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Local Xenotransplantation of Bone Marrow Derived Mast Cells (BMMCs) Improves Functional Recovery of Transected Sciatic Nerve in Cat: A Novel Approach in Cell Therapy

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ABSTRACT

Objective: To determine the effects of bone marrow derived mast cells (BMMCs) on functional recovery of transected sciatic nerve in animal model of cat.
Method: A 20-mm sciatic nerve defect was bridged using a silicone nerve guide filled with BMMCs in BMMC group. In Sham-surgery group (SHAM), the sciatic nerve was only exposed and manipulated. In control group (SILOCONE) the gap was repaired with a silicone nerve guide and both ends were sealed using sterile Vaseline to avoid leakage and the nerve guide was filled with 100 µL of phosphate-buffered saline alone. In cell treated group ([SILOCONE/BMMC) the nerve guide was filled with 100 µL BMMCs (2×10^6 cells/100 µL). The regenerated nerve fibers were studied, biomechanically, histologically and immunohiscochemically 6 months later.
Results: Biomechanical studies confirmed faster recovery of regenerated axons in BMMCs transplanted animals compared to control group ($p<0.05$). Morphometric indices of the regenerated fibers showed that the number and diameter of the myelinated fibers were significantly higher in BMMCs transplanted animals than in control group ($p<0.05$). In immunohistochemistry, location of reactions to S-100 in BMMCs transplanted animals was clearly more positive than that in control group.
Conclusion: BMMCs xenotransplantation could be considered as a readily accessible source of cells that could improve recovery of transected sciatic nerve.

Keywords: Sciatic nerve; Mast cells; Biomechanical properties; Cats.

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Introduction

The consequences of peripheral nerve injuries are disgracefully overwhelming and based on reports it is life-altering with an incidence of 13 to 23 per 100,000 persons per year [1-3]. The morbidity and unintended negative physical and psychological effects of current treatment on patients' lives necessitates the need for continued translational research to provide alternative and potentially more efficacious treatment modalities [4]. The treatment for traumatic nerve injury is variable, ranging from conservative to surgical and initial approaches may include early mobilization, physical therapy, and patient comfort. Early surgical intervention may alternatively be favored to promote nerve regeneration [4].

While many studies have investigated the benefits of cell therapies in animal models of peripheral nerve injury, clinical applications of these technologies in the form of randomized controlled trials continue to be intangible [4]. Cell-based therapy is a promising branch of regenerative medicine. The idea of using cultured Schwann cells, bone marrow mesenchymal cells and adipose derived nucleated cell fractions may be an attractive alternative to more aggressive therapies. If successful, the treatment may lead to functional improvement as well as shortened recovery times, avoiding the hurdles of additional surgeries [5-7].

Mast cells are fascinating, multifunctional, bone marrow-derived, tissue dwelling cells. They can be activated to degranulate in minutes, not only by IgE and antigen signaling via the high affinity receptor for IgE, but also by a diverse group of stimuli. These cells can release a wide variety of immune mediators, including an expanding list of cytokines, chemokines, and growth factors [8]. Mast cells have an armamentarium of inflammatory mediators interleukins such as IL-6 and IL-8, and growth factors, such as vascular endothelial growth factor, platelet derived growth factor and proteases that are released in degranulation [9]. As a result of extra cellular matrix degradation and changes in the microenvironment following initial mast cell secretion, the mast cell populations may change significantly in number, phenotype and function. There is, moreover, strong evidence that mast cells significantly influence angiogenesis [10, 11].

In a preliminary study beneficial effects of local transplantation of BMMCs on sciatic nerve repair in rat has been reported [12].

These characteristics of the mast cells has encouraged us to conduct a study to assess local BMMCs therapy in site of transection of sciatic nerve to observe whether the cells could be of benefit in sciatic nerve regeneration in cat. The aim of the present study was a single local xenotransplantation of bone marrow-derived mast cells after sciatic nerve transection and entubulation using silicone nerve guide in rat.

Materials and Methods

Study Design and Animals

Twelve male cats with approximate weight of 3.0-4.0 kg were randomized into three groups of 4 animal each (n=4). Animal care, housing and surgery were performed based on Guidelines of Animal Welfare Committee of Urmia University. The cats were premeditated with atropine sulphate [0.03 mg/kg] and anesthesia was induced by intramuscular injection of ketamine hydrochloride (50-80 mg/kg). The sciatic nerve on the right side was exposed and divided through the split muscles. Sufficient nerve was resected to create a gap of 20 mm. In Sham-surgery group (SHAM), the sciatic nerve was only exposed and manipulated. In control group (SILOCONE) the gap was repaired with a silicone nerve guide and both ends were sealed using sterile Vaseline to avoid leakage and the nerve guide was filled with 100 µL of phosphate-buffered saline alone. In cell treated group (SILOCONE/BMMC) the nerve guide was filled with 100 µL BMMCs (2×10^6 cells/100 µL).

Pokeweed Mitogen-Stimulated Spleen Cell Conditioned Medium (PWM-SCM)

Spleen cells from a donor rat were cultured at a density of 2 x10^6cells/ml in RPMI 1640 medium containing 4 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nonessential amino acids (complete RPMI1640) containing lectin (8 mg/ml) and placed in 75-cm^2 tissue culture flasks. The cells were incubated at 37-8 ºC in a 5% CO2 humidified atmosphere. After 5–7 days, medium was collected, centrifuged for 15min at 3200 g, filtered through a 0.22 µm Millipore filter and used as PWM-SCM.

Preparation of the Bone Marrow Derived Mast Cells (BMMCs)

Bone marrow of a donor male rat was used to generate mast cells based on a method described by others [13]. Briefly, the animal was anesthetized, euthanized (see above) and intact femurs were removed. Sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe. The suspension of bone marrow cells was centrifuged at 320g for 10 min, and cultured at a concentration of 0.5×10^6 nucleated cells/ml in RPMI 1640 with 10% FCS, 100 units/ml penicillin, 100 mg/ml streptomycin (Life technology), 10 mg/ml gentamycin, 2 mM L-glutamine and 0.1 mM nonessential amino acids (referred to as enriched medium). PWM-SCM 20% [v/v] was added to the enriched medium. Flasks were then incubated at 37 °C in a 5% CO2 humidified atmosphere. Non-adherent cells were transferred to fresh medium at least once a week. After 3–4 weeks, mast cell purity of >90% was achieved as assessed by toluidine blue

staining and flow cytometery.

Staining of the Mast Cells

The granularity of the mast cells was determined by Toluidine blue, Alcian blue and Gimsa stainings. In brief, the cells were cytospun, fixed with Carnoy's fluid, and in Toluidine blue staining specimens stained by either 2 minutes with acid toluidine blue (PH=2.7). Cells were examined by light microscopy. Staining procedure was the same for Alcian blue staining on cytospun. Briefly, slides were incubated in 3% acetic acid, 3 minutes alcian blue solution microwave: Hi power, 30 seconds and Washed in running water for 2 minutes, rinsed in distilled water and counterstained in nuclear fast red solution for 5 minutes, dehydrated, cleared and coversliped [14].

Characterization of Mast Cells

Mast cells were harvested, and after washing with cold PBS, the cell-surface Fc receptors were blocked with 2.4G2 (PharMingen, San Diego, CA, USA) before staining. We used a PE-conjugated anti-mouse *c-kit* (PharMingen, USA) to stain *c-kit,* and mouse FcεRI was stained with an FITC-conjugated anti-mouse FcεRI antibody (PharMingen, USA) and compared with matched isotype control antibodies. The cells were incubated with antibodies in 50 µL of PBS for 1 h at 4°C, washed with PBS, and analyzed on BD FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA). Dead cells were gated out when performing the analysis [14].

Biomechanical Testing

The nerve samples were harvested and fixed between frozen fixtures in a mechanical apparatus. The TA.XTPlus Texture Analyzer mechanical test device was used for the assessment (Stable Micro Systems, Surrey GU7 1YL, UK). After 5 min, the frozen fixtures were tightened to ensure that no slippage occurred during testing. The initial length was set to 20 mm. Each sample was stretched at a constant rate of 1 mm/min. The load and displacement were sampled 5 times per second. Each sample was stretched to complete tensile failure. Samples were kept moist during testing using a drop of normal saline solution to the nerve segments.

Histological Assessments

The regenerated nerves from all groups were isolated and 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. An image analyzing software [Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA] was used to perform morphometric analysis.

Immunohistochemical Assessments

For myelin sheath determination anti-S-100 (1:200, DAKO, USA) was used as a marker based on a method described in a previous study [9]. In brief, samples were post fixed with 4% paraformaldehyde for 2h and embedded in paraffin. Before immunreaction the samples were dewaxed and rehydrated in PBS (PH 7.4). The samples were then incubated with 0.6% hydrogen peroxide for 30 minutes. After blocking of non-specific immunoreactions, sections were then incubated in S-100 protein antibody solution for 1h at room temperature. Following washing with PBS and incubating in biotinylated anti-mouse rabbit IgG solution for 1h, horseradish peroxidase-labelled secondary antibody was applied for 1 h. The samples were then incubated with chromogene substrate solution (DAB, DAKO, USA) for 10 min. The results of immunohistochemistry were examined under a light microscope and assessed qualitatively.

Statistical Analysis

The Results were analyzed using repeated measures and a factorial ANOVA with two between-subjects factors and the Bonferroni test was used to examine the effect of time and treatments. Experimental results were expressed as means±SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA) and the significant difference was set at $p<0.05$.

Results

Staining of the Mast Cells

Findings of Mast Cell Staining

Bone marrow stromal cells of the mice were successfully harvested and cultured. In the first culture of the cells both adherent and confluent cells were observed that were appeared as heterogeneous cells. Within the first week the adherent cells were observed as confluent cells. In contrary to other common culture media, the confluent cells could live longer. In the second passage, because of limited space in the smaller flasks (T25), the confluent cells were appeared densely and on days 18 and 19 the first culture cells were appeared more homogenous. A few dividing cells were also observed. Following 3 to 4 passages and change of the culture media on day 21, the cells were homogenous enough to be harvested. The harvested cells were counted and their viability was assessed using trypan blue with Neubauer method. From each flask 12,000,000 cells with viability rate of 90% were harvested. After centrifugation, the supernatant was discarded and the pellet was resuspended in a 1 mL culture media and spread on slides. The slides were air dried at room temperature. They were fixed using carnoy and stained using toluidine blue, alcin blue and gimsa stains.The granules of mast cells were purple to red where stained with toluidine blue. These cells were matachromatic. The granules were blue and the nuclei were red where stained with alcin blue and violet where stained with gimsa (Figure 1).

Findings of Characterization of Mast Cells

To characterize the harvested cells, 100,000 cells were also assessed based on their surface markers. In the present study, for the harvested cells from differentiated bone marrow stromal cells specific markers, CD117 (c-kit) and FCεRI, were used using flow cytometers. The results showed that the cells were positive for Mast cell-related antigens for each of CD117 and FCεRI 95% and 93%, respectively, and for both of the markers 90% (Figure 2). This result was consistent with successful differentiation of the cells.

Findings of Biomechanical Testing

Biomechanical parameters including maximal load, elastic limit load, maximal displacement, maximal stress, elastic limit stress, maximal strain and elastic limit strain in experimental groups are presented in Table 1. The biomechanical findings indicated that the parameters were significantly improved in the BMMCs treated animals than non-treated ones ($p<0.05$).

Histological Studies

The animals in SILICONE/BMMC group demonstrated significantly greater nerve fiber, axon diameter, and myelin sheath thickness during the study period compared to non-treated animals (Table 2) (Figure 3) ($p<0.05$).

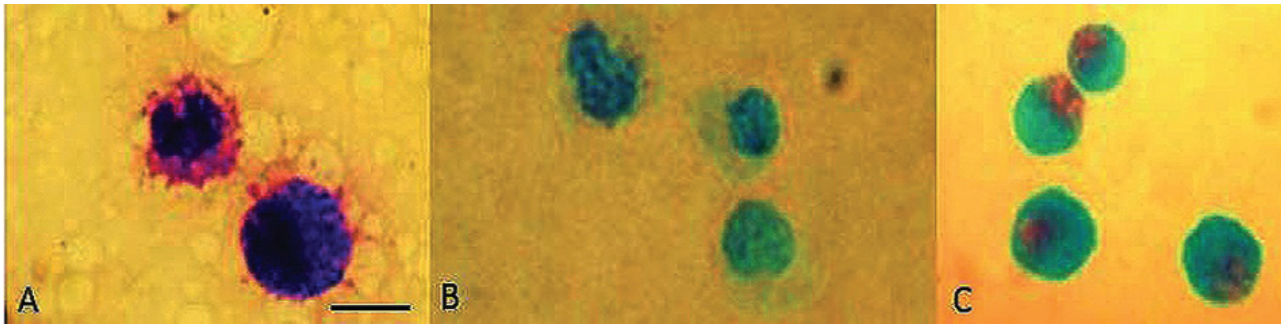

Fig. 1. Bone marrow mast cells from rat were cultured in the medium during the third week of culturing bone marrow cells. (A) Gimsa, (B)Toluidine blue and (C) Alcian blue staining. Scale bar: 10 µm

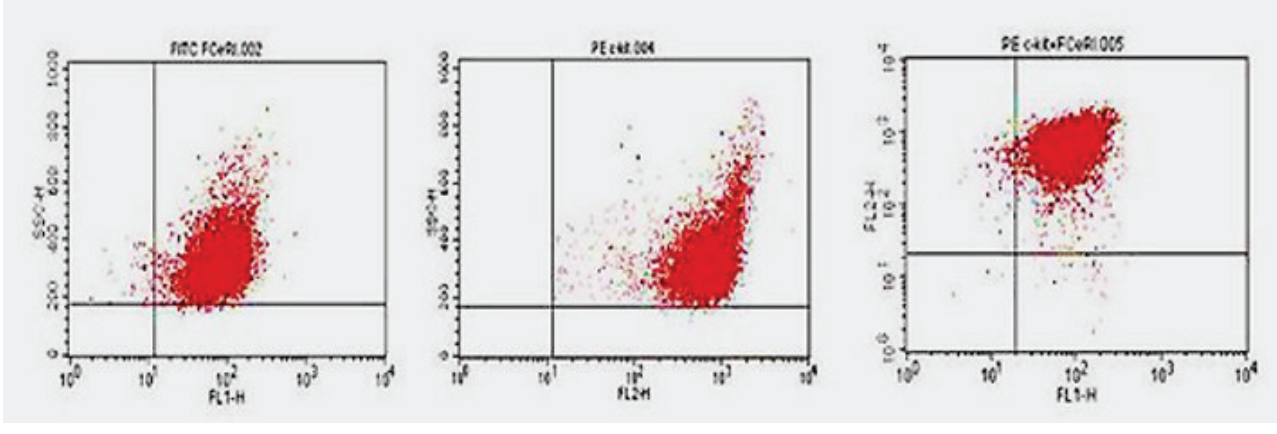

Fig. 2. Characterization of bone marrow derived mast cells (BMMC) after 3 weeks. Flow cytometric analyses of cell surface markers showed that the cells were positive for BMMC-related antigens of FCεRI (93%), CD117 (c-kit) (95%) and for BMMC-related double positive cells (90%).

Table 1. Biomechanical parameters assessed for each of the experimental groups. Values are given as mean±SEM.

Groups	Maximum Stress (N/mm^2)	Maximum Strain (%)	Maximum Displacement (mm)	Maximum Load (N)	Elastic Limit (Load) (N)	Elastic Limit (Stress) (%)	Elastic Limit (Strain)(%)
Sham	0.96±0.10	72.46±3.28	17.53±1.52	15.72±0.82	9.39±0.12	0.70±0.09	26.27±0.38
Silicone	0.58±0.09	57.15±2.23	11.15±1.13	8.17±0.36	5.14±0.18	0.56±0.37	22.19±0.82
Silocone/Bmmc	0.67±0.06[a]	67.25±2.43[a]	14.71±1.12[a]	11.35±0.17[a]	7.08±0.12[a]	0.58±0.19[a]	24.27±0.65

[a]The mean difference is significant at the .05 level *vs* SILICONE; The treated groups are compared by Student t test with other groups.

Table 2. Morphometric analyses of sciatic nerve in each of the experimental groups: Values are given as mean±SEM.

Groups	Myelinated Axons Counts (fb/mm^2)	Axon diameter (µm)	Myelin sheath thickness(µm)
Sham	6126±397	9.35±0.15	2.34±0.72
Silicone	4275±273	5.77±0.46	1.65±0.54
Silicone/Bmmc	4973±282[a]	7.43±0.24[a]	1.46±0.61

[a]The mean difference is significant at the .05 level *vs*. SILICONE group.

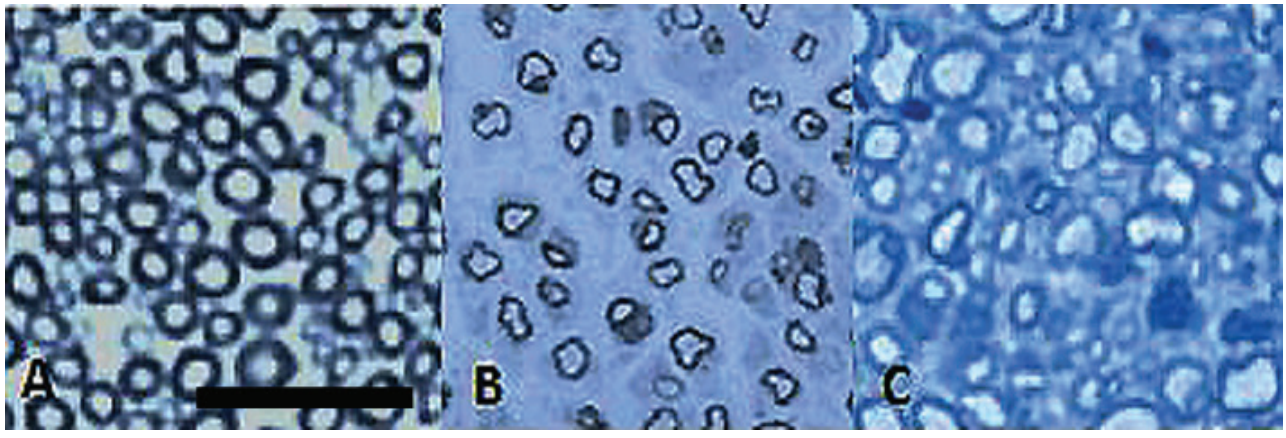

Fig. 3. Light micrograph of representative cross section taken from (A) midpoint of normal sciatic nerve (SHAM), (B) middle point of SILICONE and (C) nSILICONE/BMMC group 6 months after surgery. Toluidine blue staining. Scale bar:50µm

Immunohistochemistry

The qualitative analysis of immunohistochemistry of regenerated nerve fibers showed extensive immunoreactivity to S-100 protein in SILICONE/BMMC group. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a slight expression showing that there was Schwann cell-like phenotype around the myelinated axons (Figure 4).

Discussion

The local xenotransplantation of BMMCs improved functional recovery, biomechanical and histomorphometric indices in cats with transected sciatic nerves in the present study.

In the present study local administration of BMMCs resulted in the enhanced biomechanical properties. Peripheral nerves are remarkable tissues that not only conduct electrical impulses, however, also must bend and stretch to accommodate the movement of limbs. In order to achieve this, they have a complex structure consisting of bundles of neurons packed into fascicles and surrounded by connective tissue layers, the perineurium and epineurium. Both neural and connective tissue elements are tethered proximally at the spinal cord and have numerous branch points allowing neurons from a single nerve trunk to synapse with various target organs. The strongest connective tissue layers in peripheral nerves are the perineurium and, to a lesser extent, the epineurium. Changes in the epineurium and perineurium extracellular matrix composition are likely to have significant effects on the biomechanical properties of acellular nerve [15]. The connective tissue from the epineurium forms a layer of fiber membrane at the 3rd day postoperatively and then forms collagen at the 8th day. The key point influencing functional recovery is the number of axons throughout the suture that enhances the anti-tension capacity of the nerve [16]. Identifying the maximum tension which nerves can withstand and understanding the origin of their mechanical resilience is of great importance to improve the outcome of surgical nerve repairs. Their behaviour under loading is viscoelastic and is likely to be dependent upon a number of factors such as the internal fluid pressure maintained by the impermeable perineurium, the outer-inner layer integrity, the number and arrangement of fascicles and the molecular structural elements of the extracellular matrix such as collagen and elastin [16].

To the best knowledge of the authors, the effects of xenotransplantation of BMMCs on nerve regeneration and the biomechanical properties of sciatic nerve in cat have not previously been compared. Therefore, regarding the mechanical testing, the authors were

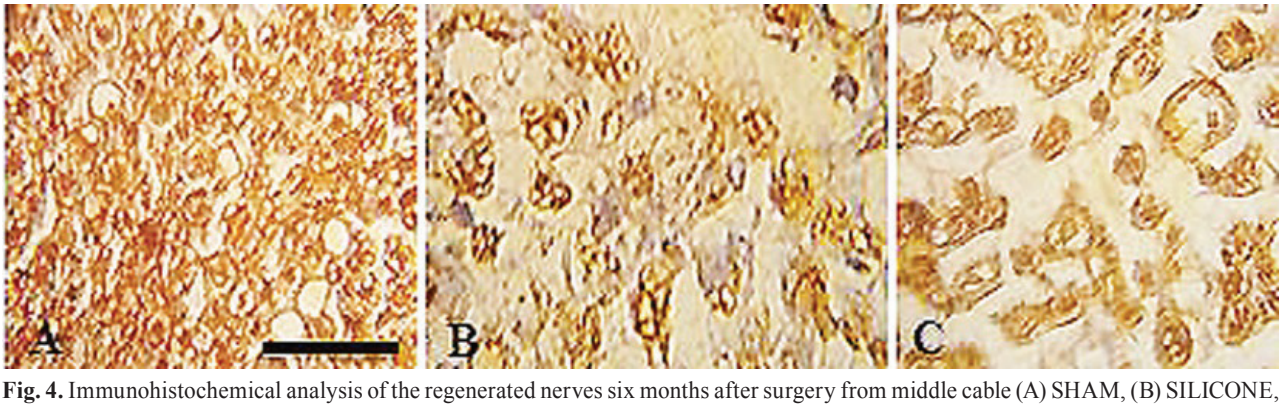

Fig. 4. Immunohistochemical analysis of the regenerated nerves six months after surgery from middle cable (A) SHAM, (B) SILICONE, (C) SILICONE/BMMC. There is clearly more positive staining of the myelin sheath-associated protein S- 100 (arrow) within the periphery of nerve, indicating well organized structural nerve reconstruction in the cell transplanted nerve. Scale bar: 50 µm

not able to compare the results of the present study to other published studies. There are few studies on cat nerve regeneration using different materials in various nerve models without dealing with biomechanical properties of the regenerated nerve fibers, however, they studied electrophysiological, histomorphometical and immunohistochemical characteristics of the regenerated nerves [17-20].

Morphometric analysis of the repaired nerve fibers indicated that there was significant difference between SILICONE/BMMC and SILICONE animals. Regarding better functional and morphometric indices in SILICONE/BMMC group, it could be stated that cell therapy both accelerated and improved the process of nerve regeneration.

In immunohistochemistry the expression of axon and myelin sheath special proteins was apparent in cell treated animals demonstrating the normal structure in histology. The response to S-100 in BMMCs treated animals was evidently more positive than in SILICONE group. This further implied that both repaired axon and Schwann cell-like cells were present and accompanied by the process of myelination and the structural recovery of repaired nerve fibers.

Depending on the mast cell phenotype and stimulus, mast cells initiate the transcription, translation and secretion of a varied array of cytokines including PDGF, VEGF. It has already been shown that PDGF, VEGF bear beneficial effects on peripheral nerve regeneration [17-20].

Mast cells have been proposed as angiogenesis promoters and the mast cell count appears to be a reliable prognostic marker in some tumors [21, 22]. Mast cells cause neovascularization by producing angiogenic factors, such as VEGF, or substances with angiogenic properties, such as tryptase, FGF, TNF, interleukin (IL)-8, histamine and heparin.

Angiogenesis is a complex process governed by many different variables. Growth factors, including VEGF, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), play important roles. Consequently, their generation within nerve conduits is vital to achieving positive clinical outcomes [23].

The regulatory influence of the mast cells is also demonstrated by the nerve repair phenomena characteristic of the proliferation stage [24]. The release of specific mediators (vasoactive amines, tryptase, IL-4 and NGF) of mast cell origin is essential to initiate regeneration of damaged nerve fibres, and lead to temporary hyperinnervation of the scar at this stage [21-29].

Mast cell- derived cytokines, including TNF, and growth factors, such as NGF, lower the threshold for activation of local neurons and promote nerve fiber growth. There is anatomical evidence for mast cell associations with peripheral myelinated and unmyelinated nerves [30]. The mast cells were introduced in the site of injury in the present preliminary study regarding this fact that changing the mast cell microenvironment alters significant changes in phenotype of the mast cells and they may act as growth factors packages that only degranulate *in situ* and do not induce inflammatory responses [26].

This preliminary study was conducted to assess effects of *in situ* xenotransplantation BMMCs at the site of peripheral nerve injury. Since mast cells bear armamentarium of inflammatory mediators, the authors aimed to assess whether the BMMCs could positively affect the nerve repair process. We aimed to use the cells as package of mediators and growth factors and we expected autolysis of the cells in situ and release of the agents. Study on proliferation and differentiation of the cells were not the case, as it remains to be studied in the future.

The major limitation of the present study was comparison of the cells with extracellular matrix, microtubules, fibroblasts, Schwan cells and other nerve segment constituents and conduits with giving the histological and molecular evidences for neuroprotective action of BMMCs. This would be considered for further studies. Therefore, the authors stress that the current investigation was conducted to evaluate a single local dose and clinical treatment potential of BMMCs on nerve repair and precise mechanisms of neuroprotective action of BMMCs in transection models remain to be investigated.

The alteration in the behavior of mast cells could be favorable in cell therapy where readily accessible and instant source of cells in large quantities are required and could be taken into consideration in the emerging field of regenerative medicine and surgery. It could be considered clinically as a translatable route towards new methods to enhance peripheral nerve repair in clinical applications.

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Conflicts of Interest: None declared.

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