Journal of Advanced Veterinary Research
(2025) Volume 15, Issue 1, 106-114

Regenerating effects of platelet-rich plasma and bone marrow aspirate on sciatic nerve injuries in dogs

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ARTICLE INFO

Recieved: 28 October 2024

Accepted: 31 December 2024

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Keywords:

Platelet rich-plasma, Bone marrow- aspirate, Regeneration, Nerve, Sciatic, Dogs

ABSTRACT

In dogs, the sciatic nerve is prone to damage from pelvic fractures due to trauma, or from internal fixation of pelvic fractures due to iatrogenic injury. It had been the goal of previous studies to find a way to accelerate nerve regeneration functionally. The present study aimed to investigate the effects of platelet-rich plasma (PRP) and bone marrow (BM) aspirate on the functional healing of surgically severed sciatic nerve in dogs after its end-to-end anastomosis. Fifteen adult healthy mongrel dogs of both sexes were included in this study. They had been anesthetized and prepared for aseptic surgery as usual. The sciatic nerve was exposed midway between the ischiatic tuberosity and the sacrococcygeal joint. It was severed and anastomosed by end-to-end fashion using three to four interrupted stitches. Dogs were then randomly allocated into three groups; A, B, and C. In group A (control), the sutured nerve was left without treatment. In group B, 1 ml of PRP was injected into the epineurium and around the nerve at the suture site. In group C, 1 ml of BM aspirate was injected into the epineurium and around the nerve at the suture site. In all groups, the muscular incision, subcutis, and skin were sutured routinely. Animals were evaluated at monthly intervals for 3 months through the recording of physiological parameters, neurological examination findings (postural reactions, and spinal reflexes), and laboratory examination, while the histopathological examination was carried out at the end of study. Data were statistically analyzed and expressed as mean ± SEM. Differences were statistically significant at $P<0.05$. The results showed a moderate degree of lameness, persistent knuckling, and dropped hock in the control group (A). The dogs of the PRP group (B) did not show lameness, knuckling, or dropped hocks. There was a slight degree of lameness, intermittent knuckling, and occasional dropping of the hock in the BM group (C). There were significant differences in hopping, extensor thrust, between groups A and both groups B and C but the righting reaction was varied substantially between groups A and C. There was not a significant variation in the biochemical tests between different groups except the creatine kinase, which was high in the BM group relative to PRP and control groups. Histopathological examination revealed variations between groups in the architectural pattern of the repaired nerves, which was better in the PRP group. It could be concluded that the addition of the PRP and BM aspirate to the injured nerve after its suturing promotes the healing microscopically with little functional improvement within months. Platelet rich plasma and Bone marrow aspirate are useful biological materials when are used in the acute injury or rupture of the peripheral nerves in animals.

Introduction

Peripheral nerve injury is a common condition that occurs due to penetrating injuries, crush, ischemia, and traction as well as surgical procedures. Severe nerve injury has a devastating impact on animals' quality of life (Forterre *et al*., 2007). The goal of peripheral nerve surgery is to restore the structural and functional integrity of the damaged nerve. Although progress in surgical techniques for nerve repair and understanding of nerve regeneration over the last 30 years is acceptable, functional recovery after a severe lesion of the major nerve trunk was often incomplete and unsatisfying. Functional recovery is particularly poor for injuries that sever or damage the nerve far from the target making the target re-innervation delayed (Yu *et al*., 2011).

Processes of nerve regeneration and target re-innervation are complex, involving a myriad of factors about neuron, growth environment, locus of the injury from the target, type of injury, the timing of nerve repair, type of nerve repair, and other factors (Diao and Vannuyen, 2000; Dvali and Mackinnon, 2003; Yu *et al*., 2011).

There are new potential therapies either chemical or biological, which aim to improve the functional regeneration of the injured nerves through an acceptable time. Recent studies have discussed the outcomes of using platelet-rich plasma (PRP) in the healing of different tissue such as tendons, ligaments, muscles, and bone (Cervelli *et al*., 2010; Lacci and Dardik, 2010). The effects of PRP on peripheral nerve regeneration had been discussed by some researchers (Sariguney *et al*., 2008; Ding *et al*., 2009). The use of bone marrow (BM) aspirate for improvement of tissue healing was reported in different previous studies (Herthel, 2001; Rolfe, 2008). It is quick, economical, and contains high levels of transforming growth factor beta (TGFβ) and platelet-derived growth factor (PDGF). Both growth factors induce collagen synthesis (Schnabel *et al*., 2006). Moreover, BM aspirate is a rich source of mesenchymal stem cells (MSCs) or stem cells. Typically, aspirated bone marrow is described to contain 40 million nucleated cells, of which 2,000 are stem cells per milliliter (or 1 stem cell per 20,000 cells) (Nathan *et al*., 2003). Several recent studies have examined the healing effects of PRP and BM aspirates on a variety of tissue types in humans and animals because of the ease of preparation and extraction. The present study aimed to investigate the effects of platelet-rich plasma (PRP) and bone marrow (BM) aspirate on the functional healing of surgically severed sciatic nerve in dogs after its end-to-end suturing.

Materials and methods

Experimental Animals and Care

The study has been approved by The Ethical Committee of The Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt according to the OIE standards for use of animals in research under the No. 06/2024/0163. It was carried out under relevant guidelines and regulations. All animals have been accommodated and cared for according to the Egyptian animal welfare law (No. 53, 1966). The experiment was carried out as a pro-

ISSN: 2090-6277/2090-6269/

spective study at the Department of Surgery, Anesthesiology, and Radiology, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt. All institutional ethical regulations were followed during the study procedures. Fifteen adult healthy mongrel dogs of both sexes (9 males and 6 non-pregnant, non-lactating females) were selected and housed in separate standard cages. The average body weight of the dogs was 15.65 kg (range =13-21 kg). The dogs were dewormed by subcutaneous injection of 1ml/50kg Ivermectin (Noromectin ® Norbrook Laboratories Ltd., Newry, Co. Down, Northern Ireland), provided daily with adequate amounts of feed and water ad libitum, and kept for two weeks to adapt.

Preparation for Surgery

In preparation for surgery, each animal was weighed to determine the dosage of anesthetics. The animals were clinically assessed to determine rectal temperature (RT), heart rate (HR), respiratory rate (RR), and capillary refill time (CRT). Dogs were fasted for 12 hours and operated on under general anesthesia by intramuscular injection of 2mg/kg Xylazine HCl 2% (Xyla-Ject, ADWIA Co., SAE, Egypt) and 10min later by intravenous injection of 10mg/kg Ketamine HCl 5% (Ketamine hydrochloride, Rotexmedica, Trittau, Germany). With the animal on left lateral recumbency, the area from the sacrum to the mid-thigh on the right side was prepared aseptically using an appropriate aseptic technique by clipping and shaving of hair and application of Povidone-iodine solution 10% (Betadine, El-Nile Co. for Pharmaceutical and Chemical Industries, Cairo, Egypt) and draped except for the surgical site.

Sciatic Neurotomy Technique

A 7-cm linear skin incision was performed midway between the ischiatic tuberosity and sacrococcygeal joint. Blunt dissection of muscles (superficial and deep fascia lata) was used to expose the sciatic nerve trunk, which presented on the greater sciatic notch of the femur. The sciatic nerve appeared as two nerve trunks that were surrounded by a nerve sheath. To ensure the sciatic nerve, the sciatic reflex was induced by slight percussion on the nerve trunk, which forced the dog to pull up the right hind limb. After exposure, the intended site of sciatic neurotomy was gently elevated using two un-tied stay stitches of Polyglycolic Acid # 6-0 (Polyglycolic Acid # 6-0, M-NATUR®, International Sutures Manufacturing Co., Egypt). A volume of 1ml Lidocaine HCl 2% (Debocaine 2%, Al-Debeiky Pharma, Egypt) was applied directly to the nerve. After 5 minutes, the nerve was severed using a scalpel blade. The nerve trunk was anastomosed in an end-to-end fashion by suturing the epineurium using 4 simple interrupted stitches of Polyglycolic Acid #6-0 (Figure 1).

Dogs were then divided into three groups A, B, and C (n=5 dogs in each group) according to the method of treatment. In group A, the control group, the nerve was not treated but left sutured as previously described. In group B, the platelet-rich plasma (PRP) group, 1 ml of PRP was injected into the epineurium and around the nerve at the suture site using a 1 ml syringe and fine needle. In group C, the bone marrow (BM) group, 1ml of BM was injected into the epineurium and around the nerve at the suture site using a 1 ml syringe and fine needle.

In all groups, the muscular layer and subcutaneous layer were sutured separately by simple continuous stitches of polyglycolic acid #2-0. The skin was then sutured by simple interrupted silk #2-0 sutures. Simple interrupted stitches were used to stitch a stint bandage to the skin over the surgical site as a form of protection.

Post-operative Care

Postoperative antibiotics were administered to dogs at a dose of 25 mg/kg. Amoxicillin and Clavulanic acid (Augmentin 1.2g vial; Glaxo Smithkline Co., Egypt) intravenously for 3 days and 0.5mg nonsteroidal anti-inflammatory Meloxicam (15 mg/2 ml; Amoun Co., Egypt) subcutaneously two times with a 24-hour interval.

Dogs were examined daily for 14 days, postoperatively, for the presence of any swellings, exudates from the operation site, dehiscence of skin stitches, or any other complications.

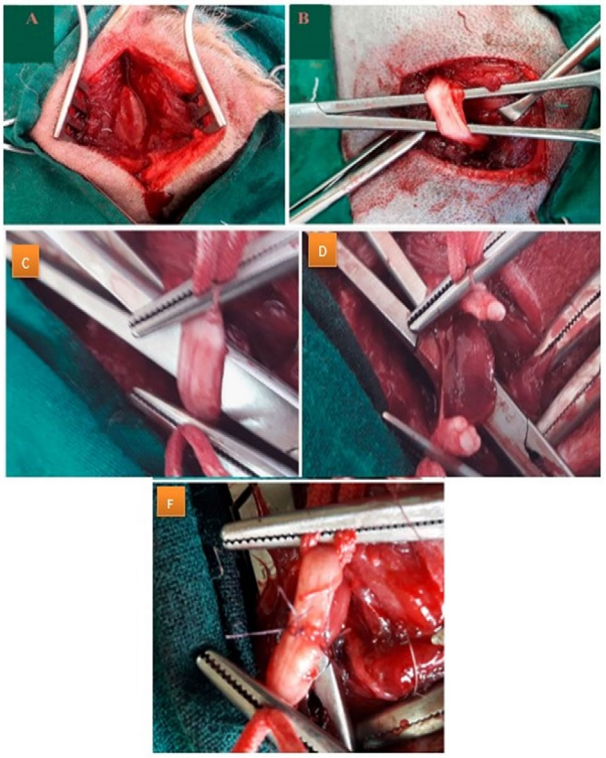

Figure 1. A to E) Surgical incision for exploration and fixation of the sciatic nerve, then its severing and end to end anastomosis using interrupted stitches and 3/0 suture material.

Platelet-rich Plasma Preparation (Dhurat and Sukesh, 2014)

A volume of 8 ml of blood was obtained from the jugular vein by venipuncture in acid-citrate dextrose (ACD) tubes. The sample was then centrifuged using a soft spin at 1800 rpm/10min. The supernatant plasma containing platelets was transferred into another sterile plain tube (without anticoagulant).

The tube was centrifuged at a higher speed (hard spin at 2500rpm/15min) to obtain a platelet concentrate. The lower third was platelet-rich plasma (PRP) and the upper two-thirds were platelet-poor plasma (PPP). At the bottom of the tube, platelet pellets were formed. After removing the PPP, the platelet pellets were suspended in a minimum quantity of plasma (2 ml).

Bone Marrow Aspiration (Bexfeld and Lee, 2010)

The skin over the iliac crest was prepared aseptically. While the patient was anesthetized and positioned in lateral recumbency a skin stab incision was done. Then a sterile 14-gauge Klima needle with stylet was advanced into the bone using firm pressure and back-and-forth twisting motion, while the bone was being stabilized with the other hand. When the needle was correctly seated in the marrow cavity, the stylet was then removed and the collecting syringe containing the sodium citrate solution was attached. The Marrow was aspirated by applying short bursts of negative pressure using a pumping action of the syringe plunger.

Evaluation Criteria

Physiological parameters including heart rate, respiratory rate, and rectal temperature were recorded pre and at 1, 2, and 3 months postoperatively. Body weight was recorded before the experiment and at the end of the study.

Gait evaluation included the presence or absence of lameness and ataxia was carried out. Dogs were video recorded for gait analysis at the end of the study. The numerical rate scaling (NRS) in dogs for grading lameness was used according to Quinn *et al*. (2007) . The scoring was clinically sound (0), barely detectable lameness (1), mild lameness (2), moderate lameness (3), severe lameness (dog might be weight bearing when standing or walking) (4), and could not be lamer (if the limb was never weight-bearing when standing or moving) (5).

Neurological examination

Neurological examination was carried out for all animals before and after surgical operation according to Abdelhakiem *et al*. (2015) and Dewey *et al*. (2016) at one-month intervals for 3 months, postoperatively. Neurological tests included a. posture reaction (knuckling, hopping, extensor thrust, hemi-walking, and righting) and b. spinal reflexes. Spinal reflexes that included; withdrawal, gastrocnemius, cranial tibialis, and sciatic reflexes were tested and recorded according to Dewey *et al*. (2016). The nociception was evaluated after the strong clamping of the lateral digit of the treated limb using a hemostat. A scoring system was used to numerically record the results of neurological tests on a neurological sheet from 0 to 3; where 0 is the absence of response or reflex, 1 is a sluggish response, 2 is a normal response, and 3 is exaggerated or hyperreflexia.

Laboratory Examination

Blood samples were collected from the jugular vein before and at one month, 2 months, and 3 months, postoperatively, on clean dry tubes without anticoagulant. Blood samples were centrifuged for 20 minutes at 2000 rpm. Sera were collected in clean and dry Eppendorf tubes and kept frozen at -20˚C until used for biochemical examinations, which included measuring aspartate transaminase (AST), creatine kinase (CK), creatinine, and urea.

Macroscopic and histopathological examination

At the end of the study, specimens of the repaired sciatic nerves were obtained for histopathological examination under the effect of general anesthesia. The skin over the operated sciatic nerve was incised. The sciatic nerve at the suture site was exposed and examined grossly for the presence of adhesions, swelling, and defect of healing. A nerve segment was then taken by cutting 1 cm above and 1cm below the suture site. Specimens were fixed in 10% neutral buffered formalin. The fixed specimens were dehydrated in graded alcohol series, cleared, embedded in paraffin, and sectioned at 3-5 µm thick sections using Richert Leica RM 2125 Microtome, Germany. The obtained sections were stained with Harris Hematoxylin and Eosin stain (H&E) (Fischer *et al*., 2008). Other stains such as Masson's Trichrome and Grimelius' Silver Stain were used. The former was used for the detection of connective tissue at the healing site. The latter has been used for studies of the cells of the peripheral nervous system. The methods utilize the reduction of silver salt to inorganic silver or silver oxide, which is deposited on specific structures (Westermark, 2015). The stained sections were examined using a LeitzDialux 20 microscope to study the histopathological features. Images were taken using a Canon digital camera (CandisonPowershot A95).

Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM). GraphPad Prism Software version 5 (GraphPad Software Inc., La Jolla, CA, USA) was used for data analysis. Comparison among groups was carried out using repeated measures of one-way analysis of variance (ANOVA) followed by the Bonferroni Posthoc test. Differences were statistically significant at $P<0.05$. Two-way ANOVA also was used to analyze the data of the spinal reflexes at different times in the different groups.

Results

Clinical Findings

Postoperative pain was tolerated by animals in all groups clinically. There was no change in their eating or drinking habits. There was a non-significant increase in the dogs' body weight (average body weight at the end of the study was 16.8 kg (range=14-22.3kg) compared to 15.65 kg (range =13-21 kg) at the beginning of the study. Physiological parameters (RT, HR, and RR) were also not significantly different between dogs of the same group at different times and between dogs of different groups pre- and post-surgery.

The animals lifted their treated limb for 3 days post-operation, and then they dragged them. The treated limbs in all animals were in a knuckled under posture. All dogs survived until the end of the experiment (3 months) except two animals, which were replaced by others. One of the two animals died 1 month from the beginning of surgery. The other animal was excluded because of the severe swelling of the paw of the treated limb. There was a mild swelling at the surgical site that was observed on the first postoperative day, but gradually reduced and completely subsided on the fourth-day post-operation.

Gait Evaluation

All animals developed a significant ($P= 0.0001$) postoperative ataxia. There were no significant differences in the degree of ataxia at different times between the groups. Regarding the presence or absence of lameness, all animals in different groups suffered from a significant ($P=0.0002$) postoperative lameness. The difference in lameness score was significant between animals of group A and those of groups B and C, but there is no significant variation in the degree of lameness between groups B and C.

Neurological Examination

Postural Reaction

Proprioceptive deficits (Knuckling) were significantly changed between groups (A) and (B), (A) and (C) ($P=0.041$), and (B) and (C) ($P=0.041$). The knuckling score was improved in dogs of the treated groups when compared to dogs of the control group. In addition, the knuckling score was improved in dogs of the PRP group (B) when compared to that of the BM group (C). There were significant differences in the hopping between groups A and both groups B and C ($P=0.00$), and there was a significant variation between groups B and C ($P=0.003$). The statistical analysis showed a significant ($P=0.0027$) variation in the hemi-walking between groups A and C, but there were no clear changes between groups A and B and groups B and C. The extensor thrust was significantly ($P=0.0032$) different between group A and both groups B and C, but the variation between groups B and C was not clear. The righting reaction was significantly ($P< 0.0001$) different between groups A and C, but there were no clear changes between groups A and B and groups B and C.

It could be briefly summarized that the dogs of the control group (A) showed a moderate degree of lameness, persistent knuckling, and dropped hock. However, dogs of the PRP group (B) showed no lameness, no knuckling, and absence of dropped hock. Dogs of the BM group (C) showed a slight degree of lameness, intermittent knuckling, and dropping of the hock.

Spinal reflexes

The results of the different spinal reflexes in different groups at different times after surgery were expressed in Figure 2. There were signif-

icant differences in the withdrawal reflex (WR) between both of BM and PRP groups and control group starting from the 4th week after operation till the end of the experiment. There were substantial changes in the WR between the BM and PRP groups at four and eight weeks after sciatic neurorraphy. There were clear differences between the PRP and both of BM and control group from two weeks till eight weeks post-operation. Gastrocnemius reflex was varied clearly between the PRP and control groups at 2, 4, 6, 8- and 12-weeks post-operation. It was a significant variation between BM and control groups at 4, 6, 8 and 12-weeks. There was highly significant variation between the PRP and BM at 2, 4, and 6 weeks, but there was a significant difference (0.0429) at 8 weeks post-operation. The cranial tibialis reflex showed a significant change at two, four and six weeks post-operatively between the PRP and both BM and control groups and at the eight and twelve weeks between the PRP and control groups. The BM group varied significantly from the control group at 4, 6, 8 and 12 weeks after operation. Regarding the sciatic reflex there were a high significant variance between the PRP and control group at 4, 6, 8- and 12-weeks post-operation. The BM group was significant (0.0144) and highly significant different from the control group at 4, 6, 8 and 12 -weeks respectively post-operatively. There was a significant difference (0.0144) between the PRP and BM groups at 4 weeks post-operation. The nociception was lost in all groups and not regained even after 3 months of the operation.

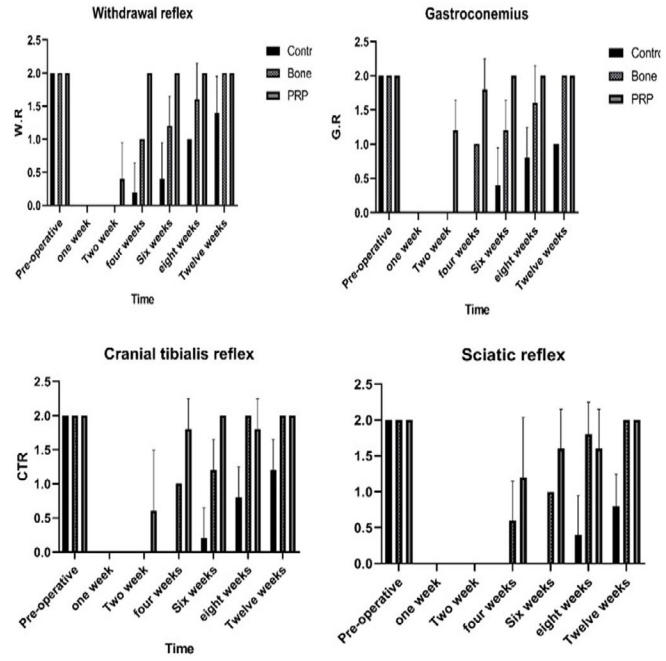

Figure 2. The results of the spinal reflexes before surgery and 1, 2, 4, 6, 8 and 12-weeks after surgery in different groups showed the improvement of the reflexes in the PRP and BM groups, especially, at the 12 weeks.

Biochemical Tests

There were non-significant (P= 0.95) changes in AST values between different groups at different times. There were significant (P= 0.0148) variations in the CK values between dogs of groups A (control) and B (PRP) versus group C (BM), but there was a non-significant change between groups A and B. There was a significant (P=0.01) increase in CK in the first-month post-surgery compared to the pre-treatment values. The variations in the Creatinine values between the different groups were not significant (P= 0.3028). Also, the values of Urea were not changed significantly (P= 0.8335) between the different groups at different times.

Macroscopic Examination

Gross examination of the operation site three months postoperatively in dogs of the control group (A) revealed the presence of an obvious and severe adhesion between the sciatic neurotomy site and surrounding structures that made dissection and isolation of the nerve difficult, in addition to the presence of marked swelling at the neurotomy site. In dogs of the PRP group (B), there was a slight swelling at the neurotomy site without adhesions to the surrounding tissues. However, the sciatic nerves of dogs of the BM group (C) showed the presence of a slight swelling at the neurotomy site with slight adhesions to the surrounding structures in all animals and the presence of ossification at the neurotomy site of one dog (Figure 3).

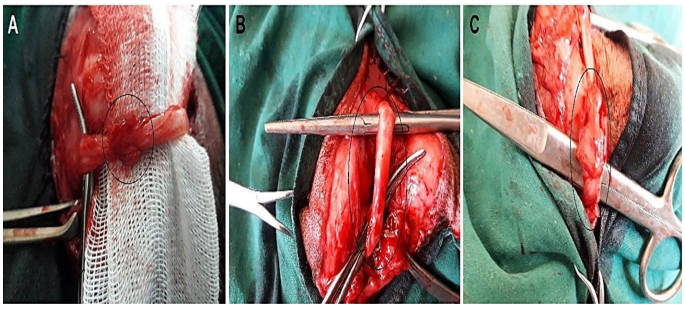

Figure 3. Gross examination of the sciatic neurotomy site 12 weeks after induction of neurotomies; adhesions and marked swelling (circle) at the neurotomy site in a dog of the control group (A), slight swelling at the neurotomy site (circle) in a dog of the PRP group (B), and ossification of neurotomy site (circle) in a dog of the BM group (C).

Microscopic Examination

Sections of the sciatic nerve that was stained by H&E

The Control Group (A)

The proximal part (just above the neurotomy site) 3 months, postoperatively, showed increased vascularization, irregular arrangement, degeneration, and necrosis of nerve fibers (Figure 4A). The intermediate part (at the neurotomy site) showed massive fibrosis and fibroblastic proliferation in addition to irregular arrangement and degeneration of the nerve fibers (Figure 4B). The distal part (just below the neurotomy site) displayed irregular configuration and severe necrosis of nerve fibers with massive fibroblastic proliferation (Figure 4C).

The PRP Group (B)

The proximal part showed Schwann cell proliferation in a regular arrangement and mild degenerated nerve fibers (Figure 4D). At the neurectomy site, there was an increase in Schwann cell proliferation in addition to newly formed nerve fibers in irregular arrangement (Figure 4E). Distal to neurotomy, there were newly formed nerve fibers with an irregular arrangement and mild fibroblastic proliferation (Figure 4F).

The BM group (C)

The proximal section demonstrated an irregular arrangement and degeneration of the nerve fibers associated with increased vascularization (Figure 4G). At the neurotomy spot, there was Schwann cell proliferation in addition to newly formed nerve fibers in irregular arrangement with vascularization (fig, 4H). Distally, there was an irregular layout of vacuolated nerve fibers with moderate fibroblastic proliferation (Figure 4I).

Sections of the sciatic nerve that was stained by Masson's Trichrome

The Control Group (A)

The proximal section of the nerve above the neurotomy site showed mild collagen formation and degeneration of nerve fibers (Figure 5A). At

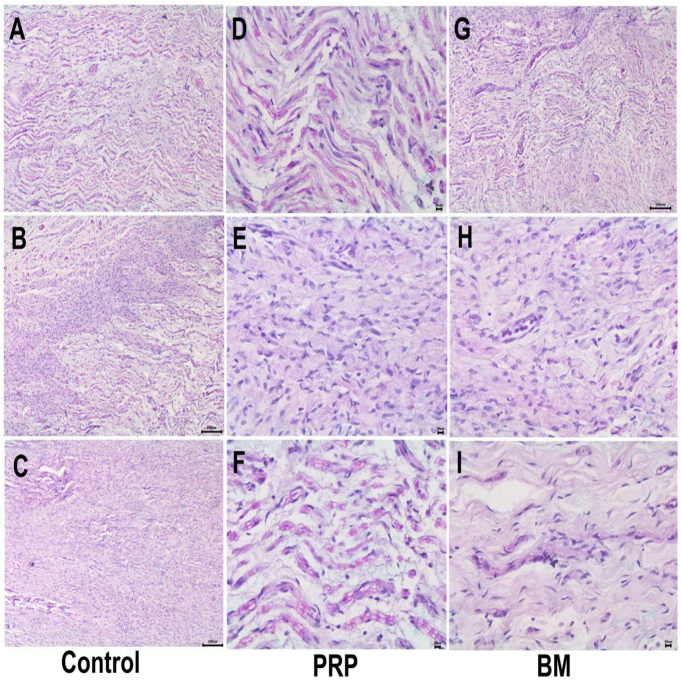

Figure 4. Longitudinal section stained by H&E from the Sciatic nerve of dogs, 3 months postoperatively: Control group; proximal part showing increased vascularization, irregular arrangement and necrotic degenerated nerve fibers (A), the intermediate part showing massive fibrosis and fibroblastic proliferation in addition to irregular arrangement and degenerated nerve fibers (B), the distal part showing the irregular arrangement and severe necrosis of nerve fibers with massive fibroblastic proliferation (C). PRP group; proximal part shows Schwann cells proliferation in a regular arrangement and mild degenerated nerve fibers (D), the intermediate part shows increase Schwann cells proliferation in addition to newly formed nerve fibers in irregular arrangement (E), the distal part shows newly formed nerve fibers with irregular arrangement with mild fibroblastic proliferation (F). PM group; the proximal part shows the irregular arrangement and degenerated nerve fibers associated with increased vascularization (G), the intermediate part shows Schwann cells proliferation in addition to newly formed nerve fibers in irregular arrangement with vascularization (H), the distal part shows the irregular arrangement of vacuolated nerve fibers with moderate fibroblastic proliferation (I).

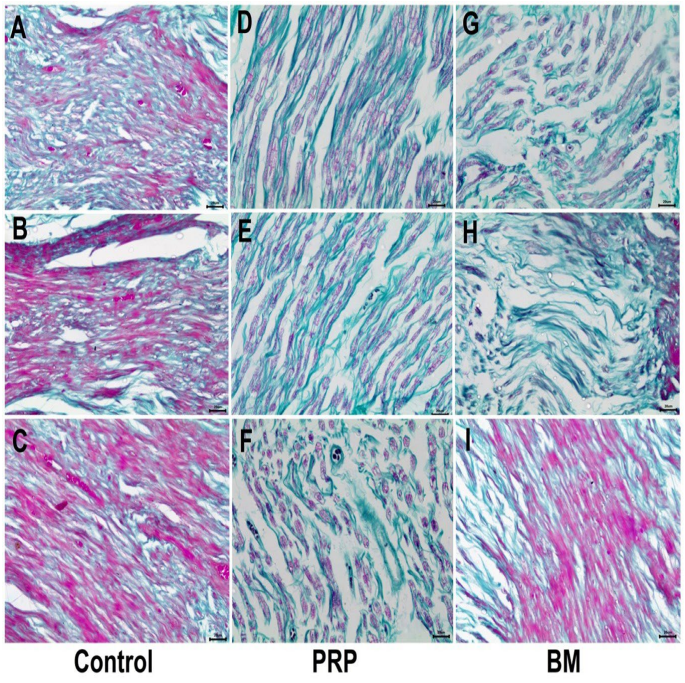

Figure 5. Longitudinal section stained by Masson's Trichrome Stain from the Sciatic nerve of dogs, 3 months postoperatively: Control group; proximal part showing mild collagen formation and degenerated nerve fibers (A), the intermediate part shows an increase in collagen fiber formation in addition to irregular arrangement and necrotic nerve fibers (B), the distal part shows the irregular arrangement and severe necrosis of nerve fibers with massive collagen formation (C). PRP group; proximal part shows Schwann cells proliferation in a regular arrangement and an increase of newly formed nerve fibers (D), and the intermediate part shows an increase in Schwann cells proliferation in addition to vacuolation of nerve fibers in irregular arrangement (E), the distal part showing newly formed of nerve fibers and increase vascularization (F). PM group; the proximal part showing Schwann cells proliferation in irregular arrangement with the presence of newly formed nerve fibers (G), the intermediate part showing minimal Schwann cells proliferation in addition to the presence of vacuolated nerve fibers in irregular arrangement (H), the distal part showing an increase in collagen formation replaced the degenerated nerve fibers (I).

the neurotomy site, there was an increase in collagen fiber formation in addition to irregular arrangement and necrosis of the nerve fibers (Figure 5B). In the distal segment, there was an irregular arrangement and severe necrosis of nerve fibers with massive collagen formation (Figure 5C).

The PRP Group (B)

The proximal histopathological section above the neurotomy site showed Schwann cell proliferation in a regular arrangement and increased newly formed nerve fibers (Figure 5D). At the neurotomy site, indicated an increase of Schwann cell proliferation in addition to vacuolation of nerve fibers in irregular arrangement (Figure 5E). Distal to the neurotomy site, there were newly formed nerve fibers and increased vascularization (Figure 5F).

The BM group (C)

Sections proximal to the neurotomy site showed Schwann cell proliferation in irregular arrangement with the presence of newly formed nerve fibers (fig 5G). At the neurotomy site, there was minimal Schwann cell proliferation in addition to the presence of vacuolated nerve fibers in irregular arrangement (Figure 5H). Distal to the neurotomy site, there was an increase in collagen formation that replaced the degenerated nerve fibers (Figure 5I).

Sections of the sciatic nerve that was stained using Grimelius' Silver Stain

The Control Group (A)

The proximal section of the nerve above the neurotomy site and the section that was taken at the neurotomy site revealed vacuolar degeneration of the nerve fibers (Figure 6 A&B). In the distal segment, there was massive disappearance of nerve axons (Figure 6C).

The PRP Group (B)

Sections proximal to the neurotomy site showed an increase in nerve axon formation in a regular arrangement and mild vacuolar degeneration in the nerve fibers (Figure 6D). At the neurotomy site, there was an increase in the axonal appearance of newly formed and vacuolated nerve fibers in irregular arrangement (Figure 6E). Distal to the neurotomy site, sections revealed the presence of nerve axons in irregular and distorted arrangement (Figure 6F).

The BM group (C)

Proximal to the neurotomy site, the histopathological section of the sciatic nerve showed an increase in nerve axons formation in irregular arrangement and vacuolar degeneration in nerve fibers (Figure 6G). At the neurotomy site, sections showed degenerated axons with vacuolated nerve fibers (Figure 6H). Distally, there was an absence of nerve axons in some areas of the nerve (Figure 6I).

The microscopic changes at the neurotomy site and above and below it after staining with different stains in the different groups were recorded and summarized in Table 1.

Discussion

The present study was conducted to investigate the effect of both platelet-rich plasma (PRP) and bone marrow (PM) aspirate on the func-

tional healing of the severed sciatic nerve in dogs. The dogs were monitored clinically and biochemically for three months, which ended with the harvesting of specimens of the healed sciatic nerve at the neurotomy site for histopathological examination.

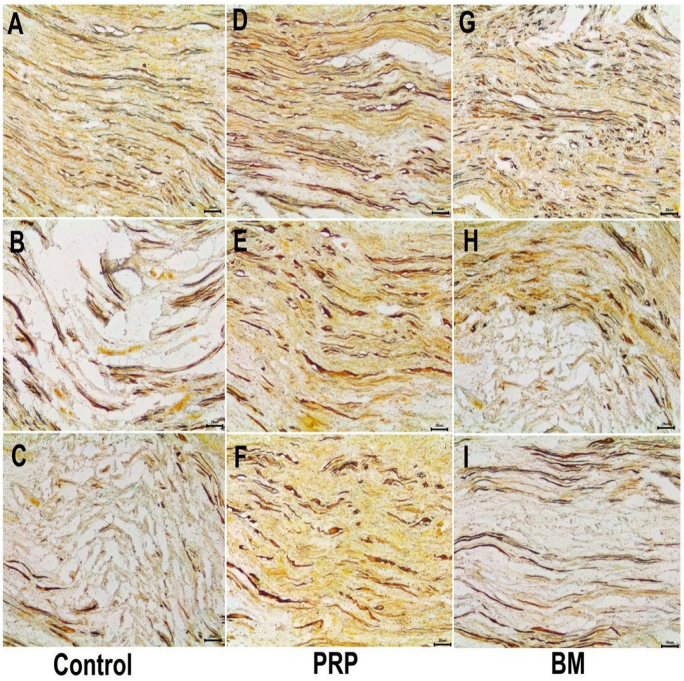

Figure 6. Longitudinal section stained by Grimelius' Silver Stain from the Sciatic nerve of dogs, 3 months postoperatively: Control group; proximal part mild collagen showing vacuolar degenerated nerve fibers (A), the intermediate part showing vacuolar degenerated nerve axons (B), the distal part showing massive disappearance of nerve axons (C). PRP group; proximal part shows an increase in nerve axons formation in a regular arrangement and mild vacuolar degeneration in nerve fibers (D), and the intermediate part shows an increase in the axonal appearance of newly formed and vacuolated nerve fibers in an irregular arrangement (E), the distal part showing the presence of nerve axons in irregular and distorted arrangement (F). PM group; proximal part shows an increase in nerve axons formation in irregular arrangement and vacuolar degeneration in nerve fibers (G), the intermediate part shows degenerated axons with vacuolated nerve fibers (H), the distal part shows the absence of nerve axons in some areas of the nerve (I).

The best definition of nerve regeneration includes the regrowth of injured axons, their myelination, restoration of synaptic connections, and recovery of physiological functions (Yu *et al*., 2011). In the present study, histopathological findings demonstrated the regrowth and healing of the severed sciatic nerve after its suturing in all groups of animals. The nerve regeneration was superior in the PRP group followed by the bone marrow group compared to the control one. Regarding the clinical evaluation, the outcomes showed that dogs of the PRP group (B) expressed better functional recovery if compared to dogs of the BM group (B) and control group (A). Considering the results of previous studies conducted by Mackinnon (1989) and Sarikcioglu *et al*. (2007)), the present study was expected to achieve acceptable results. All precautions were taken to preserve the severed nerve and its blood supply. The sharp cutting of the nerve by the scalpel during neurotomy, the gentle handling of the nerve fragments, the adequate apposition of the nerve ends (anastomosis), and the right stitching immediately following the nerve cutting were all carried out precisely. A previous study had confirmed that the timing for surgical repair of the injured nerve and repair criteria are very important in nerve regrowth and regeneration. The primary repair could be carried out within 72 hours to 7 days post-injury (Dvali and Mackinnon, 2003). According to the classification of Anatolitou *et al*. (2012), the injury of the sciatic nerve in the present study is categorized as the fifth degree of nerve injury and damage.

Two dogs did not complete this study. One of them died after one month of operation. The real cause beyond death was not fully elucidated. The second dog was excluded from the study. This was due to the severe swelling of the paw of the treated limb. It caused severe itching that lead to self-mutilation by the animal. This self-amputation of the

Table 1. Summary of the histopathological findings in the nerve sections of different groups.

Stain	Item	Control			PRP			BM		
		Proximal	Neurotomy	Distal	Proximal	Neurotomy	Distal	Proximal	Neurotomy	Distal
H&E	Vascularization	increased						increased	present	
	Nerve fibers Arrangement	irregular necrosis degeneration	irregular degeneration	irregular severe necrosis	regular mild degeneration	newly formed nerve fibers are irregular	newly formed irregular	irregular degeneration	newly formed irregular	irregular vacuolated
	Fibrosis and fibroblastic proliferation		massive	massive			mild			moderate
	Schwann cells proliferation				present	increased			present	
Masson's Trichrome	Collagen formation	mild	increased	massive						increased
	Nerve fibers arrangement	degenerated	irregular necrosis	irregular severe necrosis	increased newly formed regular	vacuolation irregular	newly formed	newly formed irregular	vacuolated irregular	degenerated
	Schwann cells proliferation				present	increased		present	minimal	
	Vascularization						increased			
Grimelius' Silver	Nerve fibers arrangement	vacuolar degeneration		massive disappearance of axons	increase in nerve axons formation regular mild vacuolar degeneration	increase in the axonal appearance of newly formed and vacuolated nerve fibers in irregular	irregular and distorted	increase in nerve axons formation in irregular vacuolar degeneration	degenerated axons with vacuolation	absence of nerve axons in some areas of the nerve

limb may occur but rarely because of nerve injury as was reported before (Crisman, 1993).

The post-operative physiological parameters in all animals did not show any significant variation from that of pre-operative values. These results might be attributed to the absence of post-operation wound infection because of using strict aseptic intraoperative procedures and the use of broad-spectrum antibiotics, post-operatively. Moreover, the use of analgesics, post-operatively, imparts to the animals the ability and tolerance to withstand pain due to nerve injury (Pypendop and Barter, 2016).

In the present study, the dogs showed clinically a knuckled under posture of the treated limb till the end of the experiment in group A and some animals of group C. However, the animals supported their weight on the treated limb. The reason for this was that the quadriceps muscle was functional due to the integrity of the femoral nerve (Chambers and Hardie, 1986). The improvement in animals of group B especially in the 3rd-month post-operation might indicate an improvement in the sensory fibers of the sciatic nerve to transmit a signal to the brain (De Lahunta and Glass, 2009).

The dogs in the present study showed post-operative ataxia. This type of ataxia is proprioceptive ataxia. The sciatic nerve is a mixed nerve (Ueyama, 1977). The injury of that nerve leads to the loss of function of both sensory and motor nerve fibers, which is manifested in the animal as proprioceptive deficit and muscle atrophy, respectively (Dewey *et al*., 2016). Ataxia is an important sign that aid in differentiation between the lesions in the peripheral nerve or the lower motor neurons (LMNs). The latter is not associated with ataxia due to the intact sensory nerve fibers (De Lahunta and Glass, 2009).

According to the findings of this study, the dogs in the PRP and BM groups showed some clinical improvement relative to the dogs in the control group through the evaluation and grading of lameness in animals. It was documented that the degree of functional recovery varies from 80% of normal in motor nerves to 50% or 60% of normal in mixed nerves (Ducker, 1972). The presence and persistence of knuckling and ataxia with an improvement of lameness in animals of this study gave evidence that the motor nerve fibers regain their function earlier than the sensory fibers in the treated sciatic nerve. The results of a previous study revealed that the good results obtained after primary nerve repair occur when the injured nerve is purely motor or purely sensory but not mixed (Townsend, 1994). This may be attributed to some motor axons in the mixed nerves may enter neurilemmal tubes of sensory neurons and vice versa, rendering them nonfunctional (Swaim, 1981).

The dogs in PRP and BM groups showed improved postural reactions gradually with time compared to the dogs in the control group. These findings and other clinical findings (gait evaluation; ataxia and lameness) verified the enhancement effects of PRP and BM when added to the treated sciatic nerve after its suturing. The findings of a previous work had revealed that PRP could promote the regeneration of the injured peripheral nerve (Yu *et al*., 2011). This is due to the growth factors in the PRP that influence the healing and regeneration of different tissues (Cho *et al*., 2010). Platelets are considered the body's natural reservoir of several growth factors, so because PRP contains more platelets than bone marrow aspirate, this also means it contains more growth factors (Herthel, 2001; Nixon, 2008; Rolfe, 2008; Schnabel *et al*., 2006). As well as bone marrow aspirate-treated tissues had a higher overall matrix metalloproteinase (MMP expression) relative to PPR. This explains why the nerve healing outcomes of dogs treated with PPR are relatively better than dogs treated with BM. On the other hand, BM aspirate's effectiveness in treating tendon and ligament injuries had been attributed to two factors; the first is the presence of growth factors as in PRP (Nixon, 2008). The second is the presence of mesenchymal stem cells (MSCs) in the BM injected into an area of a damaged tendon (Fortier *et al*., 1998). These cells might differentiate into mature tendon (or ligament) fibroblasts under the signaling influences of the tissue and produce the appropriate matrix products for repair. In the present work, the severed sciatic nerve is mainly composed of nerve fibers (axons) and free from the functional unit cells (neuron or cell body) unlike the tendons that are composed of tenocytes and extracellular matrix. Therefore, the addition of the BM aspirate to the severed nerve might improve the process of regeneration through the effect of the growth factors but not MSCs.

The spinal reflexes were scored from 1 to 2 in groups B and C, especially in the 2nd and 3rd months indicating a regain of the reflex arc which includes the peripheral nerve, nerve roots, muscles, neuromuscular junction, and the spinal segments (Dewey *et al*., 2016). Due to the spinal segments of the sciatic nerve (L6-S1) being intact so the presence of the spinal reflexes refers to the functional return of the injured sciatic nerve. The results were attributed to the components of PRP and BM as was mentioned before (Herthel, 2001; Nixon, 2008; Rolfe, 2008; Schnabel *et al*., 2006). The loss of nociception was determined by the loss of reaction of the examined dogs (turning the head toward the examiner, biting, and vocalizing) when its digits were strongly clamped by a hemostat (Abdelhakiem *et al*., 2015). In this study, all dogs in all groups did not show reactions referring to the resuming or regaining of nociception. Although the spinal cord parenchyma of the treated dogs was intact and had not been subjected to any injury or trauma, the problem was in the conduction power of the injured sciatic nerve. The authors attributed the loss of nociception to 1. The lack of functional healing of sciatic nerve (the sensory portion) 2. Most of the fibers of sciatic nerve are unmyelinated which are slow conducting of signals, as well as the brain may poorly localize the C-fiber stimuli. C-fibers within the PNS transmit acute pain but in a very slow way because they are unmyelinated fibers (Schmalbruch, 1986; Thomson and Hahn, 2012).

The results of the present work revealed no significant variation in the biochemical tests between different groups except the creatine kinase, which was high in the BM group relative to PRP and control groups. A serum CK level was measured to assess muscle damage. Elevation of this enzyme with an increase in the level of AST is strong evidence for muscle damage. CK elevation gives evidence of muscle degeneration or necrosis (Sharkey and Radin, 2010). The biochemical tests (AST, Urea) that had been evaluated in the study were not specific tests for muscle damage. AST may be elevated in case of hepatocellular damage and muscle damage. Urea is raised in the case of nephropathy or an increase in protein intake (Sharkey and Radin, 2010). Although CK is increasing in case of muscle damage, it is not specific to etiology. CK and AST may be elevated in case of muscle trauma, strenuous exercise, trembling, or intramuscular injections. Conversely, some types of muscle disease may be not associated with an increase of CK or AST (Sharkey and Radin, 2010; Stockham and Scott, 2008). In addition, the half-life of CK is short. This explains why the CK values were significantly high in the first-month post-surgery compared to the pre-treatment values. It was reported that CK activity may increase in animals with neurologic disease. Necrosis or demyelination of neural tissues may cause increased CK activity in cerebrospinal fluid samples, but neither is known to cause increases in serum CK activity (Stockham and Scott, 2008).

The results of this study showed grossly a slight swelling and an absence of adhesion with the surrounding of the sciatic nerve at the site of healing in the PRP group compared to groups A and C. This may be attributed to the anti-inflammatory effect of PRP. The results of a previous work explained that the lipoxin A4 (LXA4) levels were significantly higher in PRP compared to the whole blood suggesting that PRP may suppress cytokine release, limit inflammation, and, thereby, promote tissue regeneration (El-Sharkawy *et al*., 2007).

In the present work, the histopathological results showed that the addition of the PRP to the sutured nerve offered good results followed by the BM when compared to the control group. There was Schwann cell proliferation in addition to newly formed disoriented nerve fibers with new vascularization at the good microscopic inspection of the neurotomy site in the PRP group. According to previous published data, the signs of nerve regeneration include regenerating nerve clusters, onion bulbs,

and Schwann cells (MacKay, 2010). The outcomes of the present study displayed that the distal segment in the control group showed irregular arrangement and severe necrosis of nerve fibers with massive fibroblastic proliferation. It had been recorded that Wallerian degeneration ensues 24–48 hours after peripheral nerve injury and both distal axons and surrounding myelin degenerate (Grifn *et al*., 2013).

Wallerian degeneration develops, and slow axonal regeneration follows at a rate of 1mm/day (Grinsell and Keating, 2014). As well as the distance from the seat on nerve injury to the target has an important role in the healing and regeneration of the injured nerve. For this reason, the prognosis for return to function is good if the distance between the site of injury and the end plate is less than 10 to 15 cm, and guarded if the distance from the end plate is greater than 25 to 30 cm (Crisman, 1993). In the present study, although the sciatic nerve was cut in all dogs at the same site with an even distance from the target, there were variations between groups in the histopathological findings. These findings were better in PRP and BM groups than in the control one. These outstanding results might be attributed to the growth factors present in the PRP and BM aspirate. The growth factors that were released in the neurotomy sites promoted the process of healing. Because the PRP contains much more of growth factors than the BM aspirate (Herthel, 2001; Nixon, 2008; Pavlovic *et al*., 2016; Rolfe, 2008; Schnabel *et al*., 2006), better results were obtained with the PRP.

In the present study, the seat of neurotomy showed good healing and regeneration in all groups. This was due to the gentle handling of the nerve without its crushing, immediate repair, and suturing of the nerve ends after its cutting without leaving a gap. These factors encourage the good healing of the severed nerves (Diao and Vannuyen, 2000; Honnas *et al*., 2001; Dvali and Mackinnon, 2003).

Study Limitations

The study was carried out on a small number of animals, which is probably attributed to the loss of availability of animals, the high cost of animals, and the lack of well-prepared large spaces for hospitalizing a large number of animals who will be subjected to the same factors of the experiment at the same time. In addition, the study was conducted for three months, but the authors recommended an additional three months after displaying the histopathological findings. Also, one of the limitations of this study was the lack of dependence on some neuro-electrophysiological tests, such as motor nerve conduction velocity, F waves, and H waves for assessment of functional nerve conduction and reinnervation. This is because the lack of these facilities in our clinic.

Conclusion

The study concluded that surgical apposition and suturing of the severed nerve had an important role in the healing process of the injured nerve. Platelet-rich plasma and bone marrow aspirate are easily collected and prepared biological materials that promote nerve healing and regeneration. Nerve healing was enhanced by the addition of PRP and BM aspirate following suturing, with PRP being more effective. Although the histological findings in the PRP and BM aspirate groups were satisfactory three months after neurorrhaphy, the clinical findings did not clue the differences in their efficiency in the enhancement of nerve healing.

Conflict of interest

The authors have no conflict of interest to declare.

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