Zagazig University Medical Journal
www.zumj.journals.ekb.eg

Volume 28, Issue 6, November 2022(50-57) Supplement Issue

**Manuscript ID** ZUMJ-1910-1602 (R2)
**DOI** **10.21608/zumj.2019.18582.1602**

**ORIGINAL ARTICLE**

# Allogeneic Injectable Platelet Rich Plasma Improves Neurotization Index in a Rat Model

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**Submit Date** 2019-10-26

**Revise Date** 2019-11-26

**Accept Date** 2019-12-01

## ABSTRACT

**Background**: To detect the effect of injectable allogenic PRP in improvement of neurotization index in rat model.

**Methods**: This was Experimental and histopathological study that had included (21) Sprague-Dawley rats. Collected blood of Six rats were used as PRP source Then they were sacrificed, while (15) rats were studied. In the 15 studied rats, sciatic nerve on both sides was cut transversely and it was repaired under microscope. In all rats there was two groups Group (1): The left side (control group) we did Primary repair only but in Group (2): The Right side (PRP group): Activated PRP was injected under epineurium after primary repair.

**Results**: There was a significant relationship between the count of nerve fibers distally in both groups and a non-significant relationship proximally. In the control group G1 the neurotization index was (92.3 %) but in PRP group it was G2 (94.7%), and this statistics was considered valuable (P = .021). A reduction was noticed in the count of the new nerve fibers distal to the repair in G1 (126.6) and G2 (138) compared with that of the proximal segments of the same groups (137and 145.67respectively), these differences were statistically significant in both groups (P = 0.00).

**Conclusions**: We found in our statistics a significant differences in the count of regenerated nerve fibers in (PRP group) distally and proximally and more in the distal count. PRP has aroused as a possible treatment option for peripheral nerve injury. Because of our results, PRP can be used as adjuvant therapy that helps in peripheral nerve regeneration in cases of primary nerve repair in humans.

## INTRODUCTION

Peripheral nerve injuries' morbidity represent around 2.8% of all trauma patients, leaving many with long-term disabilities with a high societal cost **[1]**.Rregeneration power are obvious in the peripheral nervous system (PNS) after different types of injury like direct mechanical trauma or surgical resection after tumour excision. Regeneration ability differs according to different factors like age, mechanism of injury and distance of the injury to the nerve cell body. These injuries cause profound and persistent effect on the patient normal activities and usual work **[2]**.The best treatment is perfect microsurgical repair by tensionless epineurial sutures but if nerve gap is present where end to end suturing can't be done, autologous nerve graft still the best option **[3]**.

Autologous nerve graft has many complications as it sacrifices a healthy nerve and need more extensive surgery with donor site morbidity. Nerve injuries should be repaired early as possible. Delayed repair has bad prognosis. The principles in treatment of nerve injury is not changed in the last 3 decades. Despite of deep understanding of neuropath-physiology of nerve injury and regeneration, Functional outcomes still unsatisfactory **[4]**.It is clear that a purely microsurgical nerve repair will fail to pass all the complex cellular and molecular cascades of peripheral nerve regeneration. Axonal injury affects the entire length of the neuron till the brain. Many factors lead to the poor prognosis of nerve regeneration. The single most vital factor is the extensive cell death in the innervating neuronal pool **[6]**.The most important neurobiological factor to regeneration is to maintain neurons viable **[7]**. Platelet rich plasma (PRP) contains a lot of bioactive factors of plasma and the platelets alpha granules that helps in wound healing and tissue repair.It is used as an autologous cell therapy**[8]**.PRP is prepared safely from blood ample. Its preparation is rapid, simple, convenient,

and cheap **[9]**.A lot of growth factors (GFs) involved in peripheral nerve regeneration are found in PRP like (platelet-derived growth factor (PDGF) ,insulin-like growth factor (IGF), fibroblast growth factor (FGF) , vascular endothelial growth factor (VEGF), transforming growth factor-b (TGF-b) and epidermal growth factor (EGF)].It was reported in many experimental studies that PRP has a stimulating effect of on nerve regeneration **[10]**.Autologous nerve grafting are more effective than using PRP as a filler for artificial nerves or as a scaffold for nerve defect sites in many studies **[11]**.

## METHODS

We think about local injection and infiltration of allogeneic PRP in a nerve repair site increase postoperative nerve regeneration. We used six rats to collect blood for preparation of allogeneic PRP in this experiment. We did primary repair microscopically in both sides sciatic nerves then we do histological evaluations after 8 weeks from operations. Animal experiments were done after receiving approval from the Institutional Animal Care and Use Committee of the Zagazig University (ZU-IACUC). It was done in microsurgery laboratory of plastic surgery department at faculty of medicine, Zagazig University started from October, 2018 till September, 2019.The authors confirm that a high standard of ethics was applied in carrying out all aspects of the current experiment and it was done after receiving approval from the Institutional Animal Care and Use Committee of the Zagazig University (ZU-IACUC) with approval number ZU-IACUC/3/F/50/2018.

Experimental Design:This was Experimental and histopathological study that had included (21) Sprague-Dawley rats with an average weight of 250–300 gm (Table 1). Six rats were used as donors for PRP Then they were sacrificed, while (15) rats were studied. In the 15 studied rats, sciatic nerve on both sides was cut transversely and it was repaired by nylon 10/0 under microscope. In all rats, Left side was considered group (1) while the Right side was detected as group (2).

♦Group (1): The left side (control group) : Primary repair only

♦Group (2): The Right side (PRP group): Activated PRP will be injected under epineurium after primary repairPRP preparation and characterizationAbout 90 cc fresh bloods from 6 rats treated with 10% sodium citrate, anticoagulant solution was obtained in a sterile tube from inferior vena cava by open approaches after anaesthetized them. Blood was centrifuged immediately at 'soft' spin around for 10 minutes at room temperature at 3000 RPM (Fig.1 a.b.c).The upper layer was transferred with a sterile pipette to another tubes without anticoagulant and re-centrifuged in more speed (hard spin) for 5 minutes at 5000 RPM then PRP was pipetted from the base of the tubes and collected together in one tube and prepared for use after activation with 10% calcium chloride [19].

Surgical Procedure

We confirm that a high standard of ethics was applied in carrying out all aspects of the current investigation. Fifteen young adult white male rats weighing 300–350 g were used. The rats were anaesthetized by intraperitoneal and/or intramuscular injection of 0.005 mg/gm Ketamine. Hair was removed by shaving it from the mid-back and both hind limbs.The rats were fixed on a rodent operating board in prone position. A good sterilization with povidine iodine 10 %. Skin incision of about1–2 cm was started 5 mm lateral to the spine till the crista iliaca. By blunt dissection we undermined the incision until identification of the fascial line between gluteal and the biceps muscle groups. Sciatic nerve exposure by gentle blunt dissection in this fascial plane. Careful dissection with no tension to increase exposure of the nerve. The background material was put under the nerve with gentle handling of the nerve then the nerve transection was done proximal to the splitting of the nerve (Fig 2). We did immediate primary epineural repair in both sides by nylon 10\ 0 (Fig.6 a).We did nothing in the LT side sciatic nerve except the repair in group1 (G1) which considered (Control group) (Fig.3) but we inject 5ml of the previously prepared active PRP under the epineurium around the site of repair with activated allogeneic PRP only on the RT side by blunt 28 G syringe in (PRP group) (Fig.4).

Closure of the wound was done in layers by vicryl 4/0 for the muscle and prolene 4/0 for the skin in all rats and the skin was painted with povidine iodine 10 %. Dissection and re-anastomosis of the nerves in all rats done by assistance of surgical microscope and dissecting lenses to facilitate and proper performance. **Clinical follow up**

After recovery, each rat was kept in a separate cage and checked on, under the supervision, every day for the first four weeks and then every week for 8 weeks. we checked rats for feeding and cage cleaning, wound healing and daily dressing and administration of ceftriaxone 0.2 mg/gm antibiotic for 7 days once daily IM . Postoperative complications detected early and we dealed with them accordingly. Three rats died. Two rats died in the first week and one in the second week and the feet of the hind limbs were examined for any abnormal observations, such as ulceration and paralysis. After complete skin healing, rats were left to live normally in groups until the end of the 8 weeks.

**Evaluation Method**

After 8 weeks, all rats were sacrificed. A nerve

segment was resected 1 cm distal and 1 cm proximal to the repair and was sent for histological study to quantify regenerated nerve fibers number distal and proximal to the site of repair in both groups (Fig.5)Biopsy preparation and histologic evaluationEight weeks postoperatively, all rats were revised and killed with an overdose of anesthesia. We re-approached to the nerves and then we excised a 20 mm segment from each side with the repair at the center. We marked distal end by along knot. The nerve tissue was immediately fixed in formalin/saline 10% solution and samples was sent for histopathology after about 48 hours, samples fixed in paraffin wax to make blocks. We made Histological sections of 6 microns thickness and then stained with H&E and Toluidine blue as a special staining. (Fig.6, 7)We performed histological evaluation by using light microscopy then we magnified the cross sections taken to 400X. We counted the nerve fibers in both groups distally and proximally by taking the average count of the two examiners who were trained and unaware of the experiment. A neurotization index was computed as a percentage in G1 and G2 according to the formula: average number of nerve fibers in the distal segment / average number of nerve fibers in the proximal segment X 100. It indicates the number of axons that successfully crossed the repair site from the proximal to the distal segment. This index was reported to indicate the quantity of nerve regeneration after neuroanastomosis It isn't invented by us .it was used in many studies. [9, 18]

## RESULTS

### Clinical Results

Twelve rats survived the operation and there were no major complications through the follow-up period. The rats started moving in the cage slowly on their forelimbs after a short time postoperatively and they drunk water 4 hours postoperative. We removed Skin stitches after one week, and there was good healing in all rats. Mild skin infection with partial wound dehiscence happened one 10 days post-operative, were detected in 2 rats (one rat in each of G1 and G2). The infected wounds were treated as usual by washing with saline and local Betadine and then left to heal secondarily.

### Histopathologic results

There were no anastomotic ruptures in any of the rats in G1 and G2. Limited whitening and thickening of the nerve trunk were present through the area of nerve repair in both G1 and G2. Adequate regeneration was found in both groups at the end **(Fig.11)**.For inter groups Comparison of Means the histomorphometric evaluation the count of nerve fibers distally in G2 (138) was higher than of that of G1 (126.6 axons**).** This difference was statistically significant (P = 0.03) also Similar results were found in the numbers of nerve fibers of the proximal segments of both groups (145.6 and 137) with P < 0.10 **(Table 4)**.and these result was appeared in neurotization indices in both groups which were of G1 (92.3 %) and G2 (94.7%), and the difference was statistically significant with p value (P = .021) **(Table 5)**.In Intra-group comparison of the means there was a decrease in the number of the regenerating nerve fibers distal to the repair in G1 (126.6) and G2 (138) compared with that of the proximal segments of the same groups (137and 145.67respectively), these differences were statistically significant in both groups (P = 0.00) **(Table 6)**.

**Table (1):** Descriptive data of the studied rats.

| Species / Common Name | Strain/ Breed | Weight range | Sex (M, F) | Total Number | Source |
|---|---|---|---|---|---|
| Sprague-Dawley | outbred multipurpose | 300–350 gm | male | 21 | Microsurgery lab. |

**Table (2):** Inter groups Comparison of Means (Numbers of Nerve fibers proximally

| | Number of Nerve fibers Proximally (G1, Lt side) | Number of Nerve fibers Proximally (G2, Rt side) |
|---|---|---|
| **1** | **148** | **156** |
| **2** | **132** | **136** |
| **3** | **120** | **140** |
| **4** | **164** | **132** |
| **5** | **148** | **160** |
| **6** | **132** | **168** |
| **7** | **120** | **148** |
| **8** | **128** | **144** |

| | | Number of Nerve fibers Proximally (G1, Lt side) | Number of Nerve fibers Proximally (G2, Rt side) |
|---|---|---|---|
| 9 | | 132 | 136 |
| 10 | | 136 | 136 |
| 11 | | 144 | 140 |
| 12 | | 140 | 152 |
| Total | Mean | 137.00 | 145.67 |
| | Range | 120-164 | 132-168 |
| | S. D. | 12.663 | 11.244 |

**Table (3) Continue:** Inter groups Comparison of Means (Numbers of Nerve fibers distally.

| | | Number of Nerve fibers Distally (G1, Lt side) | Number of Nerve fibers Distally (G2, Rt side) |
|---|---|---|---|
| 1 | | 140 | 144 |
| 2 | | 124 | 132 |
| 3 | | 112 | 132 |
| 4 | | 152 | 128 |
| 5 | | 140 | 148 |
| 6 | | 112 | 152 |
| 7 | | 108 | 140 |
| 8 | | 124 | 136 |
| 9 | | 124 | 132 |
| 10 | | 120 | 128 |
| 11 | | 136 | 136 |
| 12 | | 128 | 148 |
| Total | Mean | 126.67 | 138.00 |
| | Range | 108-152 | 128-152 |
| | Std. Deviation | 13.248 | 8.268 |

**Table (4):** Inter-groups comparison of the mean count in (G1), (G2)

| | Distal segments | | Proximal segments | |
|---|---|---|---|---|
| | G1 | G2 | G1 | G2 |
| Mean | 126.6 | 138.00 | 137 | 145.6 |
| P value | .033* | | .10 * | |

**Table (6):** Intra-group comparison of the mean numbers of nerve fibers in the PRP-treated (G1) and non-treated (G2) groups

| | G1 | | G2 | |
|---|---|---|---|---|
| | Proximal segments | Distal segments | Proximal segments | Distal segments |
| Mean | 137 | 126.67 | 145.67 | 138.00 |
| P value | .000 | | .000 | |

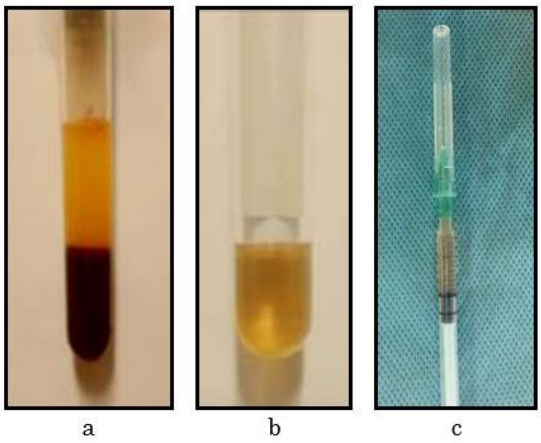


**Fig 1:** PRP preparation by double centrifugation method and activation

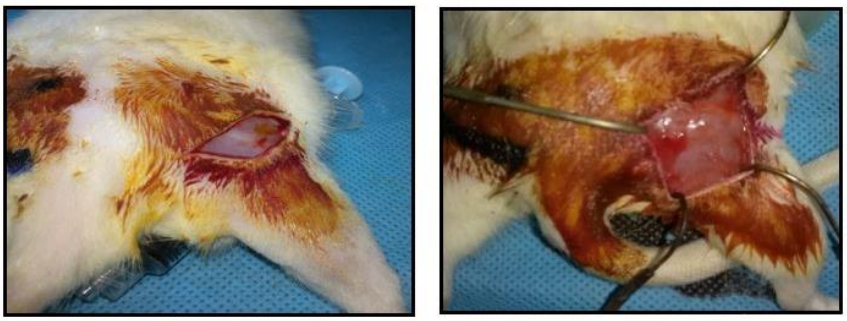

**Fig 2:** Skin incision after good sterilization and identification of fascial line between the biceps and gluteal muscle groups

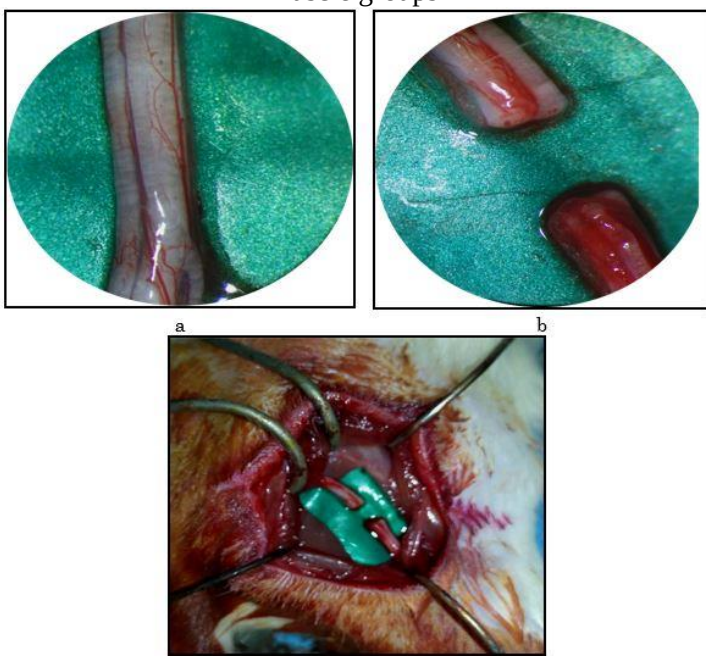


**Fig 3:** Intact sciatic nerve under microscope (A) After cutting the nerve (B)

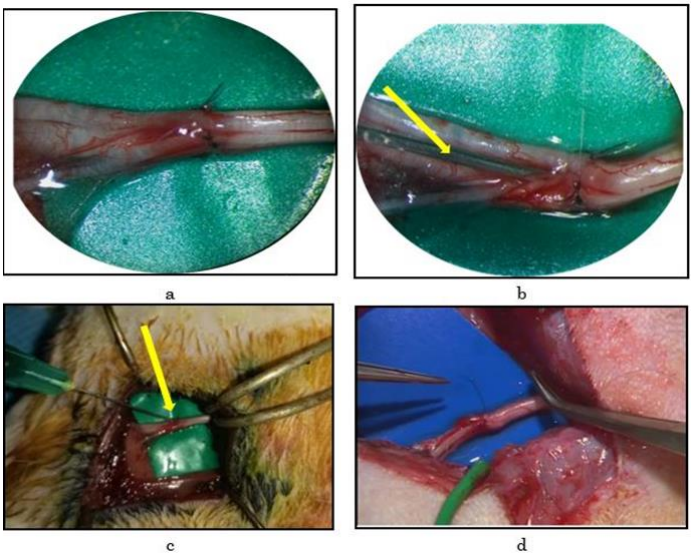


**Fig 4:** Right side sciatic nerve after good repair (a) during infiltration of PRP yellow arrow (b, c) Focusing on another nerve after good infiltration with PRP (d).

**Fig 5:** Post-operative harvesting of the nerve segment proximal and distal with the site of repair in between.

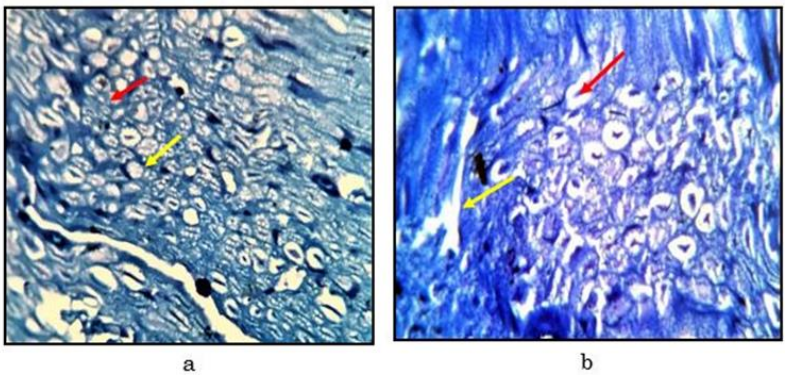


**Fig 6:** Photomicrograph showing the density of the regenerating nerve fibers of the distal segment in the PRP-treated (a) and non-treated (b) groups, toluidine blue X400(red arrow unmyelinated nerve fibre and yellow arrow myelinated nerve fibre)

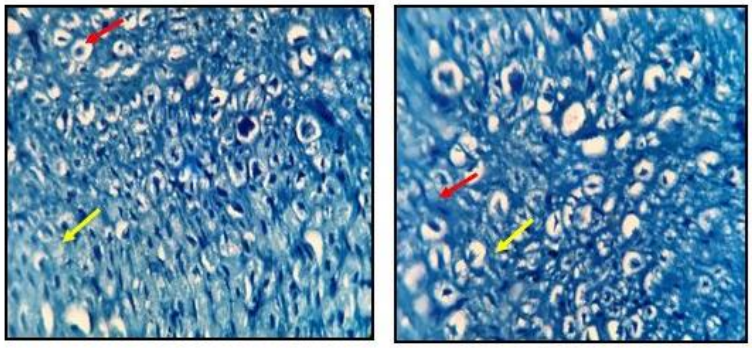


**Fig 7:** Photomicrograph showing variability in density of the regenerating nerve fibres and axonal remyelination of the proximal segment in PRP-treated (a) and non-treated (b) groups, 8 weeks postoperatively toluidine blue ,X400 (Red arrow unmyelinated nerve fibre and yellow arrow myelinated nerve fibre).

## DISCUSSION

Some trials were done to improve nerve regeneration after repair by increasing the proliferation and activation of SCs and increase blood flow around the nerve. Also attempts to increase delivery of Schwan cells with GFs in an acellular nerve allograft or artificial nerve **[12]**, and local administration of GFs to the nerve graft site **[13]**. All these studies aren't approved till now because of several disadvantages as harvesting and culture of cells, high risk of complications, costly and time-consuming processing, and various ethical problems **[14]**.We can be prepare PRP by safe procedure with high level of safety as it is prepared from autologous blood **[9]**. PRP is used in basic research and clinical applications for bone formation in the field of dental oral surgery and wound healing. The tissue repair effect of PRP is caused by various GFs in the plasma and a-granules of platelets. PRP has FGF, TGF-b, VEGF, PDGF, IGF and EGF, which stimulate cell proliferation and migration. These GFs is increase nerve regeneration in many studies. IGF stimulates nerve regeneration by stimulating synthesis of proteins and lipids necessary for nerve regeneration (in vivo). VEGF stimulates axonal elongation to stimulate the proliferation of SCs (in vitro) **[15]**. FGF, PDGF, and TGF-b act as mitogens of SCs in rats **[16]**.PRP therapy for peripheral nerve injury is studied a lot, preparation and usage of PRP, model establishment, and evaluation of outcome was not unified, Results on the role of PRP are different for each study, and allogenic PRP is usually not used in clinical settings.To detect the effect of allogeneic PRP on nerve regeneration, Rats were used in this study. There is no report that compared the difference between the sources of PRP. We focused on using allogeneic PRP in this study **[16, 17]**.Many (GFs) are found in PRP and they are highly associated in peripheral nerve regeneration. Many experimental studies approved that PRP has a good impact on nerve regeneration **[10, 15]**.

We use Pure PRP without WBCs and we prepared it by double centrifugation method and it is approved that preparation of PRP can be done by different protocols for, classified by centrifugation speed, single or double spin, and whether WBCs are included in plasma or not.The neurotization index to compare nerve regeneration in PRP-treated and non-treated groups. It indicates the number of axons that successfully crossed the repair site from the proximal to the distal segment. This index was reported to indicate the quantity of nerve regeneration after neuroanastomosis.

In our study he neurotization indices of PRP untreated group (92.3 %) and PRP treated group (94.7%), and the difference was statistically significant (P = .021) (Table 5) but in the study of Elgazzar et al., 2008 The neurotization indices of PRP treated group (91.9%) and PRP untreated group (89.5%), and the difference was also statistically significant (P = 0.008) **[9].**In this study the histomorphometric results revealed that the PRP group had a remarkable elevation in the regenerating nerve fibers count in comparison with the control groups. This supports the results recently reported by **[9, 15]**. The present results showed a statistically significant difference in end results of nerve regeneration between the PRP and control group.Also we use PRP in liquid state suitable for injection and it may be short acting although it was rapid, easy, with low cost and we use one parameter for comparison (the number of nerve fibers) so we recommend more parameters as thickness of regenerated nerve fibers, Electro-physiological evaluation, muscle weight and Immunohistological evaluation.

## CONCLUSION

A lot of positive effects of PRP on the regeneration of peripheral nerve regeneration after nerve injury by autologous supply of growth factors. Successful results in Animal studies have also showed. But there is also conflicting results about PRP and these

results should raise our attention. Techniques used to concentrate platelets and prepare PRP may influence the results. The results were reflected in the index that detect the count of nerve fibers passed the repair which was in the control group (92.3 %) and in the PRP group (94.7%), and the difference was statistically significant with p value (P = .021). The results was acceptable and showed that there is an increase in the count of regenerated nerve fibers in the PRP group especially on the distal end in comparison to the other side. PRP has aroused as a possible treatment option for peripheral nerve injury, but not completely established. We recommend use of PRP as adjuvant therapy that helps in peripheral nerve regeneration after primary repair in humans.

**Conflicts of interest. None**

**Financial disclosure. None**

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**To Cite:**

Anany, R., Aly, A., Abdel Hamid, M., Mustafa, M. Allogeneic Injectable Platelet Rich Plasma Improves Neurotization Index in a Rat Model. *Zagazig University Medical Journal*, 2022; (50-57): doi: 10.21608/zumj.2019.18582.1602