Sys Rev Pharm 2020; 11(6): 254 – 258
A multifaceted review journal in the field of pharmacy
E-ISSN 0976-2779 P-ISSN 0975-8453

Allogenous Freeze-Dried Platelet-Rich Plasma Promotes Peripheral Myelin Repair in Chronic Constriction Nerve Injury

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Article History: *Submitted: 27.02.2020* *Revised: 07.04.2020* *Accepted: 01.06.2020*

ABSTRACT

Background: Neuropathic pain is a chronic pain condition that refers to all pain that begins or is caused by a primary lesion or dysfunction or a temporary disorder in the peripheral or central nervous system (CNS). Neuropathic pain is caused by damage or injury to nerves that transfer information to the brain and spinal cord from the skin, muscles and other body parts. Platelet concentrates such as Platelet Rich Plasma (PRP) have been proven very useful for tissue regeneration since they contain high Growth Factors. PRP is tested for nerve injury to determine the potential of PRP in improving nerve.
Objective: The purpose of this study is to prove the role of PRP in increasing the thickness of myelin in the process of repairing nerves on days 14 and 21.
Methods: This study used three-month-old Rattus novergicus as experimental animals. Experimental animals were randomly divided into 7 groups, each consisting of six rats. On day 14 and 21 after treatment, rats were sacrificed. The examination of myelin thickness was obtained from Osmium Tetraoxide-Toluidine blue staining. Analysis of the data was performed with ANOVA test.
Results: The differentiation capability of FD-PRP towards the sciatic nerve was better in the single dose PRP at 21 days, as indicated by an increase in axon remyelinization compared with differentiation in the chronic ligation group. The myelin thicknes was significantly increased (p 0.000 < 0.05) in FD-PRP groups compared to ligation group at day 21, whereas myelin thicknes no significantly increased (p < 0.05) in FD-PP groups compared to control group at day 21.
Conclusion: PRP can increase axon remyelinization in neuroregeneration. Administration of PRP Single doses for 21 days show the most effective dose for neuroregeneration.
Keywords: Myelin, Platelet-Rich Plasma, Nerve, Neuroregeneration, Axon
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DOI: 10.31838/srp.2020.6.40
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INTRODUCTION

Orofacial pain includes many disorders, including temporomandibular disorders (TMD), trigeminal neuralgia, headaches and myofacial pain, and occurs in 23% of the population [1] and 7-11% of them are chronic pain.[2] Chronic pain is associated with damage to the nerve tissue itself, thus disrupting the modulation of pain in the nerve and is often referred to as neuropathic pain.[3,4] It is estimated that 37.6 million people suffer from neuropathic pain in 2005 and it is estimated that the prevalence will increase to 39.1 million people in 2011. Some forms of orofacial neuropathic pain such as *trigeminal neuralgia* and *glossopharyngeal neuralgia*, *postherpetic neuralgia* and peripheral neuropathic due to malignancy and diabetes mellitus.[5] Other forms of neuropathic pain in the orofacial can be *stomadynia (burning mouth syndrome), phantom tooth pain (atypical odontologia)* and *traumatic nerve injuries.*[2] Neuropathic pain can be accompanied by inflammatory components and effective management requires several types of drugs.

Neuroregeneration is needed for the treatment of neuropathic pain, because this pain arises due to damage to nerve tissue. Neuroregeneration or repair of nerve tissue is a regrowth or recovery from neural networks, cells, or products from cells. The mechanism that occurs is the restoration of nerve parts such as myelin and axon regeneration.

Pharmacotherapy for neuropathic pain has limitations and is generally used for symptomatic therapy to reduce pain complaints using NSAIDs and opioids.[6] Thus, new therapies for regenerating neural networks in the treatment of neuropathic pain are needed. One of the other therapies that can be used for treatment of neuropathic pain is *Platelet rich plasma (PRP). PRP* is a *platelet concentrate* derived from blood plasma and contains 4% red blood cells, 95% platelets and 1% white blood cells. PRP does not only stop the tissue degeneration and necrotic process but also increase regeneration.[7,8] By increasing the concentration of the *growth factor* at the site of the injury, it will speed up the healing process. In the latest developments it is known that *growth factors* play an important role in tissue regeneration including neural networks. This is related to receptors on the neural network, resulting in repair of the neural network.

METHODS

This research has been approved by the Ethics Commission of the Faculty of Dental Medicine, Universitas Airlangga No. 068/ HRECC.FODM /VI/2017. This research is an in vivo experimental laboratory study with *post test only control group design*. There were 7 treatment groups, each group was given the treatment of Control group, day 14 with repeated dose PRP (group A), day 14 with single dose PRP (group B), day 21 with PRP repeated doses (group C), day 21 with single dose PRP (group D), ligation for 14 days (Ligation group 14) and ligation for 21 days (ligation group 21). The tools used in this study were 4000 rpm centrifuge (Corelab BLC – 2012), freezer -80^{0}C, Ocular micrometer, magnetic stirrer, UV sterilizer *clean beanch*, freeze dryer (Virtis Benchtop 4K 4BT4K2L-105), and microscope. The materials used in this study were 10% Formaldehyde, 9% Dextrose citrate acid, 5 ml EDTA Vacutainer, xylazine ketamine, 3/0 silk thread, osmium tetraoxide, and toluidine blue.

Animals

The experimental animals were randomized and divided into 7 groups, namely: Control group, group A (day 14 with repeated dose PRP), group B (day 14 with single dose PRP), group C (day 21 with PRP dose repeated), group D (day 21 with single dose PRP), Ligation group 14 (ligation for 14 days) and ligation group 21 (ligation for 21 days). Experimental animals were adapted to the environment for seven days with basal feed feeding in all rats.

Producing Allogenic Platelet Rich Plasma (PRP)

Ten wistar rats' blood were taken from the heart, using spoit which contained *dextrose citrate acid* anticoagulants to prevent blood clots. The blood are then transferred in a sterile tube. The first centrifuged was at 4000 rpm for 10 minutes to separate red blood cells from plasma. Then by using a *disposable syringe*, the upper plasma of the tube is moved again to the dry tube. The second centrifugation was carried out at 4000 rpm for 10 minutes to move platelet-rich plasma (1/3 of the part around the tube) and a small platelet (2/3 of the upper part of the tube). The 1/3 part of the bottom of the tube is *platelet-rich plasma* (PRP).

Platelet concentrates were dissolved in PBS, collected and incubated at room temperature (30^0C) and disentrifused to remove platelet clots, extracted and frozen at -80^0C for subsequent use. Prior to use, PRP was dissolved with PBS with a ratio of 1:1. PRP is freezed-dried and dissolved with CMCNa 2% 1:1 when applied to experimental animals.

Surgical Procedure

Rat is performed with asepsis using povidone iodine in the abdominal area, then surgery is performed until white nerve fibers are found. These nerve fibers are sciatic nerves, then the sciatic nerve is loosely bonded with 4 bonds at a distance of 1 mm using 3/0 *black-silk non-absorbable*. Damage to nerve tissue occurs after the bond is left for 2x24 hours. Then the nerve injury area is ready to be treated. PRP was applied by using 27-gauge insuline syringe as much as 0.1 ml. After that, suturing is done on the wound. On the 14th day and 21st day after treatment, the rats in each group will be sacrificed using ketamine as *asphyxiation*, to observe the thickness of myelin as an indicator of nerve regeneration.

Histological analysis

The tissue that has been cut 5 mm is distal from the injury, then washed with phosphate buffer pH 7.4 three times. Then the tissue was put into 2% glutaraldehyde solution in a fixed state and left for 3 hours. Then it was transferred to phosphate buffer solution with pH 3 and stored at 4^0C.

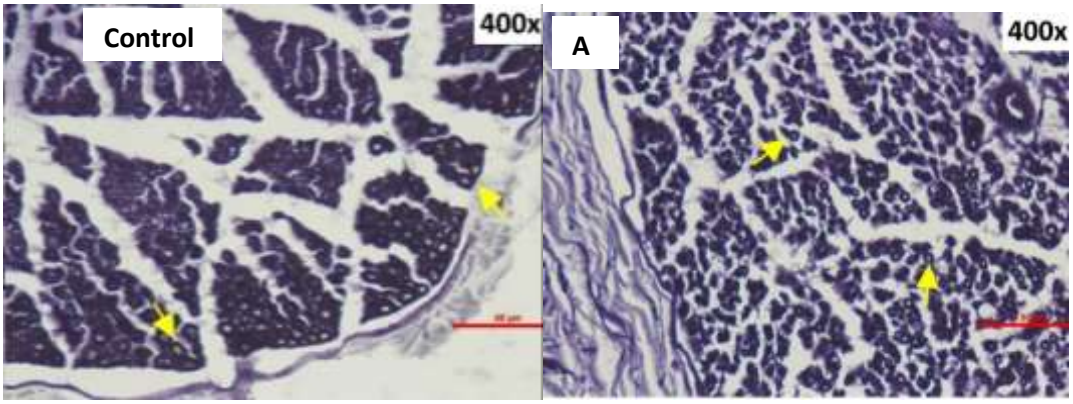
Followed by tissue processing ranging from dehydration, clearing, impregnation, embedding, tissue cutting and coloring. To see osteoblast cells staining using hematoxylin-eosin. To see the myelin thickness, Osmium Tetraoxide-Toluidine Blue was stained.

Statistical Analysis

The experimental data are presented as medians. A statistical analysis was performed using SPSS version 20 (IBM SPSS Statistics 20, IBM Corp, New York, United States). Statistical significance was assessed by the ANOVA test, values less than 0.05 were considered statistically significant.

RESULTS

From the results of staining by using Osmium Tetraoxide from the transverse section, perineureum, endoneureum, axon and myelin are seen. Compared to the control group and ligation group on day 14, in group A the myelin thickness in axons still visible, but the diameter of the axon is smaller but there is still axon thickness compared to the ligation group in the day 14, and the endoneureum distance is more far than the control group and almost the same with the ligation group on 14th day. Group B shows myelin thickness in axons, axon diameter is greater and the distance of endoneureum is smaller than the ligation group on day 14. (Figure 1)



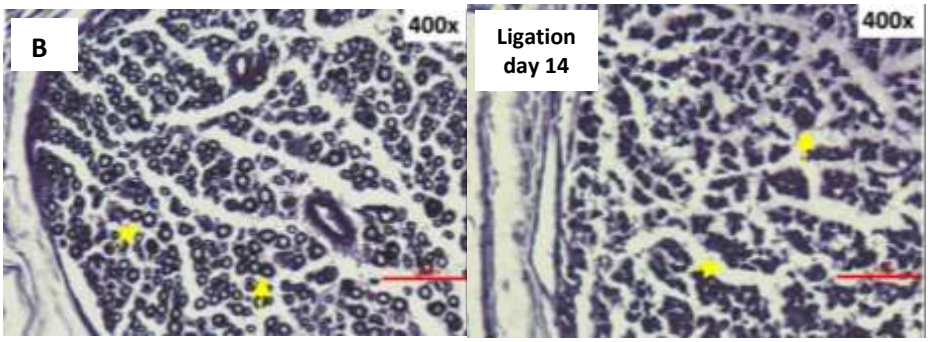


Figure 1: There is a cross-sectional view of the sciatic nerve from staining Osmium Tetraoxide-Toluidine Blue with a magnification of 100x and 400x in Control treatment, A. Repeated PRP, B. Single dose PRP and Ligation14 (ligated for 14 days) on 14th day observation. Yellow arrows in indicate of myelin.

Provision of PRP either in a single dose or with repeated doses for 21 days in the nerves that have been ligated is observed in Figure 1. From the results of staining by using Osmium Tetraoxide from the transverse section, perineureum, endoneureum, axon and myelin are seen. Compared to the control and ligation groups on day 21, in group C there is still a myelin risk in axons, axon diameter is greater than in ligation group on day 21, and the distance of endoneureum is bigger than those in control groups, but smaller than the ligation group 21st day. Group D shows myelin thickness on axons, there are still unclean myelin droplets that show wallerian degeneration and a smaller endoneurum distance than the ligation group on day 21 and are appeared to form connective tissue.

The thickness of myelin in each treatment group is calculated by calculating the myelin axon thickness from the thickest axon on the Tetraoxide Oxygen staining preparation in day 21 showed in Figure 2. Each treatment is carried out reading with a microscope 400x magnification, from the magnification the reading area is divided into 100 regions, then the myelin thickness calculation from 20 regions representing the group is carried out. From these calculations will be obtained the results of myelin thickness of each group.

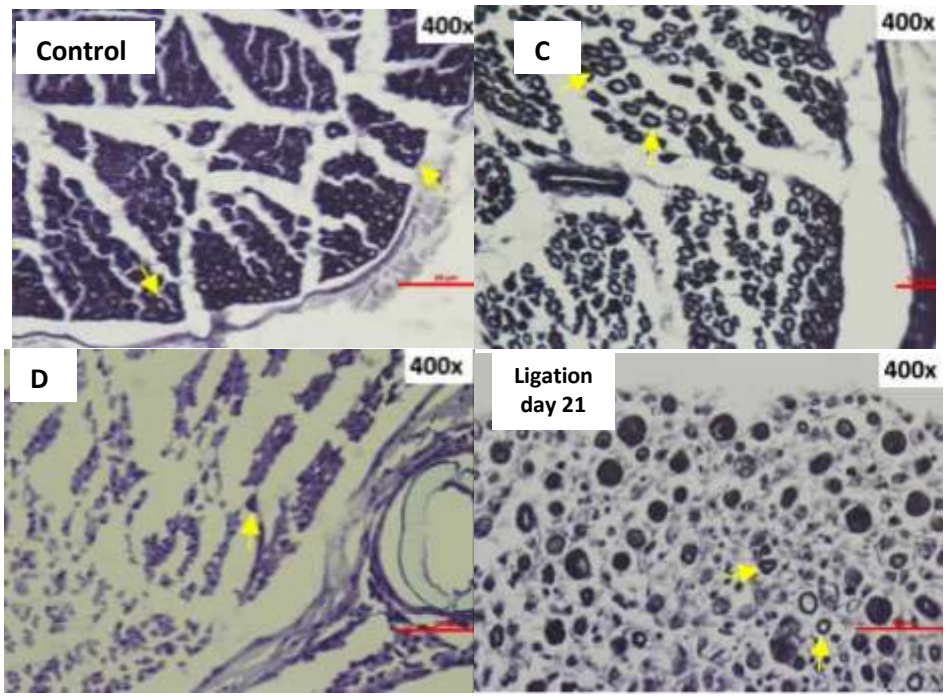


Figure 2: There is a cross-sectional view of the sciatic nerve from staining Osmium Tetraoxide-Toluidine Blue with a magnification of 100x and 400x in Control , C. Repeated PRP, D. Single dose PRP and Ligation 21 (ligated for 21 days) on 21st day observation. Yellow arrows in indicate of myelin.

From the myelin thickness measurement (Table 1), each group shows the best average myelin thickness and close to myelin thickness in control nerves is group D and the lowest mean is in group A. Myelin thickness of all treatment groups is better than the ligation group either on the 14th or 21st day.

Table 1: The mean table of myelin thickness in each treatment group

Group	Mean±SD	MANOVA test	Post hoc Bonferoni						
			Control	A	B	C	D	Ligation day 14	Ligation day 21
Control	1,96 ± 0,23		-	0,005*	0,328	1,000	1,000	0,000*	0,000*
A	1,35 ± 0,24		-	-	1,000	0,191	0,007	0,045*	0,000*
B	1,58 ± 0,30		-	-	-	1,000	0,427	0,001*	0,000*
C	1,76 ± 0,38		-	-	-	-	1,000	0,000*	0,000*
D	1,95 ± 0,24	0,000	-	-	-	-	-	0,000*	0,000*
Ligation day 14	0,85 ± 0,18		-	-	-	-	-	-	1,000
Ligation day 21	0,58 ± 0,16		-	-	-	-	-	-	-

From the results of statistical data analysis, *Kolmogorov-Smirnov Test* issued to get a Sig (2-tailed) value of 0.447> 0.05, thus it can be said that the data is normally distributed. Followed by homogeneity test using *lavene's test* results in sig> 0.05, which means that the data is homogeneous or has the same variance, hence the data is valid for parametric tests using Independent t-test. From the results of different tests using the MANOVA test and post hoc bonferoni is found that the value of Sig. 0.000 <0.05 which means that there are significant differences.

DISCUSSION

The selection of allogeneic PRP materials is expected to be applicable to humans (*human allogeneic PRP*) in the future. It can be a favorable option other than allogeneic PRP that it is not applicable and efficient for patients who require PRP applications with immunocompromised conditions especially with thrombocytopenia, where not all patients have an adequate platelet count.[9] Based on the research conducted by Rachmawati, Astuti, Purwati (2015) Allogeneic PRP with freezed dried preparations has the same potential in *fresh autologous* conditions of PRP and does not cause immunological hypersensitivity response in rabbit experimental animals, as evidenced by no increase in humoral immune response (immunoglobulinM/Ig M) after injection of FD-PRP in the form of a liquid, but still has the function of increasing TGF β1 like fresh PRP. Freezed dried (FD) is processed with a temperature of -83^{o}C for 24 hours and dried which are thought to have deactivated the *Host/Human Protein Antigen* (HPA) on platelet membranes.[10]

Nerve injury performed on experimental animals as a neuropathic pain model is to use ligation in the sciatic nerve.[6,11,12] This treatment causes damage to peripheral nerve tissue. Immediately after an injury to the peripheral nerve, it will be followed by a process of degeneration in the nerve endings. Histological changes mainly involve physical fragmentation of axons and myelin. Neurotubules and neurofilament will be disconnected and the axon shape becomes irregular, axon continuity will disappear and impulse conduction will not occur and myelin disintegration will occur.

Neuroregeneration or repair of nerve tissue is a regrowth or recovery from neural networks, cells, or products from cells. The tip of the axon appears from the proximal end and develops to the distal part. Its development is regulated by chemotactic factors secreted by Schwann cells.[13,14] One of the neuroregeneration parameters that can be seen from this study is the thickness of myelin formed.[15]

The myelin thickness measurements of each group appear to be the best average thickness of myelin and close to myelin thickness in control nerves is group D and the lowest average is in group A. The myelin thickness in group D is the same as the control group and group C and is significantly different from the group ligation 21. Because the growth factors found in *Platelet-Rich Plasma* play a role in increasing *Schwann* cell proliferation and differentiation of several neurotropic factors that can accelerate nerve regeneration.[16] In the repeated dose group C causes recurrent inflammation which slows down the healing process from nerve injury. The neutrophils contained in PRP has more than 40 enzymeshydrolytic and toxic molecules in the granules so that they can become oxidants such as superoxid anions, hydrogen peroxides, and hypochloric acid.[17] These toxic molecules can cause tissue damage which will interfere with nerve regeneration. Although a single dose given to group D, CMC material as a PRP carrier scaffold functions as a scaffold that supports PRP, it can continue to be in the location of damaged peripheral nerves so that the growth factors in PRP can be continued at the wound site on an ongoing basis.[18]

Mielin is formed because of the stimulation of neurotrophin factors released by schwan cells, thus to find out the presence of neuroregeneration is by observing the number of schwann cells in each treatment group. Group A is the same as group ligation 14, the mean value of Schwann cells in this treatment is the lowest compared to all groups. Group B is significantly different from the control group, and the same as the group ligation 14 because it is still dominated by inflammation. Two weeks at the beginning of neuroregeneration, there is activate inflammatory reaction to stimulates Wallerian Degeneration. Group C is different from the control group and group ligation 21. And group D is the same as the control group and was different from the group ligation 21. Because group D is given a single dose of PRP application, so the inflammation caused by surgery is minimal compared to group C. In group C repeated surgery is performed to give repeated PRP, so

inflammation continue and last longer. Because for 21 days PRP can increase *Schwann* cell proliferation, *Schwann* cell migration, and extracellular matrix synthesis such as collagen which has benefits in nerve regeneration. The peripheral nerve healing process begins and is controlled by bioactive proteins found on platelets and plasma.[19] The increased bioactive proteins such as TGF-β, PDGF, IGF act as catalysts to accelerate peripheral nerve regeneration. *Schwann* cells and neurons express PDGF and PDGF receptors function as mitogens and survival factors for *Schwann* cells. The bioactive protein TGFβ-1 has a role in *Schwann* cell proliferation and differentiation of several neurotropic factors.[20]

CONCLUSION

FD-PRP induced neuroregeneration in chronic constriction nerve injury which was characterized by increased myelin thickness in day 21. Remyelination of regenerated axon is the simples aspect of peripheral nerve repair. Recently, some study focus on biomaterial development to promote axonal regeneration and remyelination. In this study, FD-PRP showed the ability to stimulate nerve tissue formation in the initial stage of neuroregeneration. We hope that this study will be beneficial for developing effective therapy for nerve repair, especially in neuropathic pain teraphy.

ACKNOWLEDGEMENT

The authors would like to thank the Stem Cell Center and Tissue Bank of Dr. Soetomo Hospital, Surabaya for its support.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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