Original research

Repair of nerve defect with chitosan graft supplemented by uncultured characterized stromal vascular fraction in streptozotocin induced diabetic rats

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Abstract

Regenerative properties of stem cells at the service of nerve repair have been initiated during recent decades. Effects of transplantation of characterized uncultured stromal vascular fraction (SVF) on peripheral nerve regeneration were studied using a rat sciatic nerve transection model. A 10-mm sciatic nerve defect was bridged using a chitosan conduit filled with SVF. In control group, chitosan conduit was filled with phosphate-buffered saline alone. The regenerated nerve fibers were studied 4 weeks, 8 weeks, and 12 weeks after surgery. In sham-operated group, the sciatic nerve was only exposed and manipulated. Behavioral and Functional studies confirmed faster recovery of regenerated axons in SVF transplanted animals than in control group (P < 0.05). Gastrocnemius muscle mass in SVF transplanted animals was found to be significantly more than that in control group. Morphometric indices of the regenerated fibers showed the number and diameter of the myelinated fibers were significantly higher in SVF transplanted animals than in control group. In immunohistochemistry, location of reactions to S-100 protein in SVF transplanted animals was clearly more positive than that in control group. SVF transplantation combined with chitosan conduit could be considered as a readily accessible source of stromal cells that improve functional recovery of sciatic nerve.

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1. Introduction

Organ reinnervation and functional recovery following peripheral nerve injury still remains a major challenge and return of functional recovery to the preinjury level rarely occurs. Recent therapeutic advances in the control of diabetes mellitus and diabetic neuropathy have renewed the interest in the rate and quality of nerve regeneration in this chronic disease. Although measurable improvements may follow better control of blood sugar and administration of aldose reductase inhibitors, complete recovery is dependent on the regeneration of damaged axons and the reestablishment of fully functional connection with their targets. To achieve maximum functional recovery various techniques are being used. Employment of regenerative properties of stem cells at the service of nerve repair has been initiated during recent decades. Widely accepted method by most surgeons is bridging the defect with an autologous donor nerve. Different graft equivalents have also been applied to bridge the nerve stump and regulated through the interaction of a variety of protein and cell signals. Biodegradable nerve guides as a temporary scaffold are better than non-degradable biomaterials because the latter remain in situ as a foreign body and ultimately result in limiting recovery of nerve function. Nevertheless, the resistance to biodegradation can be a cause of chronic nerve compression in the long run and a second surgery may therefore be required for its removal. Beneficial effects of chitosan as a conduit in promoting nerve regeneration have already been documented and it seems chitosan as a natural polymer has excellent properties including biocompatibility, biodegradability, non-toxicity and adsorption properties, and might be a suitable functional material for peripheral nerve regeneration.

The original and classical view of adipose tissue as a rather specialized passive storage organ has changed dramatically. The
adipose tissue has several properties that are advantageous for neuronal sprouting and direction and has been used in different areas of surgery in recent decades. Apart from adipocytes and pre-adipocytes adipose tissue contains microvascular endothelial cells, smooth muscle cells, resident monocytes, lymphocytes and stem cells. In the last few years, it has been identified that adipose tissue possesses a population of multi-potent stem cells which can be differentiated to a Schwann cell phenotype and may be of benefit for treatment of peripheral nerve injuries and promoting neurite outgrowth in vitro. It has also been reported that differentiated adipocyte-derived cells enhance peripheral nerve regeneration. Beneficial effects of cultured uncharacterized omental adipose derived stromal vascular fraction have already been reported by authors.

However, to the best knowledge of the authors literature is poor concerning effects of combination of chitosan conduit and characterized uncultured SVF on peripheral nerve regeneration in vivo in diabetic rats. The objective of this study reported here was to evaluate effectiveness of characterized uncultured SVF as a readily accessible source of stromal cells on peripheral nerve regeneration using a diabetic rat sciatic nerve transaction model. Therefore, a chitosan conduit was prepared and filled with uncultured SVF. Assessment of the nerve regeneration was based on behavioral, functional (Walking Track Analysis), muscle mass measurement, histomorphometric and immunochemical (Schwann cell detection by S100 expression) criteria 4, 8 and 12 weeks after surgery.

2. Materials and methods

2.1. Experimental design

Sixty male diabetic White Wistar rats weighing approximately 300 g were divided into four experimental groups \( n = 18 \), randomly: Sham-operated group (Sham), transected group (TC), control group (CHIT) and SVF group (CHIT/SVF). Each group was further subdivided into three subgroups of five animals each. Four donors were also assigned to SVF isolation and preparation. Two weeks before and during the entire experiments, the animals were housed in individual plastic cages with an ambient temperature of \( 23 \pm 3 \) °C, stable air humidity, and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water. For insulin-deficient diabetes, rats were fasted overnight before receiving a single intraperitoneal injection (50 mg/kg in 0.9% sterile saline) of streptozotocin (STZ). Hyperglycemia (15 mmol/l or greater) was confirmed 2 days later by measurement of tail-vein blood glucose concentration ( Ames Glucostix; Myles, Elkhart, IN). The rats underwent grafting procedures three days after induction of diabetes.

2.2. Collection of omental adipose tissue, isolation of SVF

The entire abdomen was prepared aseptically and after ventral midline incision approximately 4–5 g omentum were harvested from donor animals. The donor animals were then euthanized by over dose of the anesthetics. The technique of SVF isolation is described elsewhere. In brief, the way the harvested omentum was rinsed with HANKS-buffered saline (HBS), trimmed, minced with two scalpels into very small pieces, and aspirated into a 10-ml pipette; then the tissue was transferred into a 50-ml Erlenmayer flask containing 1500 U/mL collagenase type II (Sigma Chemical Co). The ratio was 1 g of omental tissue to 2 mL of collagenase. The suspension of omental tissue and collagenase was incubated for 40 min in a 37 °C water bath at 100 shaking minutes per minute. The digested tissue was homogenized by repetitive pipeting, transferred into a 15-ml tube, and centrifuged twice at 100 g for 5 min. The supernatant contained mainly adipocytes and the collagenase solution. The cell pellet was resuspended in 10 mL phosphate-buffered saline (PBS), filtered through a 150-μ pore-size mesh to remove non digested large tissue fragments, and then washed two times with HBS. The SVF pelat was resuspended in sterile PBS solution as 10-μl aliquots (2–107 cells/ml), each loaded into sterile syringes. The syringes containing PBS solution and SVF were shipped chilled to the investigators for immediate injection.

2.3. Flow cytometric analysis and characterization of SVF

Rat omental adipose tissue-derived stromal cells were analyzed by flow cytometry for the expression of typical stromal cell markers. Cells were stained using specific monoclonal antibodies against rat surface markers, anti-CD90 PE, anti-CD44 FITC, anti-CD106 biotin followed by streptavidin FITC and anti-CD45 FITC. Mouse IgG2a K PE and mouse IgG1 K FITC were used as isotype controls. Flow cytometry was performed with a PAS flow cytometer (Partec GmbH, Germany). Cell Quest software was 123 used for data analysis. Cells were positive for MSC-related antigens of CD44 (43.87%), CD90 (88.82%), and negative for CD106 (VCAM-1) (7.28%) and hematopoiesis-related antigen of CD45 (3.47%) (Fig 1).

2.4. Preparation of chitosan conduit

Chitosan solution was prepared by dissolving molecular weight, crab shell chitosan (~400 kDa, 85% deacetylated) (Fluka, Sigma-Aldrich St. Louis, MO, USA) in an aqueous solution (1% v/v) of glacial acetic acid (Merck, Darmstadt, Germany) to a concentration of 2% (w/v) while stirring on a magnetic stirrer-hot plate. The solution was stirred with low heat (at 50 °C) for 3 h. The resultant chitosan solution was filtered through a Whatman No. 3 filter paper then vacuum filtration to remove any undissolved particles. To overcome the fragility of chitosan, glycerol (Sigma Chemical Co., St. Louis, MO, USA) was added as 30% (w/w) of the total solid weight in solution. Chitosan conduit was made according to the method described by others by gentle injection of the prepared solution into a home-made mold. The prepared conduit was 2 mm in external diameter, 1.8 mm in internal diameter and 10 mm in length. This internal diameter complies with optimal function in rat models.

2.5. Grafting procedure and transplantation of SVF

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain. The University Research Council approved all experiments. Following surgical preparation in the sham-operation group (Sham) the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with resorbable 4/0 sutures, and the skin with 3/0 nylon. In the TC group the left sciatic nerve was exposed through a gluteal muscle incision and transected proximal to the tibio-peroneal bifurcation where a 7 mm segment was excised, leaving a gap about 10 mm due to retraction of nerve ends. Proximal and distal stumps were each sutured to adjacent muscles. In CHIT group after transection and excision of 7 mm of the nerve, both proximal and distal stumps were inserted 2 mm into a chitosan conduit, 2 mm in diameter and 14 mm in length, and 2/0 nylon sutures were placed at each end of the cuff to fix the graft in place and to leave a 10-mm gap between the stumps. The conduit was filled with 10 μL phosphate-buffered saline solution and sterile Vaseline was used to seal the
of the sciatic nerve. The SFI was a negative value and a higher SFI meant the better function assessed in the NC group and the normal level was considered as 0.

2.6. Behavioral testing

Functional recovery of the nerve was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for rat hind limb motor function. Although BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated that it could be most useful in assessment of never repair processes in peripheral nerve injuries. Scores of 0 and 21 were given when there were no spontaneous movement and normal movement, respectively. A score of 14 shows full weight support and complete limbs coordination. BBB recordings were performed by a trained observer who was blinded to the experimental design. The testing was performed in a serene environment. The animals were observed and assessed within a course of a 4-min exposure to an open area of a mental circular enclosure. BBB scores were recorded once before surgery in order to establish a baseline control and again weekly thereafter to assess functional recovery during 16 weeks.

2.7. Functional assessment of reinnervation

2.7.1. Sciatic functional index (SFI)

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method of others. The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula:

\[
SFI = -38.3 \times \frac{EPL - NPL}{NPL} + 109.5 \times \frac{ETS - NTS}{NTS} + 13.3 \times \frac{EIT - NIT}{NIT} - 8.8
\]

In general, SFI oscillates around 0 for normal nerve function, whereas around –100 SFI represents total dysfunction. SFI was assessed in the NC group and the normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

2.7.2. Static sciatic index (SSI)

SSI is a time-saving digitized static footprint analysis described by others. A good correlation between the traditional SFI and the newly developed static sciatic index (SSI) and static toe spread factor (TSF), respectively, has been reported by others. The SSI is a time-saving and easy technique for accurate functional assessment of peripheral nerve regeneration in rats and is calculated using the static factors, not considering the print length factor (PL), according to the equation:

\[
SSI = \left(\frac{108.44 \times TSF}{C2} + \frac{31.85 \times ITSF}{C0}\right) - 5.49
\]

Where:

\[
TSF = \frac{ETS - NTS}{NITS}
\]

\[
ITSF = \frac{EIT - NIT}{NITS}
\]

Like SFI, an index score of 0 was considered normal and an index of –100 indicated total impairment. When no footprints were measurable, the index score of –100 was given.

2.8. Measurement of gastrocnemius muscles mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance. Two independent observers unaware of the analyzed group made all measurements.

2.9. Histopathological preparation and quantitative morphometric studies

Middle cable of grafts in Sham, TC, CHIT and CHIT/SVF groups were harvested and fixed in 2.5 percent glutaraldehyde. The nerves were post fixed in OsO4 (2%, 2 h), dehydrated through an ethanol series and embedded in Epon. Semi thin transverse (5 µm) sections were next stained with toluidine blue and examined under light microscopy. Morphometrical analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity, systematic random sampling and two-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases.
2.10. Immunoistochemical analysis

In this study, anti-S-100 (1:200, DAKO) was used as maker for myelin sheath. Specimens prior to immunohistochemistry were post fixed with 4% paraformaldehyde for 2 h and embedded in paraffin. Then the nerve sections were dewaxed and rehydrated in PBS (pH = 7.4). They were incubated by 0.6% hydrogen peroxide for half an hour to neutralize endogenous peroxide. After that the sections were incubated with normal swine serum (1: 50, DAKO, Germany) for blocking of non-specific immunoreactions and then were incubated in S-100 protein antibody solution for 1 h at room temperature. They were washed three times with PBS and incubated in biotynilated anti-mouse rabbit IgG solution for 1 h. Horseradish peroxidase-labelled secondary antibody (1:100 swine anti-rabbit diluted in 5% normal rat serum) was applied for 1 h. All sections were then incubated with diaminobenzidine tetrahydro-chloride chromogen (DAB, DAKO) substrate solution for 10 min. The results of immunohistochemistry were examined under a light microscope.

2.11. Statistical analysis

Experimental results were expressed as means ± SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial ANOVA with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were considered significant when P < 0.05.

3. Results

3.1. BBB recovery

In order to assess hind limb recovery the open field locomotor was used. Fig. 2 shows BBB scores compared to the baseline. All experimental groups, except for sham, showed the greatest degree of functional deficit one week after surgery. The T3 treated group showed significant improvement in locomotion of the operated limb compared to the control group during the study period (P < 0.05).

3.2. Recovery of sciatic nerve function and reinnervation

3.2.1. SFI outcome

Fig. 3 shows sciatic function index (SFI) values in experimental groups. Prior to surgery, SFI values in both groups were near zero. After the nerve transection, the mean SFI decreased to –100 due to the complete loss of sciatic nerve function in all animals. At the end of the study period, animals of SVF transplanted group achieved a mean value for SFI of –34.4 ± –3.16 whereas in control group a mean value of –46 ± –2.2 was found. The statistical analyses revealed that the recovery of nerve function was significantly different between CHIT and CHIT/SVF groups (P < 0.05) except for myelin sheath thickness that did not show significant difference between CHIT and CHIT/SVF groups. The mean ratios of gastrocnemius muscles weight were measured. There was statistically significant difference between the muscle weight ratios of CHIT/SVF and CHIT groups (P < 0.05). The results showed that in CHIT/SVF group muscle weight ratio was bigger than CHIT group and the gastrocnemius muscle weight loss was improved by transplantation of SVF (Fig. 5).

3.3. Gastrocnemius muscles mass measurement

3.4. Histopathological and quantitative morphometric findings

Figs. 6–8 show quantitative morphometric analyses of regenerated nerves for each of the experimental groups. Statistical analysis by means of a one-way ANOVA test showed that 4 weeks after surgery, CHIT/SVF group presented significantly greater nerve fiber, axon diameter and myelin sheath thickness compared to CHIT animals (P < 0.05). Although CHIT presented regeneration patterns, the morphometric indices in CHIT/SVF both after 8 and 12 weeks were significantly higher than in CHIT group except for myelin thickness that did not show significant difference between CHIT and CHIT/SVF groups (P > 0.05) at the end of study period. Using Factorial ANOVA analysis with two between-subjects factors
(Group × time); in CHIT group axon diameters did not show significant difference between 8 and 12 weeks (P > 0.05). Thickness of myelin sheath showed an interaction across time in CHIT and CHIT/SVF groups. Growth in mean thickness of myelin sheath did not show statistical difference between 8 and 12 weeks inside CHIT and CHIT/SVF groups (P > 0.05).

3.5. Findings of immunohistochemical analysis

The cross sections of regenerated nerve segments indicated extensive immunoreactivity to S-100 protein. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression representing that Schwann cell-like phenotype existed around the myelinated axons (Fig. 9). In both groups, the expression of S-100 was similar to those of the histopathological findings.

4. Discussion

A few reports have been carried out about Wallerian degeneration after nerve injury in experimental diabetes postulating that Wallerian degeneration is delayed in diabetes.25,26 Despite much effort to introduce ideal therapeutic drugs for diabetic neuropathy, aldose reductase inhibitors, have been shown to be the most established compounds among potent drugs. However, although experimental data on aldose reductase inhibitor shave been very promising, their clinical efficacy seems limited even for mild degrees of diabetic neuropathy. Neurotrophic factors appear less effective than such conventional drugs; no extensive trials have shown their efficacy and a considerable number of adverse effects are also problematic.27 Cell or tissue engineering technology combined with molecular biology could provide ideal therapies for nerve regeneration by enhancing axon extension and by modulating either axon–Schwann cell or axon–extra cellular matrix interaction, although there are still many difficulties to overcome. Furthermore, huge amount of unknown mechanisms about cell molecular biology and signal pathways may contribute to the process of nerve regeneration. Their exploration may provide us with beneficial clues for the treatment of diabetic neuropathy with reference to nerve regenerative capacity as well as cell biology. Although the present study showed the neuroprotective action of
local transplantation of SVF in peripheral nerve injuries in diabetic rats, data regarding the molecular mechanisms leading to its neuroprotective action remain to be investigated in depth. The authors have not provided the histologic and molecular evidence for the neuroprotective action of SVF, which may be considered a limitation of this study.

The choice of applicable evaluation methods to determine functional recovery continues to be a challenge and the most widely used model for evaluation of motor and sensory nerve function is the rat sciatic nerve model.22,28 Walking is a coordinated activity involving sensory input, motor response and cortical integration.42 Therefore, walking track analysis (sciatic function index) is a comprehensive test. The results of present study showed that characterized uncultured SVF when loaded in a chitosan conduit resulted in a quicker and noticeable acceleration of functional recovery of the sciatic nerve.

As the posterior tibial branch of the sciatic nerve regenerates into the gastrocnemius muscle, it will regain its mass proportional to the amount of axonal reinnervation.43 In the present study 12 weeks after surgery the muscle mass was found in both experimental groups. However, SVF transplanted animals showed significantly greater ratios of the mean gastrocnemius muscle weight than CHIT group indicating indirect evidence of successful end organ reinnervation.

Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor.12–14 Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling13,14 do not necessarily predict the reestablishment of motor and sensory functions.31,33

Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery.22–30 Therefore, research on peripheral nerve injury needs to combine both functional and morphological assessment.

In the histopathological studies, quantitative morphometrical indices of regenerated nerve fibers showed significant difference between CHIT and CHIT/SVF groups indicating beneficial effect of characterized uncultured SVF on the sciatic nerve regeneration.

Immunohistochemical studies showed the expression of axon and myelin sheath special proteins was evident in both groups which indicated the normal histological structure. The location of reactions to 5–100 in SVF transplanted animals was clearly more positive than in CHIT group. This further implies that both regenerated axon and Schwann cell-like cells existed and were accompanied by the process of myelination and the structural recovery of regenerated nerve fibers.

Adipose tissue has been identified as possessing a population of multi-potent stem cells which can be differentiated to a Schwann cell phenotype. It has been concluded that adipose tissue could be of benefit for treatment of peripheral nerve injuries and promoting neurite outgrowth in vivo.13,14 Schwann cells have great importance in organizing the structure of the peripheral nerve because they produce a basement membrane containing extra cellular matrix (ECM) proteins that support axonal growth and form the endoneurial tubes through which regenerating axons grow.27

We indicated in other work that chitosan conduit could be a useful conduit in sciatic nerve transaction injuries.38 In the present study we used a chitosan as a conduit to provide a scaffold for characterized uncultured SVF to facilitate Schwann cells migration and subsequent acceleration of nerve repair in vivo.39

Adipose tissue can be harvested from several sites such as subcutaneous fat depots and omentum. Morphological studies revealed substantial differences between the subcutaneous and omental fat depots.10 The omental adipose tissue contains more blood vessels and sympathetic nerve fibers than the subcutaneous depots indicating a greater metabolic activity in the former. In addition, more monocytes/macrophages are present in omental as compared to subcutaneous adipose tissue.40

It has been demonstrated that omentum contains growth factors, interleukines, cytokines and chemokines. Adipocytes as well as pre-adipocytes and macrophages appear to be involved in the local production of these proinflammatory proteins.21 It is been confirmed that nerve growth factors have beneficial effects on nerve regeneration.41 Some types of macrophages in peripheral nerves produce interleukin-1, which stimulates nerve growth factor synthesis in Schwann cells, with subsequent stimulation of

![Fig. 8. Line graph shows the quantitative results of mean diameter of axon. The mean diameter of axon in sham-operated group was nearly 7.08 ± 0.22 (mean ± SD). Both groups of CHIT and CHIT/SVF showed the lower mean diameter of axon than the sham-operated group even at the end of the study. From 4 to 8 weeks, CHIT/SVF group had significantly more mature axons than CHIT group and this kind of significant difference increased in favor of CHIT/SVF group in the later period. Diameter of axon in both control and treatment groups did not increase after 8 weeks. *P < 0.05, CHIT/SVF group is compared with CHIT group.](image)

![Fig. 9. Immunohistochemical analysis of the regenerated nerves. Representative cross section taken from midpoint of (A) TC, (B) SHAM, (C) CHIT/SVF and (D) CHIT groups. In SVF transplanted animals there is clearly more positive staining of the myelin sheath-associated protein S-100 (arrows) within the periphery of nerve. Scale bar:10 μm.](image)
regeneration of sensory axons. These point towards a possible explanation for significant improvement of functional recovery of the sciatic nerve in SVF transplanted animals of our study. Further studies are warranted to compare beneficial effects of stem cells derived from subcutaneous and omental adipose tissues on peripheral nerve regeneration.

The value or necessity for isolating and proliferating specific adherent cell lines from each tissue source, and the impact of additional growth factors contained at unknown quantities in adipose tissue or digested derivatives, are poorly understood. Additionally, regulatory authorities (e.g., the FDA) allow autologous minimally manipulated cell therapy when the procedures do not appreciably change the cells (i.e., differentiation), whereas more manipulative methods, such as differentiation, or sorting, may require formal approval as a drug before clinical use. Adipose tissue provides an alternative source of multipotent cells in the form of concentrated nucleated cell populations, many of which may be clinically relevant when compared with other sources.

It has been demonstrated that differentiated adipose-derived stem cells could enhance regeneration distance similar to differentiated bone marrow stem cells. Thus the choice of uncultured SVF as a more readily accessible and instant source of multipotent cells instead of culture differentiation might seem more favorable for cell therapy. Results of the present study showed that SVF improved the sciatic nerve regeneration in a similar manner to undifferentiated cultured bone marrow stem cells that was performed by authors elsewhere.

In conclusion, the uncultured SVF resulted in improvement of functional recovery of the sciatic nerve. Omental adipose tissue can provide a readily accessible source of cells in large quantities that contribute to nerve regeneration in the emerging field of regenerative medicine especially where a traumatic injury is dealt with. Isolation of an injectable cell pool derived from uncultured SVF has distinct advantages with regard to timeliness, compared to the time needed for cultured multi-potent stem cells derived from other tissue sources. Morbidity associated with harvest of SVF is mild, cell yield is high compared with results for cultured stromal cells derived from other sources.

Ethical approval

None.

Funding

None.

Author contribution

Rahim Mohammadi: Study design and writing.
Negin Sanaei, Sima Ahsan, Hawdam Rostami, Sedighe Abbaspour-Dalivand: Grafting procedures and Data collection.
Keyvan Aminii: Data analysis.

Conflict of interest

There are no conflicts of interests.

Acknowledgments

The authors would like to thank Dr. Pouya Malekhtetabi, Department of Cellular and Molecular Biotechnology and Mr. Jaafary, Urmia Pathobiology Center, for their technical help.

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