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Original Article

Electrophysiological and histopathological effects of mesenchymal stem cells in treatment of experimental rat model of sciatic nerve injury

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Abstract: Aim: The aim of this study was to evaluate electrophysiological and histopathological effects of mesenchymal stem cells in treatment of sciatic nerve injury. Material and methods: Thirty-two female Spraque-Dawley rat were used in this study. Eight rats were used as a reference group in electrophysiological analysis for evaluation of non-injured nerve recordings (Control Group). Twenty-four rats were used for experimental evaluation. Twelve rats were anastomosed without treatment with mesenchymal stem cells (Sham Group) and twelve other rats were anastomosed and treated with mesenchymal stem cells (Stem Cell Group). Surgicel and bioglue were used in anastomosed line in both Groups. Eight weeks after the surgery, electrophysiological evaluation of rats was performed and, then, rats were decapitated under anesthesia and specimens including sciatic nerves and anastomosed line were taken for histopathological evaluation. Electromyography and nerve conduction velocity testing and histopathological scoring including rate of Wallerian degeneration, and neuroma and scar formation were evaluated for both Groups. Results: There were no statistically significant differences between Sham and Stem Cell Groups with respect to histopathological evaluation. However, nerve conduction velocity showed significant difference between groups (P = 0.001). Nerve conduction velocity was significantly improved in Stem Cell Group when compared to Sham Group. Conclusion: In this study, based on nerve conduction velocity data, it was concluded that treatment with mesenchymal stem cells during end-to-end anastomosis improves functional regeneration.

Keywords: Mesenchymal stem cells, experimental sciatic nerve injury, nerve conduction velocity, scar tissue

Introduction

Although peripheral nerve injuries are common, reconstruction of transected nerves often results in incomplete or non-functional recovery [9, 12, 21]. The functional reconstruction is possible with primary nerve repair or with the use of nerve autograft, both of which are still accepted as golden standard methods for treatment [1, 2, 5, 9]. Primary nerve repair requires direct approximation of proximal and distal stumps, which is often not successful due to large gap in between; and, therefore, autografts are the preferred treatment for severe injuries [9]. The problems with autografts, on the other hand, are the donor-site morbidities such as denervation distal to the donor site, scarring, and neuroma formation, and the limited options for the selection of a suitable site for nerve harvesting [9, 36]. That is, new treatment modalities are definitely required for reconstruction of severely injured nerves.

Progenitor cells and stem cells were widely studied in handling ischemic stroke and neurodegenerative diseases of central nervous system. The possible use of stem cells in reconstruction of peripheral nerve injuries is currently under investigation. Mesenchymal stem cells are successfully used in hematopoietic system and autoimmune-inflammatory (autoinflamma-

tory) diseases, and regenerative medicine. Being immunosuppressive and non-immunogenic, they are mostly preferred for treatments where HLA matching is not required [10, 34].

In this study, we investigated the regenerative, histopathological and electrophysiological effects of treatment with mesenchymal stem cells in reconstruction of experimental rat model of sciatic nerve injury.

Material and methods

Study design

This experimental animal study was approved by the Ondokuz Mayis University Ethics Committee for Animal Experiments. All experiments were carried out in accordance with international guidelines and local requirements at the Experimental Animal Implementation and Research Center of Ondokuz Mayis University.

Thirty-two adult female Spraque-Dawley rats, weighing 200 to 300 g, were used in the study and randomly divided into three groups. Eight rats were separated to be used as a reference group in electrophysiological analysis for evaluation of non-injured nerve recordings (Control Group). Twelve rats were anastomosed without treatment with mesenchymal stem cells (Sham Group) and twelve other rats were anastomosed and treated with mesenchymal stem cells (Stem Cell Group) for evaluation of electrophysiological and histopathological effects of treatment with mesenchymal stem cells after experimental sciatic nerve injury.

Mesenchymal stem cell acquisition

Mesenchymal stem cells (MSC) from 4-week-old green fluorescent protein (GFP) transgenic Sprague Dawley rats used in the study were provided by the Molecular Biology Department of Genetics Division of Ondokuz Mayis University. The animals were deeply anesthetized, the femurs and tibias were dissected and the bone marrow was plated on Petri dishes containing Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and Primocin. Cells were allowed to adhere, and nonadherent cells were removed after 48 hours by replacing with fresh medium. Adherent cells were then cultivated at 37°C in a humidified atmosphere containing 5% CO_2 with refreshing the medium twice a week. The cells were harvested by a Trypsin/EDTA solution when reached near confluence. After two to three passages, the cells were labeled with SPION and transplanted into animals. Cells were characterized as MSC by their spindle-shaped morphology and adherence to plastic, while their multipotency was confirmed by their differentiation into adipocytes, osteoblasts, and chondroblasts according to a standard differentiation protocol [11].

MSC identification with flow cytometry

The flow cytometry studies were undertaken according to the criteria defined by Dominici et al. in 2006 [11]. Mononuclear cells harvested in interphase were placed in 3 ml vials and 6 vials were used for each cell line. Tubes were labeled as surface markers and control group, respectively, and 100 µl cell suspension containing 1×10^6 cells were added to each tube. Ten µl surface markers were added to the tubes number 1 to 5. None of the surface markers were added to the tube number 6. All of the tubes were vortexed and incubated at 4°C in dark for 15 minutes. One ml of washing solution was added and tubes were centrifuged at 1400 rpm for 5 minutes. Supernatant was removed before adding 0.5 ml washing solution to the residual cell sediment and cell identification was performed by flow cytometry. The outcome of using HSC (hematopoetic stem cell) and MSC markers CD11b/c, CD44, CD45, CD90, CD106 were CD11b/c [%97 (+)], CD44 [%1 (-)], CD45 [%99 (+)], CD90 [%34 (+)] and CD106 [%11 (-)].

Surgical procedure

Rats were allowed free access to water and food before and after surgery. Cefazolin sodium (10 mg/kg, Cefozin® Bilim İlaç) was applied presurgery via intramuscular injection for prophylaxis. The rats were anesthetized with an intraperitoneal injection of ketamine (50-150 mg, Ketalar® Parke Davis) and xylazine (5-10 mg/kg, Rompun® Bayer). Rats were then placed in the prone position and left femoral and gluteal areas were exposed. Surgical areas were shaved and scrubbed with 10% povidone-iodine solution for antisepsis. The sciatic nerve was exposed by 3 cm surgical incision parallel to femur and dissection of extensor and flexor group of the thigh muscles. In Sham Group rats, the sciatic nerve was divided using a microsurgical technique, and end-to-end anastomosed by 3 sutures with 8-0 prolene suture

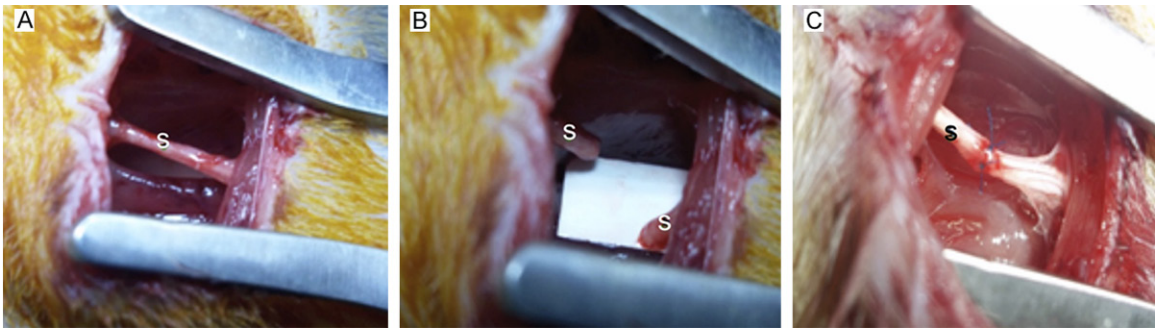

Figure 1. Dissection (A, B) and surgical reconstruction (C) of experimental sciatic nerve injury (S: sciatic nerve).

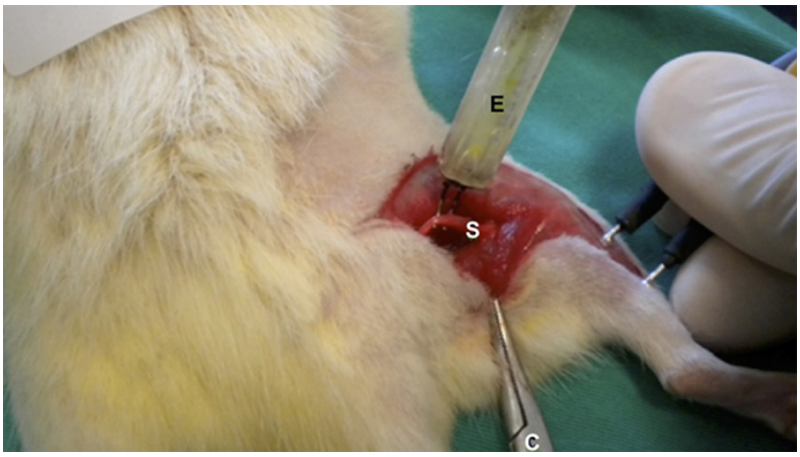

Figure 2. EMG testing (S: sciatic nerve, C: cla-mp, E: electrode).

under the microscope (**Figure 1**). Surgicel (Surgicel® ETHICON) and bioglue (BioGlue® CryoLife) were used in anastomosed line to eliminate other factors affecting the healing. The muscle fascia was sutured with 3-0 Vicryl suture, and the skin was closed anatomically. In Stem Cell Group rats, the sciatic nerve was also divided and end-to-end anastomosed by 3 sutures with 8-0 prolene suture under the microscope (**Figure 1**). MSC (2×10^6 cells) injected to anastomosed line by an insulin pen, and surgicel (Surgicel® ETHICON) and bioglue (BioGlue® CryoLife) were used in anastomosed line to stabilize MSC in the surgical area. The muscle fascia was sutured with 3-0 Vicryl suture, and the skin was closed anatomically. Rats were treated with paracetamol (2 mg/ml) added to drinking water for 3 days after the surgery for postoperative analgesia. There were no animal lost during the study. Eight weeks after the surgery, electrophysiological evaluation of rats was performed and, then, rats were decapitated under anesthesia and specimens were taken for histopathological evaluation.

Electrophysiological evaluation

Electrophysiological evaluation studies were performed with PowerLab/4SP (ADInstruments, Australia) and analyzed by Scope program. Basal nerve electrophysiological recordings were evaluated with control group rats without experimental sciatic nerve injuries. Eight weeks after surgery, Sham and Stem Cell Group rats were anesthetized and the sciatic nerves were exposed to evaluate nerve conduction velocity (NCV) and electromyography (EMG) testing.

Nerve conduction studies were performed by stimulating sciatic nerve with supramaximal impulse and measuring the action potential on the same nerve. Stimulating electrode was attached 5 mm proximal to the sutured nerve and recording electrode was to the injury distal. The amplitude and latency values of action potentials of the impulse and the distance between electrodes were measured. NCV was calculated as mm/s with dividing the distance between the electrodes to initial latency.

For EMG testing, stimulating electrode was inserted 5 mm proximal to the sutured nerve, the compound electrode to the gastrocnemius muscle, and a reference cap electrode to the knee joint (**Figure 2**). Threshold supramaximal

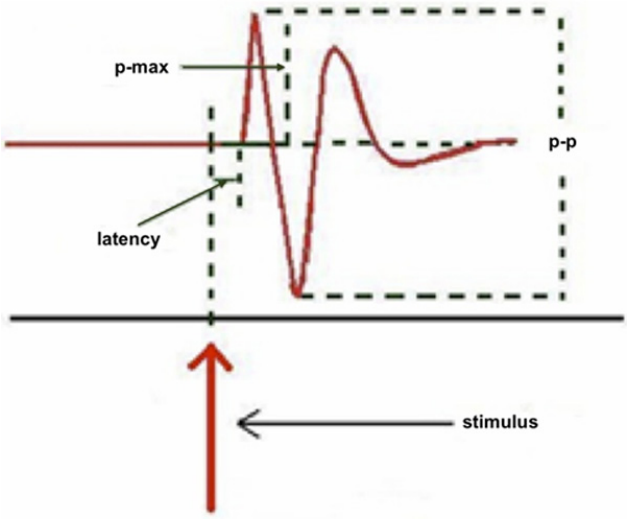

Figure 3. Parameters used for EMG testing.

Table 1. Histopathological scoring criteria

Score	Criteria
Scar formation	
Grade 1	<100 fibroblasts in ×40
Grade 2	100-150 fibroblasts in ×40
Grade 3	>150 fibroblasts in ×40
Neuroma formation	
1	Absent
2	Present
Rate of Wallerian degeneration	
1	Slow degeneration
2	Medium degeneration
3	Fast degeneration

impulse currents were adjusted for each rat before EMG recordings. For each rat, three independent measurements for the compound muscle action potential in the gastrocnemius muscle was recorded and averaged. Time to deflection (latency), peak-to-peak amplitude (p-p) and maximum peak values (p-max) were used as parameters for evaluating EMG recordings (**Figure 3**).

Histopathological evaluation

Rats were decapitated eight weeks after the surgery and specimens were taken from 1 cm proximal and 1 cm distal regions of repaired sciatic nerve and fixed with 10% formaldehyde. Sciatic nerve specimens including suture line and its proximal and distal regions, were embedded in paraffin. Three blocks of five-micrometer-thick sections were stained with hematoxylin and eosin (H&E) stain to calculate scar and neuroma formation and with toluidine blue to calculate rate of Wallerian degeneration under light microscopy. Histopathological evaluation was scored as given in **Table 1**.

Statistical analysis

Statistical analysis was performed with the SPSS software package (Statistical Package for Social Sciences, version 13.0, SPSS Inc., Chicago, Illinois, USA). Electrophysiological recordings were analyzed by ANOVA. Histopathological evaluation results and categorical variables were analyzed by chi-square and Fisher's exact test. The normality of quantitative variables were analyzed by Kalmogorov-Smirnov test and normally distributed variables were compared with variance analyses and Hoc Tukey HSD tests. A *p* value less than 0.05 was considered as statistically significant.

Results

Electrophysiological recordings

Effects of treatment with mesenchymal stem cells after experimental sciatic nerve injury on electrophysiological recordings of Control, Sham and Stem Cell group rats were summarized in **Table 2**. Comparison of EMGs were based on time to deflection (latency), peak-to-peak amplitude (p-p) and maximum peak values (p-max). Results indicated statistically significant improvement in EMG, p-p and p-max values between Sham and Control, and Stem Cell and Control Groups after surgical sciatic nerve dissection and repair (**Table 2**, 0.05 for both). Statistical comparison of EMG, p-p and p-max recordings between Sham and Stem Cell Groups, on the other hand, indicated no significant difference (P > 0.05). NCV record-ings of Sham and Stem Cell Groups when individually compared to control indicated no statistically significant difference; whereas, treatment of experimental sciatic nerve injury with MSC significantly improved NSV recordings in Stem Cell Group compared to Sham group (**Table 2**, P = 0.001). The comparison of com-pound muscle action potential results between the Groups indicated no statis-

Table 2. Results of electrophysiological evaluation of experimental rat model of sciatic nerve injury

	Control Group (n =8)	Sham Group (n =12)	Stem Cell Group (n =12)	*p* value[a]
EMG (ms)	2.08	4.01	3.36	$<0.05^1$; $<0.05^2$; $>0.05^3$
p-p (mV)	29.81	8.14	11.82	$<0.05^1$; $<0.05^2$; $>0.05^3$
p-max (mV)	19.25	5.77	8.63	$<0.05^1$; $<0.05^2$; $>0.05^3$
NCV (m/s)	42.36	36.77	41.86	$<0.05^1$; $>0.05^2$; 0.001^3
Compound muscle action potential (mV)	26.56	25.84	26.77	$>0.05^1$; $>0.05^2$; $>0.05^3$

EMG, electromyography; p-p, peak-to-peak amplitude; p-max, maximum peak value; NCV, nerve conduction velocity. [1]*p* value between Control and Sham Groups; [2]*p* value between Control and Stem Cell Groups; [3]*p* value between Sham and Stem Cell Groups. A *p* value <0.001 ([a]) was considered statistically significant.

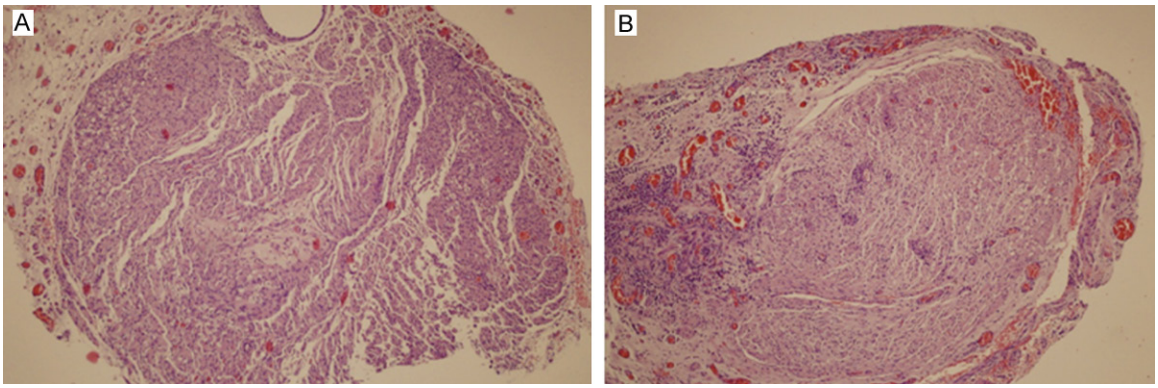

Figure 4. Scar formation visualized by hematoxylin and eosin (H&E) staining. A. Grade 1 scar formation (×100 magnification); B. Grade 2 scar formation (×10 magnification).

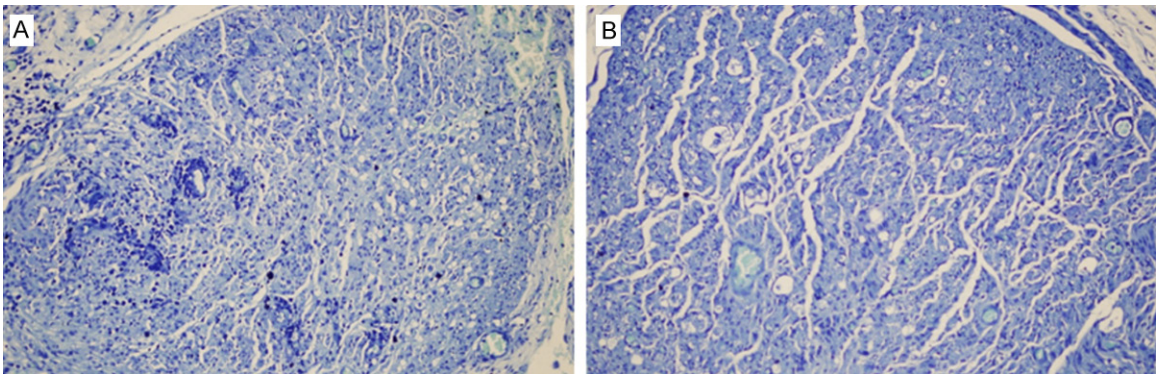

Figure 5. Wallerian degeneration visualized by toluidine blue staining (×200 magnification). A. Alow degeneration; B. Medium degeneration.

tically significant change in the outcomes (**Table 2**, P > 0.05 for all).

Histopathological scoring

The analyses of histopathological effects of MSC in treatment of experimental rat model of sciatic nerve injury included determination of scar formation, neuroma formation and rate of Wallerian degeneration. The degree of pathological changes were scored according to **Table 1**. Statistical comparison of results showed no significant differences in between Sham and Stem Cell Groups with respect to scar formation, neuroma formation, and rate of Wallerian degeneration (**Figures 4**, **5**; **Table 3**).

Discussion

Several surgical techniques can be used for the treatment of nerve injuries with no clinical superiority to each other. Factors such as com-

Table 3. Results of histopathological evaluation of experimental rat model of sciatic nerve injury (P > 0.05 for all)

	Sham Group (n = 12)	Stem Cell Group (n = 12)	Total (n = 24)
Scar formation (n, %)			
Grade 1	4 (33.3)	5 (41.7)	9 (37.5)
Grade 2	8 (66.7)	7 (58.3)	15 (62.5)
Grade 3	0	0	0
Neuroma formation (n, %)			
1	12 (100)	12 (100)	24 (100)
2	0	0	0
Rate of Wallerian degeneration (n, %)			
1	7 (58.3)	7 (58.3)	14 (58.3)
2	5 (41.7)	4 (33.3)	9 (37.5)
3	0	1 (8.3)	1 (4.2)

pliance of proximal and distal ends, tension in anastomotic line, and use of atraumatic suture technique are known to positively affect the regeneration. Treatment of peripheral nerve injuries, however, is still a major problem due to the limitations in nerve regeneration, which necessitate development of alternative surgical approaches [12, 36, 40, 41]. For this respect, stem cells appear to be a promising treatment option for severe nerve injuries [6, 13, 28].

Stem cells became commonly used in peripheral nerve surgery studies from 2000 and on [7, 8, 13, 25]. Schwann cells, which provide trophic support for neurons, play a key role in peripheral nerve regeneration [1]. Both syngeneic and allogeneic Schwann cell transplantation were proven to improve regeneration [13, 17, 23]. Acquiring enough Schwann cells, on the other hand, is difficult; therefore, neuronal progenitor cells which can differentiate to sustentacular cells similar to Schwann cells are preferred for the treatment of peripheral nerve injuries [25]. Murakami et al. [25], for example, reported results of treatment of peripheral nerve injury with neuronal progenitor cells with improved axonal regeneration. Cuevas et al. [8] studied the effect of bone marrow stromal cells implanted to peripheral nerve injury, and reported the enhancement in new fiber regeneration. Transplantation of vein graft cultured bone marrow stromal cells was reported to improve peripheral nerve regeneration based on statistically significant increase in axon numbers in 4, 8 and 12 weeks after the surgery [7]. Zarbakhsh et al. [40, 41] investigated indi-vidual and synergistic effects of transplantation with Schwann and bone marrow stromal cells in axonal regeneration in experimental rat model of peripheral nerve injury, and showed the effectiveness of both approaches in functional recovery in 12 weeks. Adipose-derived stem cells differentiated to Schwann cell-like phenotype *in vitro* were successfully used as engineered neural tissue in treatment of rat sciatic nerve with a 15 mm gap [13]. Recent molecular biology and regenerative medicine studies have also indicated the potential of mesenchymal stem cells (MSC) for medical treatment. MSC are pluripotent immature cells which can be differentiated to bone, cartilage or fat cell depending on the purpose [28]. Undifferentiated MSC does not recognized by immune system and, thereby, cause an immune reaction; therefore MSC transplantations can be effectively used in treatment of several diseases [6, 34].

In this study, we investigated the effect of treatment with MSC in surgical reconstruction of experimental rat model of sciatic nerve injury. The evaluation based on electrophysiological and histopathological comparison of two main group of rats; Sham Group where no MSC were used in surgical treatment and Stem Cell Group treated with MSC. The surgical factors that can affect the sciatic nerve regeneration or electrophysiological and histopathological evaluation results are mainly the number of sutures used in anastomosis and the use of bioglue. The number of sutures used for surgical reconstruction in Sham and Stem Cell Groups were determined based on the literature. Martins et al. in 2011 [22] compared collagen 1 and collagen 3 amounts in treatment of experimental rat model of sciatic nerve injury with 3 or 6 epineural nerve sutures 8 weeks after the surgery, and reported less collagen amount and less epineural scar tissue in rat group anastomosed by 3 sutures. Bioglue, which was used in this

study to stabilize MSC in the anastomosed line during the surgery, is commonly used in nerve repair [3, 4, 24, 27, 35]. It is a surgical adhesive with no thrombocytes, and it was proven not to cause any clot formation or act like a barrier in axonal progression during nerve regeneration [31].

We used both electrophysiological (EMG, p-p, p-max, NCV and compound muscle action potential) and histopathological (scar formation, neuroma formation and rate of Wallerian degeneration) testing for the evaluation of the effects of treatment with MSC in surgical reconstruction of experimental rat model of sciatic nerve injury. Electrophysiological comparisons allow objective and numeric evaluation of nerve regeneration, and therefore, widely used in peripheral nerve injuries. In this study, the electrophysiological comparisons of Sham and Stem Cell Group rats were performed 8 weeks after the surgery, based on the literature suggesting sciatic nerve regeneration 4 to 8 weeks after surgical reconstruction, and functional regeneration in 8 weeks [13, 16, 29]. No statistically significant differences were observed in comparison of Sham and Stem Cell Groups with respect to EMG evaluation parameters (EMG, p-p, p-max, **Table 2**, $P > 0.05$ or all); whereas NCV was statistically improved in treatment with MSC during the surgery (**Table 2**, $P = 0.001$).

Histopathological comparison of the effects of Sham and Stem Cell Group rats after surgical reconstruction of sciatic nerve injury with or without use of MSC included determination of scar formation, neuroma formation and rate of Wallerian degeneration. One of the main problems in surgical reconstruction of peripheral nerve injuries is the scar formation in anastomosed line. A severe nerve injury causes fibroblasts to aggregate on the injured area. Collagen secretion in endoneurium is provided by both fibroblast and Schwann cells. The accumulation of collagen in the injured nerve causes thickening of endoneural tube and tightens the area for axonal regeneration [15]. Several agents can be used to reduce epineural and extraneural scar formation, such as aprotinin, ADCON-T/N, cis-hydroxyproline, estrogen-progesterone, and methylprednisolone acetate [14, 26, 32, 33]. In their studies, Gorgulu et al. [15] showed that low-dose exter-nal beam radiation decreased scar formation on extraneural scarring after peripheral nerve surgery in rats. In this study, we investigated the effect of MSC in reducing scar formation after sciatic nerve surgery. The comparison of Sham and Stem Cell Groups indicated no statistically significant difference with respect to scar formation (**Table 3**, $P > 0.05$).

Neuroma is one of the reasons for posttraumatic or postoperative chronic pain after nerve injuries. The distal ends can swell due to peripheral nerve injury, and neuroma can form in connective tissues [37]. Neuromas usually appear in long term, several months, even years, after trauma or surgery; therefore, early detection and treatment of neuroma formation is not possible. In this study, the histopathological evaluation of neuromas 8 weeks after surgical reconstruction of experimental rat model of sciatic nerve injury indicated no neuroma formation in both Sham and Stem Cell Groups (**Table 3**, $P > 0.05$), supporting that neuroma is a pathology developing in the long term.

Severe injuries of peripheral nerve, except neurapraxia, leads to Wallerian degeneration in the distal nerve, which is caused by Schwann cells [9]. Regeneration starts from proximal to distal line in injured nerve after completion of Wallerian degeneration [38]. In our study, histopathological comparison indicated medium rate of Wallerian degeneration in both Sham and Stem Cell Groups (**Table 3**, $P > 0.05$). The similar rate of Wallerian degeneration in both Groups suggested similar regeneration rate in both treatments; however, a long term comparison is still required to obtain final results considering the functional regeneration may take up to a year.

Conclusion

There are several studies in the literature reporting the positive effect of stem cells on regeneration in peripheral nerve injuries [6, 9-11, 18, 19, 25, 28, 30, 36, 39]. Kanaya et al. [20] reported incompatibility in parameters used to evaluate peripheral nerve regeneration and suggested the evaluation of several parameters together. In this study, we investigated the effect of MSC in surgical reconstruction of experimental rat model of sciatic nerve injury with both electrophysiological and histopathological evaluation 8 weeks after the surgery to

observe and compare morphological and physiological regeneration. Our results indicated no statistically significant difference in treatment with MSC with respect to parameters evaluated in histopathological scoring, namely, scar and neuroma formations, and rate of Wallerian degeneration; whereas among electrophysiological parameters, NCV, which demonstrates the physiological regeneration, significantly improved in Stem Cell Group, similar to other studies [30, 39]. The results obtained in this study indicated the potential of mesenchymal stem cells for clinical use.

Disclosure of conflict of interest

None.

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