Peripheral nerve regeneration: A comparative study of the effects of autologous bone marrow-derived mesenchymal stem cells, platelet-rich plasma, and lateral saphenous vein graft as a conduit in a dog model

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Abstract

Background: The quality of healing of peripheral nerve injuries remains a common challenge causing pain and poor quality of life for millions of people and animals annually.

Aims: The objectives of this study were to evaluate the healing quality of facial nerve injury in a dog model following local treatment using an autologous injection of platelet-rich plasma (PRP) or bone marrow-derived mesenchymal stem cells (BM-MSCs) at the injury site in combination with the application of an autologous saphenous vein graft as a conduit.

Methods: 20 apparently healthy adult Mongrel dogs were randomly divided into 4 equal groups. Dogs in groups 1, 2, and 3 were subjected to facial nerve neurectomy and saphenous vein conduit graft implantation at the site of facial nerve injury. Dogs in groups 2 and 3 received 1 ml of autologous PRP and BM-MSCs, respectively. Injections were administered directly in the vein conduit immediately after nerve injury. Dogs in group 1 (grafted but not treated; control) received only an autologous vein graft, and those in group 4 (normal control) received no graft and no PRP or BM-MSCs treatment. The dogs were monitored daily for 8 weeks after surgery. Clinical evaluation of the facial nerve, including lower eyelid, ear drooping, upper lip, and tongue functions, was carried out once per week using a numerical scoring system of 0–3. At the end of the study period (week 8), the facial nerve injury site was evaluated grossly for the presence of adhesions using a numerical scoring system of 0–3. The facial nerve injury site was histopathologically assessed for the existence of perivascular mononuclear cell infiltration, fibrous tissue deposition, and axonal injury using H&E-stained tissue sections.

Results: Clinically, BM-MSCs treated dogs experienced significant ($p < 0.05$) improvement in the lower eyelid, ear, lip, and tongue functions 4 weeks postoperatively compared to other groups. Grossly, the facial nerve graft site in the BM-MSCs treated group showed significantly ($p < 0.05$) lesser adhesion scores than the other groups. Histopathologically, there was significantly ($p < 0.05$) less perivascular mononuclear cell infiltration, less collagen deposition, and more normal axons at the facial nerve injury site in the BM-MSCs treated group compared to the other groups.

Conclusion: This study showed clinically significant enhancement of nerve regeneration by applying autologous BM-MSCs and autologous vein grafting at the site of facial nerve injury. However, further clinical trials are warranted before this application can be recommended to treat traumatic nerve injuries in the field.

Keywords: Neuropathies, Pain, Nerve regeneration, Pluripotent cells, Nerve graft.

Introduction

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are pluripotent progenitor cells capable of undergoing differentiation into different cells (Khan and Newsome, 2019). In nervous tissues, BM-MSCs have been shown to promote structural and functional nerve regeneration by differentiating into Schwann cell-like cells and inducing the production of local neurotrophic growth factors necessary for axonal growth and conversion of stem cells into myelinating cells (Cooney *et al.*, 2016). Platelet-rich plasma (PRP), on the other hand, is known to contain high concentrations of many neurotrophic growth factors that enhance the healing of injured nerve tissues, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), fibrin, fibronectin, and vitronectin (Arnoczky *et al.*, 2011; Wasterlain *et al.*, 2012). Furthermore, it has been shown that PRP provides neuroprotection against cell apoptosis, stimulates angiogenesis, enhances axonal regeneration and growth, and improves the local microenvironment by overcoming inflammatory responses (Sanchez *et al.*, 2017).

Peripheral nerve injuries (PNIs) remain a common challenge in both human and veterinary medicine, causing pain and poor quality of life for millions of people and animals annually (Martinez *et al.*, 2011; Daly *et al.*, 2012). Unlike healing of nerve tissues in

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the central nervous system, the regeneration of the peripheral nervous system is considerably more efficient (Gaudet *et al.*, 2011). Unfortunately, this remarkable healing ability is often hampered by significant nerve gaps due to traumatic injuries (Gaudet *et al.*, 2011). The sural nerve graft remains the most common autograft donor that is used to bridge nerve gaps induced by injuries (Ryu *et al.*, 2011). Other methods such as bio-tubes and conduits were also used previously (Sinis *et al.*, 2011). Conduits, which are tubes made of silicone, have been demonstrated to induce rapid functional recovery and enhance microscopic healing when used in nerve reconstruction of acute, subacute, and chronic injuries (Sinis *et al.*, 2011). Recent studies using autologous vein grafts have shown significant improvement in the healing of injured nerves with less formation of fibrous tissues and adhesions (Mathieu *et al.*, 2012). In this study, an autologous vein graft harvested from the lateral saphenous vein was used for the first time as a conduit for the treatment of PNI of the facial nerve using either BM-MSCs or PRP in a dog model.

Materials and Methods

Animals

A total of 20 apparently healthy adult Mongrel dogs weighing between 15 and 20 kg were used in the study. The dogs were purchased from a local breeder and allowed 2 weeks of acclimatization. Before the analysis, the dogs were subjected to a complete general physical examination, including neurological evaluation. Special attention was paid to evaluating facial expression and cranial nerve functions, including masseter muscles symmetry, size, and tone, palpebral reflex, corneal examination, tongue tone and function, and nostril and labial symmetry and function. In addition, complete blood cell count and serum chemistry profile were carried out for all animals. Only dogs with normal findings were allowed to be enrolled in the study.

During the acclimatization period, the dogs were treated for internal parasites (Ascaten; Alfasan, Holland) with 1 tablet per 5 kg and vaccinated against canine distemper virus, canine adenovirus type 1, canine adenovirus type 2, canine parainfluenza virus, canine parvovirus (Vanguard Plus 5, Zoetis, USA), and *Leptospira* spp. (MSD Animal Health, Ireland). The dogs were identified using neck collars and housed individually in cages. Dogs were fed twice daily using locally produced dog pelleted feed and offered fresh drinking water *ad-lib*.

Study design

The dogs were randomly divided into four equal groups (five dogs each). Dogs in groups 1, 2, and 3 were subjected to facial nerve neurectomy and saphenous vein conduit graft implantation at the site of facial nerve injury. Dogs in groups 2 and 3 received 1 ml of autologous PRP and BM-MSCs, respectively. Injections were administered directly in the vein

conduit immediately after nerve injury was created. Dogs in group 1 (grafted but not treated; control) received only an autologous vein graft. Those in group 4 (normal control) received no facial nerve neurectomy, vein graft, and injections. All dogs were monitored daily for 8 weeks after surgery.

Anesthesia and analgesia

The dogs were fasted for 12 hours, and water was withheld for 6 hours before the induction of general anesthesia. General anesthesia was induced by intramuscular injection of xylazine (Xylaject 20%; Adwia, Egypt) at 1.1 mg/kg and ketamine (Ketamine HCl 10%; Alfasan, Holland) at 5 mg/kg. Maintenance of anesthesia was achieved by repeated injections of xylazine and ketamine, intramuscularly as needed. Intravenous isotonic fluids were administered during the entire time the dogs were maintained under general anesthesia at 10 ml/kg/hour.

PRP preparation

Autologous PRP was collected and prepared according to previously described methods (Daradka *et al.*, 2016). Briefly, approximately 20 ml of whole blood was collected aseptically via cephalic vein puncture using vacutainer needles and placed immediately in ethylenediamine tetraacetic acid-containing tubes. The PRP was collected using double centrifugation cycles at 200 and 400 *g* at 22°C for 10 minutes, respectively. The number of platelets in PRP was then adjusted to approximately 5×10^6/ml using sterile phosphate-buffered saline (PBS) and used immediately to treat the nerve injury.

Bone marrow aspiration and stem cell preparation

Bone marrow aspiration was carried out 2 weeks before creating facial nerve injury to allow adequate time for stem cell preparation. With the dogs under general anesthesia, bone marrow aspiration was carried out according to previously described methods with modifications (Muir *et al.*, 2016). Approximately 20 ml of bone marrow was aspirated using an 18-gauge needle attached to a 20 ml syringe containing traces of heparin from the iliac crest. Collected bone marrow was transferred to the laboratory immediately to isolate the stem cells according to previously described methods (Somasundaram *et al.*, 2015). Briefly, approximately 40 ml of sterile PBS was added to 20 ml of bone marrow aspirate and placed in glass tubes containing Ficoll-Paque solution (Merck, Germany). The glass tubes were then centrifuged and washed twice using PBS at 400 *g* for 10 minutes. The mononuclear cell layer (buffy coat) was aspirated and rewashed using PBS to remove Ficoll-Paque residues. After that, the erythrocytes portion in the mononuclear cell suspension was removed by lysis using 0.7% NH_4Cl solution for 3 minutes. The lysis reaction was abruptly stopped by 0.9% ice-cold NaCl solution. The mononuclear cell suspension was centrifuged again at 400 *g* for 10 minutes at room temperature. The cell pellet was suspended in Minimum Essential Medium **α**

(Mediatech, USA) containing 10% fetal bovine serum, 1× nonessential amino acids, 4 mM L-glutamine, 100 U/ml penicillin, and 0.01 mg/ml streptomycin sulfate (Life Technologies, USA). The media was left at room temperature for 24 hours and nonadhered cells were removed by changing the media. The media was cultured at 37°C under 5% CO_2 and 95% humidity for 1 week. The bone marrow cells were then used for the autologous treatment of the nerve injury. At treatment time, the BM-MSCs were detached from culture flasks using trypLE (Life Technologies, USA), counted using a hemocytometer, and adjusted to 1×10^6 per ml of saline. Each dog in group 3 received 1 ml BM-MSCs at the facial nerve injury site under general anesthesia.

Lateral saphenous vein graft harvesting
While the animal was still under general anesthesia, the lateral side of the right hind leg just above the proximal hock joint was prepared for aseptic surgery. An approximately 3 cm long segment from the right lateral saphenous vein was collected by no-touch technique (Papakonstantinou *et al.*, 2016). Briefly, an approximately 5–6 cm long skin incision was made over the saphenous vein. The skin and soft tissues were dissected and reflected using a self-retaining retractor. After the vein was adequately exposed, 2-0 absorbable suture (Atramat, Mexico) was used to ligate both ends of the proposed section of the vein using double ligation, and the vein was severed between the two ligatures. The collected vein segment was immediately placed in a sterile Petri dish containing cold, sterile saline containing 10% gentamicin.

Creation of facial nerve injury, grafting, and treatment protocol
The right side of the face was prepared for aseptic surgery under general anesthesia. An approximately 4–5 cm long vertical skin incision was made anterior to the ear and parallel to the mandibular ramus. The ventral buccal branch of the facial nerve was then carefully dissected and exposed. The nerve was straightened (without tension), and 1 cm long segment was surgically removed using a scalpel blade. After that, the circular autologous saphenous vein graft was placed around the severed nerve ends, covering approximately 0.5 cm proximal and 0.5 cm distal to the cut ends of the nerve. In a simple interrupted pattern, the graft was sutured to the perineurium using 4-0 monofilament suture material.

Dogs in groups 1, 2, and 3 received 1 ml saline, 1 ml of PRP, or 1 ml of BM-MSCs, respectively. The treatment was carried out immediately after the nerve injury was created. The treatment solutions (saline, PRP, and BM-MSCs) were injected into the conduit to fill the lumen with the injected material. The subcutaneous tissues and skin were then closed routinely using a simple interrupted pattern and 2-0 absorbable suture material (Atramat; Mexico). Animals in groups 1, 2, and 3 were subjected to saphenous vein harvesting and facial nerve neurectomy, while animals in group 4 were not operated on and received no treatments, and served as normal control.

Postoperative care
All animals were allowed to recover from anesthesia under close monitoring in their cages. Dogs were then monitored daily for abnormal clinical signs of fever, anorexia, and depression. The surgical sites, including the saphenous vein collection site and the facial nerve treatment site, were also monitored daily for abnormal local signs such as swelling, pain, or discharge. During the immediate postoperative period (5 days), all animals were treated using amoxicillin (Clamoxyl; Zoetis, USA) at 15 mg/kg orally twice per day and meloxicam (Metacam; Boehringer Ingelheim, Germany) at 0.2 mg/kg once orally.

Clinical evaluation of facial nerve functions
All dogs were subjected to careful evaluation of cranial nerve functions once a day for 8 weeks postoperatively. Any abnormal signs related to facial nerve injury, including eyelid position, lip deviation, degree of salivation, and tongue position, were evaluated using a numerical scoring system of 0–3 (Table 1). The scoring system was developed, pretested, and validated before the study was initiated using three pilot dogs. The evaluation was carried out by a blinded and trained researcher.

Gross evaluation
At the end of the observation period (8 weeks), animals were humanely euthanatized using an intravenous anesthetic injection overdose (Leary *et al.*, 2013). Immediately following euthanasia, the animals were subjected to complete necropsy. Special attention was given to examining the site of facial nerve injury. The facial nerve was carefully dissected and inspected. The degree of adhesion formation involving the nerve injury site was assessed using a score of 0–3 where 0 indicates normal tissue, 1 indicates mild adhesion formation, 2 indicates moderate adhesion formation, and 3 indicates extensive adhesion formation. A trained and blinded researcher carried out the necropsy and gross evaluation procedures.

Histological evaluation
A total of three tissue samples, 0.5 mm in length, were collected from each nerve. One sample from the injury site, including the conduit, one sample from the nerve segment immediately proximal, and one distal to the conduit site to ensure normal tissues were included in the sample. Tissue samples were placed directly in 10% buffered formalin solution and transported to the laboratory for routine histopathological processing. Fixed tissue samples were embedded in paraffin, cut into 5 µm thick sections, and stained using hematoxylin and eosin (H&E) staining. Stained tissue sections were examined by a pathologist who remained blinded to the treatment group using a light microscope with 10× magnification lenses.

The severity of cellular infiltration was estimated by counting the total number of perivascular mononuclear

Table 1. Scoring system used to assess clinical signs related to facial nerve injury in dogs.

Clinical observations	Score	Description
Lower eyelid	0	Eyelid is in normal position
	1	Eyelid skin is slightly loose.
	2	Eyelid is dropped about 2–3 mm
	3	Eyelid is dropped more than 4 mm
Ear drooping	0	Normal position of the ear
	1	The ear is slightly drooped
	2	The ear is moderately drooped
	3	The ear is severely drooped
Upper lip	0	Normal position of the upper lip
	1	The upper lip is slightly deviated to the contralateral side of the nerve injury
	2	The upper lip is moderately deviated to the contralateral side of the nerve injury
	3	The upper lip is severely deviated to the contralateral side of the nerve injury
Salivation	0	No to minimal saliva dripping
	1	Slight saliva drooling is present
	2	Moderate saliva drooling is present
	3	Severe saliva drooling is present
Tongue	0	The tongue lies completely inside the mouth when the animal is not panting
	1	The tongue lies inside the mouth but sometimes protruding outside
	2	The tongue is protruding out of the mouth most of the time and unable to retract
	3	The tongue lies outside of the mouth all the time and unable to retract

Table 2. Scoring system used to assess the histopathological features at the site of facial nerve injury in dogs.

Histological features	Score	Description
Perivascular mononuclear infiltrate	0	Normal tissue
	1	Minimum mononuclear cells
	2	Moderate mononuclear cells
	3	Severe mononuclear cells
Collagen deposition	0	Normal tissue
	1	Minimum
	2	Moderate
	3	Severe
Nerve axons	0	Normal tissue
	1	Few scattered injured axons
	2	Mild to moderate axonal injury
	3	Scattered severe axonal injury

cell infiltrates in H&E-stained sections. The degree of cellular infiltrate was then given a score of 0–3 where 0 indicates normal tissue, 1 indicates minimum perivascular cell infiltrate, 2 indicates moderate perivascular cell infiltrate, and 3 indicates severe perivascular cell infiltrate (Table 2). The severity of the axonal injury was assessed using a scale of 0–3 where 0 indicates normal tissue, 1 indicates few scattered injured axons, 2 indicates mild to moderate axonal injury, and 3 indicates scattered severe axonal injury (Table 2). Fibrous tissue formation was assessed by assessing the degree of collagen deposition using a score 0–3 where 0 indicates normal tissue, 1 indicates minimum collagen deposition, 2 indicates moderate collagen deposition, and 3 indicates severe collagen deposition (Table 2).

Statistical analysis

Data are presented in median ± standard error. All clinical, gross, and histopathological evaluations were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney test. Statistical analysis was carried out using Statistical Package for the Social Sciences IBM statistical software version 22 (IBM Statistics, USA). Statistical differences were considered significant at p-value < 0.05.

Ethical approval

All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Jordan University of Science and Technology (JUST-ACUC-05-2016).

Results

Clinical evaluation

Significant differences (p < 0.05) were detected in the clinical scores of the lower eyelid, ear position, upper lip deviation, degree of salivation, and tongue position in BM-MSCs treated group starting at week 4 and which remained significant throughout the study period. No significant differences were noted between the groups in any clinical parameters during weeks 1–3. Clinically, dogs in the BM-MSCs treated group experienced improved lower eyelid function, less ear drooping, less upper lip deviation, less saliva drooling, and improved tongue tone and position after week 4 in comparison to all other groups.

Gross evaluation

Examination of the facial nerve injury site at week 8 of the study revealed statistically significant differences (p < 0.05) in the scores of fibrosis and adhesion formation and revascularization in BM-MSCs treated dogs compared to all other groups (Figs. 1 and 2). The facial nerve injury site in BM-MSCs treated group showed significantly less fibrosis and lower adhesion scores, while revascularization was substantially more at this site in this group as compared to other groups.

Histological evaluation

Analysis of the histopathological scores at the facial nerve injury site showed statistically significant differences (p < 0.05) between groups regarding inflammatory cell infiltration, fibrous tissue deposition, and continuity of the myelin sheaths. In the BM-MSCs treated group, there was significantly less cellular infiltration and less fibrous tissue deposition with complete continuity of the nerve axon and myelin sheaths compared to the other groups (Figs. 3 and 4).

Discussion

Traumatic PNIs cause pain and suffering and represent an excellent treatment challenge (Ciaramitaro *et*
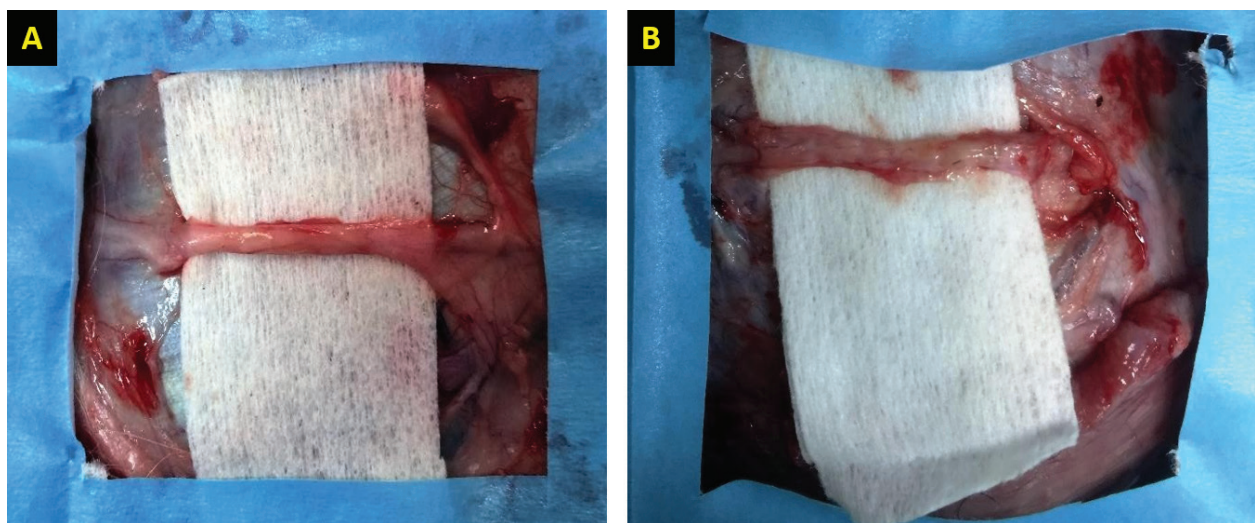

Fig. 1. Gross evaluation of the facial nerve injury site in control groups. Group 1 (A) received an autologous lateral saphenous vein graft only, while dogs in group 4 (B) received no autologous lateral saphenous vein graft, no autologous BM-MSCs and lateral saphenous vein graft, and no platelet-rich plasma. Note the abnormal shape and size of the nerve indicating poor healing with extensive fibrosis.
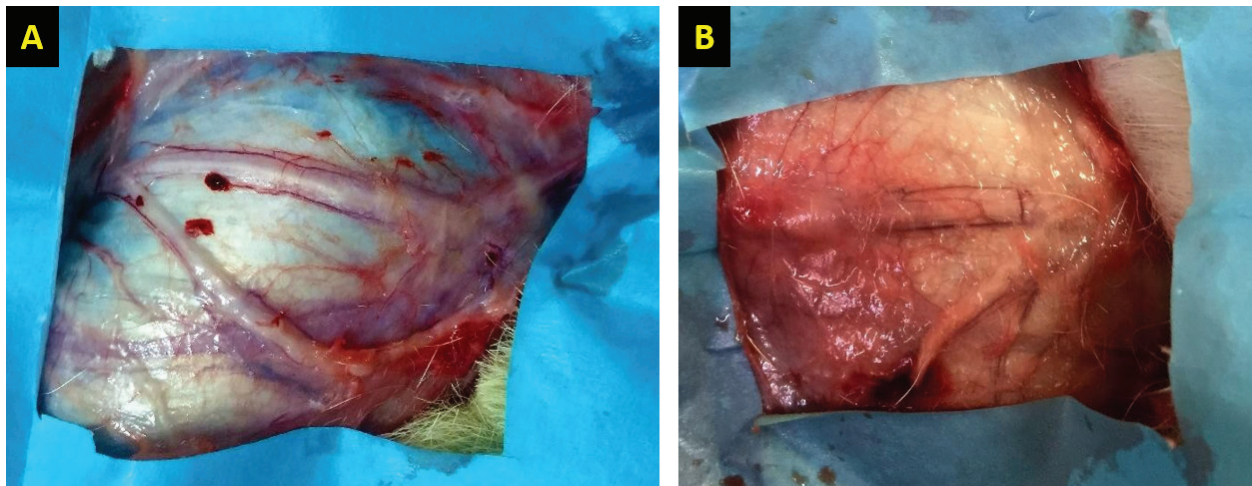
Fig. 2. Gross evaluation of the facial nerve injury site following treatment using autologous BM-MSCs and lateral saphenous vein graft (A), platelet-rich plasma, and autologous lateral saphenous vein graft (B). Note the significant improvement of nerve shape and size with significantly lower degree of fibrosis and adhesions in group A as compared to other groups.
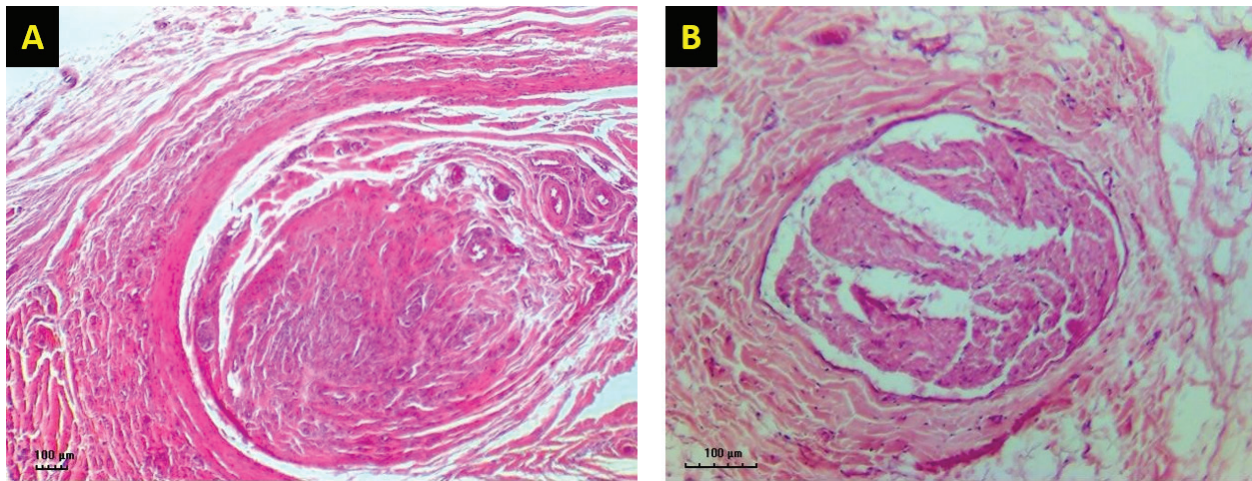

Fig. 3. H&E-stained tissue sections obtained from the facial nerve injury site in control groups. Group 1 (A) received an autologous lateral saphenous vein graft only, while dogs in group 4 (B) received no autologous lateral saphenous vein graft, no autologous BM-MSCs, and no platelet-rich plasma. Note the increased cellular infiltration and deposition of fibrous tissue, scattered and poorly myelinated fibers. (Bar = 100 µm).

al., 2010). Complete nerve disruption, also called neurotmesis injuries (Sunderland type V), results in total loss of nerve fiber structures and continuity (Caseiro *et al.,* 2016). Generally, these injuries require highly complex reconstructive microsurgery techniques to ensure acceptable results (Gartner *et al.*, 2014; Pereira *et al.*, 2014; Cartarozzi *et al.*, 2015). Although many surgical techniques have been used, including bi-conduits and nerve and vein grafts, healing of severely affected nerves remains suboptimal (Caseiro *et al.,* 2016). Several limitations have been associated with the use of these techniques, including donor and recipient site morbidity in addition to nerve and fiber diameter problems.

Therefore, the search for new models and techniques is still widely pursued (Caseiro *et al.,* 2016). In this study, the vein graft did not only provide a scaffold or a conduit to bridge the gap in the injured nerve, but also served as a reservoir where the experimental material was instilled. Although mild inflammatory cell infiltrate at the site of the vein graft was noted in the control group, there was no evidence of tissue rejection. It was clear that the vein graft provided a permeable barrier that allowed transportation of fluids and experimental material (BM-MSCs and PRP) to the surrounding tissues and promoted healing of injured nerves. In addition, it perfectly connected the distal and proximal segments of the transected nerve.
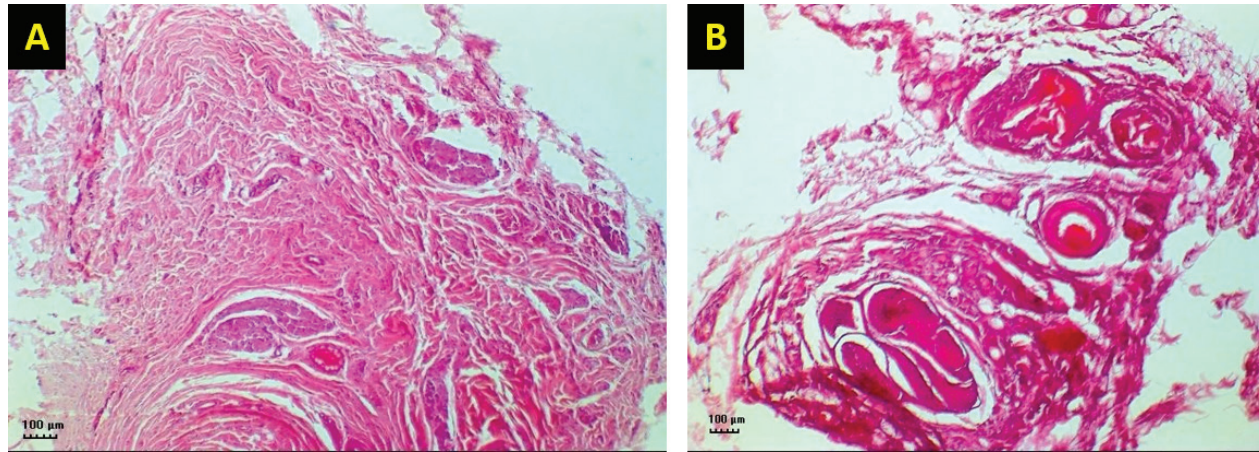
Fig. 4. H&E-stained tissue sections obtained from the facial nerve injury site showing significantly lesser inflammatory cell infiltrates, less fibrous tissue deposition, normal shape and size of the nerve axons, thin but continuum myelin sheaths in BM-MSCs, and lateral saphenous vein graft treated group (A) compared to platelet-rich plasma and autologous lateral saphenous vein graft treated group (B). (Bar = 100 μm).

The increased cellular infiltrates in the transplanted control group are thought to be part of the normal postsurgical inflammatory reaction and not related to graft rejection or infection.

Autologous BM-MSCs used in this study showed superior results compared to PRP. BM-MSCs treated animals showed faster and improved clinical recovery of facial nerve functions. These results agree with previous findings in a sciatic nerve transection model in rats treated by the application of MSCs with fibrin scaffold and nerve tubulization (Cartarozzi *et al.*, 2015). It has been reported that BM-MSCs express a neuroprotective function at the injury site by secreting paracrine factors, stimulating cell division, delaying or preventing apoptosis (Nieto *et al.*, 2018). In addition, BM-MSCs promote neurotrophic substances, including NGF, ciliary neurotrophic factor, BDNF, and GDNF, and NGF-B all of which are known to facilitate axon regeneration and remyelination (Nieto *et al.*, 2018).

In a previous study, intraneural injection of PRP had improved the functions of the chronically injured radial nerve (de Cortazar *et al.*, 2018). Although the results in this study indicate substantial clinical improvement in PRP-treated dogs compared to the control, the histological evaluation of the nerve injury site showed the presence of a significantly increased fibroblast population. These results are congruent with previously reported findings in the PRP perineural treatment of patients with diabetic neuropathy (Hassanien *et al.*, 2020).

In general, the clinical improvement demonstrated in this study was similar in all groups for the first 3 weeks, after which a significant difference was noted in dogs treated by BM-MSCs. Histological evaluation of the BM-MSCs treated group showed the least amount of fibrosis and significantly more normal looking fibers than other groups. These results are similar to previously reported findings (Wang *et al.*, 2015).

Conclusion

The results of this study provide a readily accessible alternative to the use of nerve graft for nerve injury treatment. Collection and application of an autologous lateral saphenous vein graft as a conduit to bridge a significant gap in this model of nerve injury and the application of BM-MSCs in the lumen of the grafted vein, which acted as a reservoir, proved to be a valid approach in the management of injured peripheral nerves. However, further clinical studies are warranted to evaluate this approach in treating naturally occurring traumatic PNIs.

Conflict of interest

The authors declare that they have no competing interests.

Authors' contributions

Mousa H. Daradka designed and coordinated all the experimental work. Mohammad A. Irsheid MAI performed the experimental work, handled the animals, collected samples, and drafted the manuscript. Zuhair Bani Ismail analyzed the data, interpreted the results, and edited the manuscript.

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