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# 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 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.[1] Recent therapeutic advances in the control of diabetes mellitus and diabetic neuropathy[2] 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.[3] 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.[4] 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.[5] 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.[6] 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.[7–9]

The original and classical view of adipose tissue as a rather specialized passive storage organ has changed dramatically.[10] The

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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.[11,12] Apart from adipocytes and pre-adipocytes adipose tissue contains microvascular endothelial cells, smooth muscle cells, resident monocytes, lymphocytes and stem cells.[13] 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*.[14] It has also been reported that differentiated adipose-derived cells enhance peripheral nerve regeneration.[15] Beneficial effects of cultured uncharacterized omental adipose derived stromal vascular fraction have already been reported by authors.[16]

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 transection 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 immuohistochemical (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.[16,17] 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 Erlenmeyer 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 motions 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 SFV pellet was resuspended in sterile PBS solution as 10-μl aliquots ($2–10^7$ 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 medium 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.[18] Chitosan conduit was made according to the method described by others[24] 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.[19]

### 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.[20] 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 two 10/0 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

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