 25-75] of repaired tendons at three weeks after surgery.

	No.	Collagen type III expression	Collagen type I expression
Controls	5	1.000 (1.000-2.250)	0.000 (0.000-1.000)
Treatments	5	3.000 (2.750-3.000)	1.000 (0.750-1.250)

No statistically significant differences were found in terms of Col III and I expression between the groups.

Table 3. Mechanical properties (mean ± SD) of repaired tendons at three weeks after surgery.

	No.	Ultimate load (N)	Energy absorption (N.mm)	Stiffness ($N.mm^{-1}$)
Controls	5	3.02 ± 0.91 *	3.77 ± 1.912 *	65.66 ± 8.84
Treatments	5	9.35 ± 2.88 *	15.69 ± 2.53 *	75.23 ± 8.47

* Values with same symbol in each column are significantly different ($P < 0.05$).

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