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Effects of different platelet-rich plasma administration methods on peripheral nerve regeneration: A histomorphometric study

Meutia MAULINA [1,*], Dessy RAKHMAWATI EMRIL [2], Endang MUTIAWATI [2], Etriwati ETRIWATI [3], Sri HASTUTI [2], Safrizal RAHMAN [4], Zulkarnain ZULKARNAIN [5]

[1]Department of Histology, Faculty of Medicine, Universitas Malikussaleh, Aceh, Indonesia
[2]Department of Neurology, Faculty of Medicine, Universitas Syiah Kuala-Dr. Zainoel Abidin Hospital, Aceh, Indonesia
[3]Department of Pathology, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Aceh, Indonesia
[4]Department of Orthopedic and Traumatology, Faculty of Medicine, Universitas Syiah Kuala-Dr. Zainoel Abidin Hospital, Aceh, Indonesia
[5]Department of Physiology, Faculty of Medicine, Universitas Syiah Kuala, Aceh, Indonesia

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Abstract

Peripheral nerve damage is a common medical problem that frequently leads to significant functional limitations and long-term disabilities. Platelet-rich plasma (PRP), a growth factor-rich substance, promotes axonal regeneration in peripheral nerve injuries. This study aimed to investigate the effect of PRP on axonal regeneration in Wistar rats (*Rattus norvegicus*) after peripheral nerve injury via histomorphometric analysis. This study used a post-test-only control-group design. Twenty-five white rats were randomly assigned to the control or treatment groups (n=5). The control group consisted of the sham-operated and saline groups. The treatment groups consisted of local, intraperitoneal, or combined PRP. A single dose of PRP was administered after axonotmesis of the sciatic nerve. After 21 days, a surgical procedure was performed to extract sciatic nerve tissue, which was then stained with Luxol fast blue for histomorphometric analysis. The mean diameter of the fibers, diameter of the axons, number of axons, and myelin sheath thickness were analyzed using MANOVA ($p<0.05$). Compared with the saline group, the PRP treatment group presented a greater number of regenerating myelinated axons with larger fiber and axon diameters, and thicker myelin ($p<0.05$). The myelinated axons in the sham-operated group showed a typical morphology, whereas those in the saline group showed a decrease in myelinated axons, smaller fiber and axon diameters, and thinner, irregular myelin sheaths. Injection of a combination of PRP, both intraperitoneally and locally, promoted the regeneration of damaged nerves in rats.

Keywords: platelet-rich plasma, peripheral nerve injury, nerve regeneration, axonotmesis

1. Introduction

Peripheral nerve injuries occur frequently in clinical settings and can have various causes, such as violent acts with sharp objects or gunshots, recreational pursuits, motor vehicle accidents, and incidents that occur during surgical procedures (1–3). Traumatic peripheral nerve injuries are relatively common, occurring in approximately 2.8% of the population and affecting people under the age of 40 years. Almost 70% of patients fall into this age group (4). Among these patients, men were more frequently affected than women were, with an incidence rate of 1.52. The upper extremities were the most frequently affected regions (42.1%). In particular, the ulnar nerve (18%) and median nerve (12.8%) are frequent sites of injury in the upper extremities, whereas the sciatic nerve (10.8%) is a common site of injury in the lower extremities (2, 4, 5).

Peripheral nerve damage can lead to axonal demyelination, degeneration, or a combination of both. From a clinical perspective, demyelination and axonal degeneration can cause impaired motor and sensory functions or a combination of both. Damaged peripheral nervous systems have substantial structural and functional regenerative capacities (3, 6, 7). These injuries can significantly affect an individual's quality of life, functional ability, and work capacity (8).

Platelet-rich plasma (PRP) has been studied as a potential method to accelerate tissue regeneration. The advantages of PRP in the regeneration of injured nerves have been previously demonstrated (9–12). PRP was obtained from autologous blood by centrifugation. PRP is rich in growth factors, including PDGF, VEGF, and IGF-1, which are involved in degranulation of endothelial growth factor, and insulin-like growth factor (10, 13).

Several neurotrophic factors in PRP can accelerate axonal regeneration after peripheral nerve injury (14). Furthermore, PRP contains essential cytokines that regulate cell processes and cell differentiation and has neuroprotective effects. TGF-β, found in PRP, is a versatile cytokine that contributes to a

*Correspondence: meutia.maulina@unimal.ac.id

variety of phenotypic changes in Schwann cells, enhances the intrinsic growth capacity of nerves, and modulates the permeability of the blood-nerve barrier, thereby promoting increased nerve regeneration (15).

The progression of peripheral nerve regeneration can be evaluated by histological analysis, with histomorphometric evaluation as a key component. Histomorphometry is used to measure cells or tissues and assess changes in cell shape. This involves assessing parameters such as the cell number, volume, thickness, length, and width (16). To evaluate axonal regeneration, histomorphometric analysis includes measuring variables, such as the number of axons, fiber diameter, and axon diameter within each nerve fascicle.

Local injection of collagen sponge-activated PRP and cytidine 5-phosphocholine improved mental nerve regeneration after injury, as demonstrated by histomorphometric analysis. These changes include increases in fiber diameter, axon diameter, and myelin thickness (17). Previous studies have demonstrated neuroprotective, neurogenic, and neuroinflammatory effects of PRP in the treatment and regeneration of peripheral nerves after local administration (18–20). However, the effects of PRP on post-axonomic axonal regeneration using various methods of administration have not been fully elucidated. This study examined the effects of PRP administered via different routes, specifically local, intraperitoneal, or a combination (local and intraperitoneal), on histomorphometric parameters of axonal regeneration after peripheral nerve injury.

2. Materials and Methods

2.1. Animals

Male Wistar rats (*Rattus norvegicus*) aged 3-4 months and weighing 250-300 grams were obtained from the Laboratory of Experimental Animals of the Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia. Throughout the study, the animals were kept under controlled conditions at room temperature with *ad libitum* access to standard food and water. The rats were acclimatized for two weeks before initiation of the experiments. In the post-test control-only group design, 25 male Wistar rats were randomly assigned to five groups (n=5). The sham-operated and saline groups served as controls, whereas the treatment groups consisted of local PRP, intraperitoneal PRP, or a combination of local and intraperitoneal PRP.

2.2. PRP collection and preparation

Ten donor rats were anesthetized with ether after 5 ml of intracardiac blood was collected in a tube containing 0.3 ml of anticoagulant. After whole blood was centrifuged twice, first at 1600 rpm for 10 min and then at 2000 rpm for another 10 min, the blood was effectively separated to obtain PRP. This method allows for the clear separation of blood into three distinct components: the lower layer consisting of erythrocytes, the upper layer comprising the supernatant, and the PRP layer placed in the middle. After the initial centrifugation, the erythrocyte layer was carefully removed. The subsequent centrifugation step resulted in the separation of the supernatant layer, producing 3 ml of pure PRP. PRP is activated by direct application of light and vibration activators shortly before each administration to animals (21).

2.3. Induction of peripheral injury by axonotmesis of the sciatic nerve

Axonotmesis injury to the sciatic nerve was induced by minor surgery, using a previously described protocol (22–24). White rats were anesthetized intraperitoneally with ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight). The gluteal area of each rat was shaved, and the skin was sterilized with iodine solution (povidone) and prepared by draping at the site of the operation. An incision was made in the skin and superficial gluteus muscle to locate the sciatic nerve from the sciatic notch to the popliteal branch, identifying the right biceps femoris muscle. The injured nerve was marked with a 0.8 nylon suture at the epineurium. The nerve was then clamped using an arterial clamp for 60 seconds and placed approximately 1.5 cm from the sciatic foramen. The location of axonotmesis was marked with a 0.4 silk suture in the muscle, according to the site of axonotmesis of the sciatic nerve.

2.4. Experimental design

The saline and PRP control groups underwent sciatic nerve axonotmesis injury, whereas the sham-operated group did not. The sciatic nerve at the axonotmesis site in the saline control group was covered with a gelatin sponge that had been saturated with 0.2 ml of 0.9% saline solution. In contrast, the sciatic nerve in the treatment group was bandaged with a gelatin sponge that had been moistened with 0.2 ml of PRP. The group assigned to receive intraperitoneal treatment received only intraperitoneal PRP without the use of a gelatin sponge, which was administered five minutes after the axonotmesis procedure had been completed. In the sham control group, the sciatic nerve was exposed without axonotmesis, which is a form of acute compression injury. The wound was sutured using a thread. The entire procedure was performed by the same operator, using microsurgical instruments and a sterile environment. The animals that underwent treatment were subsequently relocated to their home cages, where they were observed for 21 days (25, 26).

2.5. Histomorphometric analysis of regenerated nerves

On day 22, rats were sacrificed by cervical dislocation. The sciatic nerves were subsequently surgically extracted. The nerve was divided into two separate sections, with the first segment beginning 5 mm proximal to the axonotmesis site and extending 10 mm distal. The second segment was obtained by cutting it 5 mm distal to the first section. To prepare histological specimens, 5-millimeter-thick nerve segments (segment 2) were immersed in 10% BNF solution. Sciatic nerve tissue sections measuring 5 µm in thickness were stained with Luxol fast blue (LFB).

Histomorphometric analysis was used to quantify axonal

regeneration by counting the axons and measuring the diameters of the fibers and axons within the nerve fascicles. Histological characteristics were assessed using a BX-51 microscope (Olympus, Japan) with a DP80 camera system (Olympus, Japan) and the Cell Sens software (Mitani Corp., Japan). Images were captured in three fields of view at 100x magnification, and the overall fiber diameter, axon diameter, and number of axons in each field of each group preparation were recorded.

2.6. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Multivariate analysis of variance (MANOVA) was performed using SPSS software (version 25.0; IBM Corp.). Statistical significance was set at $p< 0.05$.

3. Results

The results of histomorphometric analysis of the sciatic nerve are presented in Table 1. The PRP-treated groups presented the highest mean values for all the evaluated parameters; however, these values were significantly lower than those observed in the sham-operated group. Although there were no significant differences in the mean diameter of the nerve fibers between the sham-operated and combined PRP treatment groups ($p>0.05$), there were significant differences in the diameter of the axon and myelin sheath thickness ($p<0.001$). No significant differences in the number of axons or myelin sheath thickness were observed between the local and intraperitoneal injection groups ($p>0.05$). Histomorphometric analysis of the sciatic nerve in the sham-operated group revealed myelinated axons with a normal morphology. In contrast, the saline control group showed a reduction in the number of myelinated axons, exhibiting signs of atrophy characterized by small fiber and axon diameters as well as thin and nonhomogeneous myelin (Fig. 1).

Table 1. Histomorphometric analyses of the sciatic nerve among the experimental groups

Groups	Fibre Diameter (µm)	Axon diameter (µm)	Axon counts (fb/mm^2)	Myelin sheath thickness (µm)
Sham	4.35±0.21^a	2.06±0.26^a	83.13±6.06^a	2.29±0.23^a
Saline	2.22±0.15^b	1.24±1.27^b	47.07±5.89^b	0.93±0.19^b
Local PRP	3.15±0.24^c	1.46±0.15^c	58.93±1.36^c	1.69±0.33^c
Intraperitoneal PRP	3.62±0.08^d	1.69±0.08^d	64.73±3.57^c	1.93±0.03^c
Combined PRP	4.20±0.22^a	1.98±0.03^a	71.53±4.16^e	2.16±0.36^d

Data presented as mean±SD. Note: $^{a-e}$ Within a column, means without a common superscript statistically differ ($p<0.05$)

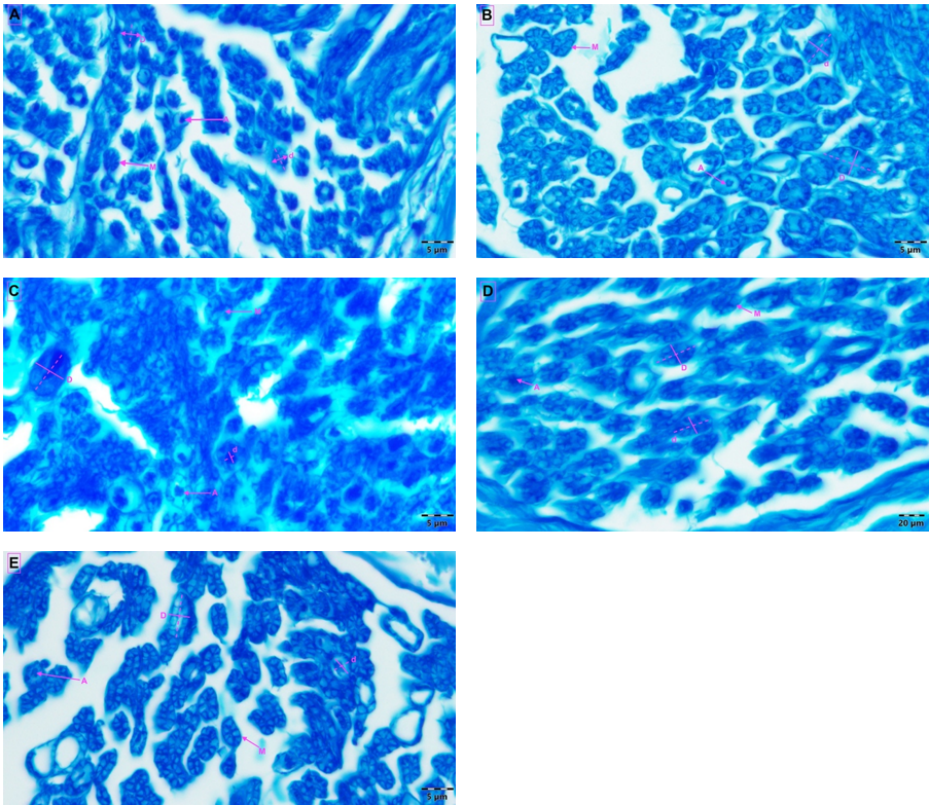

Fig. 1. Photomicrographs of histomorphometric analysis of the sciatic nerve after axonotmesis stained with LFB (100x objective lens). (A) saline group, (B) sham-operated group, (C) local PRP group, (D) intraperitoneal PRP group, and (E) combined PRP group. A: axon; M: myelin sheath; D: fiber diameter; d: axon diameter

4. Discussion

This study investigated the effects of PRP administered through various routes, namely, the intraperitoneal route and combined local and intraperitoneal routes, on axon regeneration after peripheral nerve injury. The number of axons in the saline group was the lowest among all groups. A

higher number of axons indicates a better regeneration process, accompanied by a larger axon diameter. However, when regeneration is poor, a considerable number of axons may be present, but their diameter may not be normal. The presence of a significant number of small-diameter axons was indicative of abnormal sprouting. Thus, it is crucial not only to assess the number of axons but also to assess the diameter and homogeneity of the myelin sheath. Excessive but suboptimal regeneration is defined by an abundance of axons with relatively small diameters, thin myelin sheaths, low density, and inconsistent distribution. The smaller number of axons in the saline group can also be caused by axons that sprout in an unorganized growth direction (not toward one target). This can be characterized by an increased distance between axons in nerve tissue sections (23).

Compared to the saline group, the PRP treatment group showed a higher number of regenerating myelinated axons with larger fiber and axon diameters and thicker myelin. A previous study showed that local administration of PRP leads to increases in both the diameter and number of regenerative axons in the distal portion (18, 19). Intracavernosal administration of PRP in rats after cavernous nerve injury leads to the concurrent acceleration of myelinated axon regeneration, reduced apoptosis, and increased proliferation of corpus cavernosum smooth muscle cells in the early stages (27). Histological analysis of nerve regeneration also revealed an increase in the number of inflammatory cells and endoneurial vacuoles in the PRP group (20). Furthermore, the administration of PRP to rats after injury significantly improves nerve conduction and increases the number of axons in the sciatic nerve (28).

Compared to local administration, intraperitoneal PRP has more effective systemic effects. This is due to the extensive surface area and the numerous blood vessels present in the abdominal cavity. This route of administration accelerates the direct absorption of PRP into the bloodstream, thereby facilitating its rapid absorption (29). According to Karakas et al., intraperitoneal administration of PRP demonstrated superior effectiveness in reducing postoperative peritoneal adhesions. This is achieved through tissue separation, reduction of inflammation, inhibition of matrix metalloproteinase and plasminogen activation, and obstruction of subsequent stages of adhesion formation (30).

Compared with the local or intraperitoneal administration of PRP alone, the use of PRP through intraperitoneal and local injections has shown superior results. Our findings showed that the combined PRP group exhibited a higher mean fiber diameter, axon diameter, axon count, and myelin sheath thickness than the local or intraperitoneal PRP groups. Furthermore, the mean fiber diameter, axon diameter, and myelin thickness in the combined PRP group were not significantly different from those in the sham-operated group. The group that received local and intraperitoneal PRP exhibited the thickest myelin sheath, which was similar to that observed in the sciatic nerve 21 d after axonotmesis. This significant result occurred because greater axon myelination results in faster transmission of neural impulses and better clinical outcomes (31).

In the combined group, the nerves showed an almost normal structure, although some nerve fibers still showed a thin myelin sheath. Our findings are consistent with those of a previous study that demonstrated that local and intraperitoneal administration of PRP after sciatic nerve injury effectively mitigated symptoms of neuropathic pain and improved motor function in mice, as indicated by the extensor postural thrust test (EPT) (32). Muttaqien reported that the combined application of local and intraperitoneal platelet-rich plasma (PRP) resulted in the greatest improvement in motor function in mice, as determined by the toe-out angle (TOA) test after sciatic nerve injury, exceeding the effects of administering PRP locally or intraperitoneally separately (26). The combination of local and intraperitoneal PRP not only exerts immediate effects on the injured sciatic nerve, but also triggers systemic effects by circulating through the blood vessels in the abdominal region. The use of PRP can increase the levels of several circulating growth factors such as IGF-1, VEGF, and FGF by activating specific biological pathways, thereby increasing the benefits of PRP (33, 34).

PRP shows substantial potential to improve wound healing and tissue regeneration owing to the rejuvenating abilities of platelets. When PRP is administered at the site of injury, it activates platelet-release factors that exhibit anti-inflammatory properties and prevent tissue degradation (35). The ability of PRP to promote axonal regeneration after injury is attributed to its ability to secrete growth factors, cytokines, and extracellular matrix components. This secretion facilitates the migration, proliferation, stabilization, and differentiation of various cell types, including Schwann cells, endothelial cells, fibroblasts, and mesenchymal cells (13). Furthermore, PRP has been found to induce nerve regeneration in injured nerves, leading to an increase in myelin thickness and a significant increase in the number of regenerating axons. PRP also induces the regeneration of injured nerves, leading to increased myelin thickness and a large number of regenerating axons (36). Furthermore, evidence suggests that PRP can promote nerve regeneration through neuroprotection, prevention of neuronal apoptosis, stimulation of vascular regeneration, promotion of axonal regeneration, and regulation of inflammatory responses in the microenvironment (37).

According to Moegni et al., PRP can be administered safely to patients with different pathological conditions without causing allergic reactions, infections, coagulation problems, or life-threatening situations. When administered systemically, calcium can be used as a PRP activator to prevent thromboembolism. This has been attributed to the ability of calcium to activate platelet granules, thereby suppressing

platelet activation (38, 39). PRP promotes peripheral nerve regeneration via various mechanisms. The following mechanisms are involved: increasing the capacity for axonal outgrowth, promoting the formation of new blood vessels, providing neuroprotection, preventing cell apoptosis, addressing the inflammatory environment, and reducing muscle atrophy in the denervated target areas. These results suggest that PRP plays a complex role in the restoration of peripheral nerves and that Schwann cell proliferation is a fundamental step in this regenerative process (40).

In this study, the type of nerve injury examined was characterized by axon transection or axonotmesis, which are more severe than neuropraxia. This type of injury results in damage and loss of axonal continuity as well as demyelination of the axon, damage to the endoneurium and perineurium, and Wallerian degeneration. After injury, nerve fibers undergo degeneration and regeneration. The diameter of the nerve fibers is an indicator of a successfully regenerated nerve, which is close to the normal size (23, 41). Axonotmesis-induced regeneration of the sciatic nerve highlights the neuroprotective and neuroregenerative properties of PRP, which contains an abundance of growth factors and cytokines that accelerate nerve regeneration.

In conclusion, PRP has been shown to be highly effective in improving the fiber diameter, axon diameter, myelin sheath thickness, and axon count in the sciatic nerve of Wistar rats (*Rattus norvegicus*) after sciatic nerve injury. Our study demonstrated that combined intraperitoneal and local PRP injections improved axonal regeneration in a rat model of sciatic nerve injury following axonotmesis.

Ethical Statement

All the experimental procedures were approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia (Ref. no: 168/KEPH/IX/2022, 14 September 2022).

Conflict of interest

The authors declare that they have no competing interests.

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Authors' contributions

Concept: M.M., Design: D.R.E., Data Collection or Processing: M.M., E.M., Analysis or Interpretation: M.M., E.E, Z.Z., S.R., Literature Search: D.R.E., E.M., Writing: M.M., S.H.

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