Impact of Platelet-Rich Plasma on Sciatic Nerve Injury in Rats: HSP 70 Expression and Histological Changes in the Anterior Horn of the Spinal Cord

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ABSTRACT
Peripheral nerve injuries are a significant and growing cause of disability worldwide, as reported by the World Health Organization (WHO). These injuries initiate complex cellular and molecular responses, including the activation of protective pathways that upregulate proteins such as Heat Shock Protein 70 (HSP 70), which plays a crucial role in neuroprotection and the inhibition of neuronal apoptosis. This study investigates the effects of Platelet-Rich Plasma (PRP) on peripheral nerve regeneration, focusing on neuron density, Nissl body presence, and HSP 70 expression as key indicators of recovery. The study utilized 36 rat spinal cord samples, divided into two main groups based on the evaluation time points, day 7 and day 42 post-injury. Each time point group was further subdivided into three: control, sciatica injury, and sciatica injury treated with PRP. Results demonstrated that PRP treatment significantly enhances peripheral nerve regeneration. This was evidenced by increased neuron density, improved Nissl body formation, and a modulation of HSP 70 expression levels, leading to accelerated recovery of motor function, particularly noticeable by day 7 post-injury. These findings suggest that PRP could be a promising therapeutic option for enhancing nerve regeneration and functional recovery after peripheral nerve injuries.

1. Introduction

Peripheral nerve injury is a common illness with mild to severe symptoms that can cause motor abnormalities, sensory disorders, or both. Sciatic nerve injuries are one type of nerve damage that can result in chronic health conditions and potentially cause permanent disability. Incomplete injuries, or those that only affect a portion of the neuron structure, are the most common sorts of instances in Indonesia and other nations. According to WHO data from 2011, up to 35.4 million people under 60 had disabilities as a result of accidents in developing nations.1,2 Depending on the degree of compression, the process that results in nerve injury can range from complete damage to the connective tissue of the neuron structure to loss of myelin at the injury site and axon damage.3,4 The first phase of injury is characterized by disruption to the cell membrane around the wounded axon. This allows a large number of Ca2+ ions to enter the cell and activates signaling pathways known as retrograde signaling that propagate to the neuron cell body and cause cytoskeleton disarray.

Axons will begin to degenerate proximal to the lesion; this loss of axon interaction with Schwann cells is known as Wallerian degeneration. Retrograde signaling towards the cell body causes morphological changes such as the nerve cell body hypertrophy, chromatolysis or the removal of Nissl bodies, the nucleus moving to the edge, and the neuron cell body starting to synthesizesynthesise proteins. When a neuron is injured, its signaling pathway shifts to one that up-regulates several genes involved in growth and the synthesis of cytoskeleton proteins, while down-regulating genes linked to neuronal activity and neurotransmission to promote the expression of pro-regeneration genes. In order to increase the innervation of distal axons to their target organs, the re-myelination process and re-innervation of motor neuron synapses depend on the peripheral nerve regeneration process's success.5,6 Chaperone proteins play an important role in the axon regeneration process, especially HSP 70, where this protein mediates cytoprotective functions, namely by ensuring post-translational processes in terms of protein folding run correctly, stabilization, and protein translocation to prevent aggregation of polypeptide chains that are not folded properly due to stress on these neurons.7,8,9. Although peripheral nerves may typically regenerate through the formation of new axons or collateral growth, this remains a health concern because increasing motor nerve function is frequently hampered. Platelet-rich plasma (PRP) is one therapy that can be used in

conjunction with or as an adjuvant to surgical intervention to modify regeneration following peripheral nerve damage. 18 PRP is an autologous product made up of sticky proteins, microparticles, and other macromolecules. Growths that are created by platelet and plasma activation due to fibrinolysis in tissue, such as VEGF, BDNF (Brain-Derived Neurotophic Factor), ILβ-1 (Interleukin β-1), and PDGF (Platelet-Derived Growth Factor), are stored in a fibrin matrix and bonded by heparan sulfate fibrin domains.10,11,12. Judging from the different problems that can occur due to peripheral nerve injury there are still limitations in prior studies addressing the molecular mechanisms in terms of regeneration given PRP therapy after peripheral nerve injury. It therefore motivates researchers to study how PRP injection affects the regeneration of peripheral nerves by examining Hsp70 expression, and histological parameters such as neuron density and nissl body density in the anterior horn of the spinal cord.

2. Methods

Location and Time of Study

This study was conducted in the Department of Anatomy and Histology, Faculty of Medicine, University of Indonesia, over the course offor four months, from July 2023 to October 2023.

Research Sample

This study used samples in the form of stored biological material, namely spinal cord paraffin blocks from male Wistar rats with a body weight of 200 - 300 grams totaling 36 samples. Next, the mice will be divided into 2 large groups based on their termination time, namely termination on day 7 and termination on day 42 after injury. For each termination group, there were three small groups, namely control, sciatica or injury group and sciatica or injury group given Pure-PRP.

Sample Preparation

In the tissue preparation process, after making paraffin blocks from each tissue, namely the spinal cord, it is then cut using a microtome knife. The glass object containing the tissue is left to dry, so that the paraffin tape is firmly attached to the glass object and the preparation is ready to be used for further examination. We used a Pure-PRP as the intervention in this study. Then, the next step prepares the PRP liquid solution after completing the tissue preparation. We made PRP using allogeneic PRP, which is made from donor mouse heart blood. Next comes purification, which involves centrifuging the blood from the puncture into a tube. The amount of protein, particularly NGF, in the PRP is measured using an ELISA assay. The concentration of NGF in the prepared PRP was discovered to be 17.039 pg/ml.

Examination Used to Assess the Regeneration of Peripheral Nerves

Histological Assessment using Hematoxylin Eosin

The sample that has been embedded in paraffin is then cut using a microtome with a thickness of 4 microns. After that, stain the preparation using the hematoxylin-eosin staining procedure. This was done using a double-blind procedure with multiple observers, averaging 400x magnification across all samples. This study examined the Nissl body density value, which is the number of Nissl bodies divided by the area of the neurons in the cytoplasmic area of the α motor neuron cell bodies in Lamina Rexed IX of the anterior horn of the Spinal Cord. This study also examined the neuron (α motor) in Lamina Rexed IX of the anterior horn of the Spinal Cord. Then, it was analyzed using the Image Raster application, which had previously been calibrated based on the magnification of the microscope lens, namely 400x magnification used.

Assessing HSP 70 Expression Using Immunohistochemistry

The samples, encased in paraffin blocks, underwent a 3-micron thickness incision and were subsequently examined using immunohistochemistry staining analysis. The primary antibody used was Rabbit anti-mouse HSP-70 (GTX111088) with a dilution result of 1:800. The N-Histofine

Simple Stain MAX PO (Multi) detection kit was presented. It contains Universal Immuno-peroxidase Polymer, Goat anti-Rabbit Immunoglobulin, and two drops (100 μL) that are incubated for 30 minutes at room temperature 25oC. HSP-70 expression was identified in Rexed Laminae IX the anterior horn of Spinal cord on both sides. The Image-J application was used to compute the expression findings, and the H-Score (Histochemical Scoring Assessment) was computed to ascertain the staining intensity outcomes for every specimen. The H-Score is calculated using the following formula:

$$\textbf{H-Score}=(0\times P0)+(1\times P1)+(2\times P2)+(3\times P3)$$

The staining intensity (i) and the percentage contribution of colored cells at each intensity level (Pi) combine to provide the H-Score evaluation findings. The staining intensity is represented by the i value, which ranges from 0 (no staining evidence) to 3 (intense staining). In the meantime, ImageJ will be used to extract the Pi value, which ranges from 0% to 100%, from the analysis findings. The range of the H-Score is 0 to 300.13

Statistical Analysis

The two-way ANOVA hypothesis test will be used to evaluate the data from this study because it contains two nominal independent variables from more than two groups and one numerical dependent variable. Analysis of 1 independent variable which has 2 groups which are nominal and 1 dependent variable which is numerical, namely using the independent T-test. The data processing uses the SPSS (Statistical Product and Service Solution) program version 25.0.

3. Results and Discussion

Neuron Density with HE Staining

There was a significant difference between the control group and the sciatica group (LSD-test P= 0.000) and sciatica +PRP (LSD-test P= 0.007), motor neuron density between the sciatica group and sciatica + PRP also showed a significant difference (LSD-test P = 0.000). The results of α motor neuron density in the sciatica + PRP group showed the highest results compared to the control group and the sciatica group. Similar results also occurred in the 42nd day termination group, there were significant differences between the three groups ($p<0.05$) with normally distributed data ($p>0.05$). In the control group there was a difference in mean neuron density between the sciatica group (LSD-test P= 0.000) and sciatica + PRP (LSD-test P= 0.005), motor neuron density between the sciatica group and sciatica + PRP also showed a significant difference (LSD- test P= 0.000). The sciatica + PRP group had the highest neuron density results compared to the control and sciatica groups.

Based on termination day comparisons, the neuron density results in the sciatica group between day 7 and day 42, as determined by the Independent T-test, demonstrated significant differences ($p<0.05$), with an increase in neuron density values on day 7. Meanwhile, in the sciatica + PRP group, the 42nd day termination group showed an increase in neuron density compared to 7th day group, but based on the Independent T-test, it did not show a significant difference ($p>0.05$). Therefore, given that there was no statistically significant difference in neuron density between the 7th and 42nd day, it may be concluded that the motor neuron density value increased more quickly in the sciatica + PRP group, specifically on the 7th day.

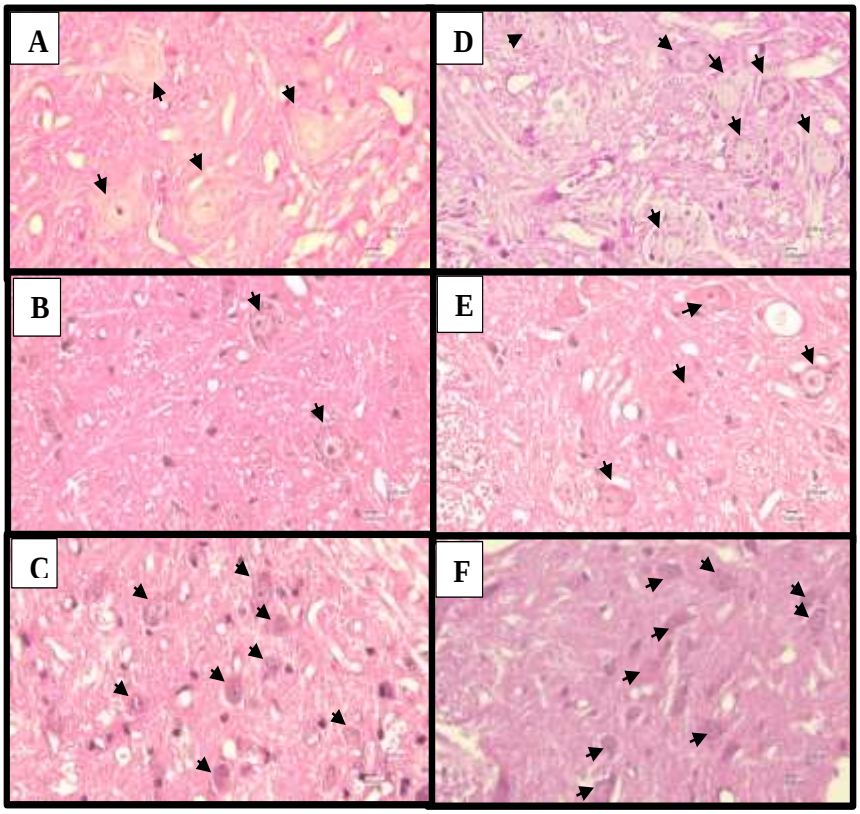

Figure 1. Anterior horn of the spinal cord histological picture of neuron cell bodies (field of view 400x). TIt is evident that the control groups H-7 (picture A) and H-42 (image D) have a greater distribution of α motor neuron cell bodies. The H-7 sciatica group (picture B) and the H-42 sciatica group (image E) had lower numbers of α motor neurons. H-7 (picture C) and H-42 (image F) in the sciatica + PRP group showed an increase in the quantity of α motor neurons.

Nissl Body Density with HE Staining

Based on test ANOVA with normally distributed data ($p>0.05$), the evaluation of Nissl body density in α motor neurons in the anterior horn of the spinal cord in the day 7 and day 42 termination groups revealed significantly different results ($p<0.05$) between the three groups (control, sciatica, and sciatica + PRP). In the day 7 termination group, there was a significant difference between the control group and the sciatica group (LSD-test P= 0.000) and sciatica + PRP (LSD-test P= 0.015), Nissl body density between the sciatica group and sciatica + PRP also showed differences significantly (LSD-test P= 0.000), the density of Nissl bodies in the injured group, whether given or given PRP, decreased in density compared to the control group. The results were not much different in the day 42 termination group, there was a difference in mean Nissl body density between the control group and the sciatica group (LSD-test P= 0.020) and there was no significant difference between the control group and sciatica + PRP (LSD-test P = 0.161), the Nissl body density results between the sciatica and sciatica + PRP groups showed a significant difference (LSD-test P= 0.001).

The Nissl body density results between day 7 and day 42 in the sciatica and sciatica + PRP groups showed significant differences ($p<0.05$) based on the Independent T-test results, which examined the comparison of termination days between the two groups. It appears that in both the sciatica and sciatica + PRP groups, the Nissl body density value of α motor neurons experienced a statistically significant increase at the time of day 42 termination. Thus, at the time of termination on day 42, the Nissl body density value in the sciatica + PRP group grew significantly, and at that point, the Nissl body density increased in both groups the sciatica and the sciatica + PRP groups.

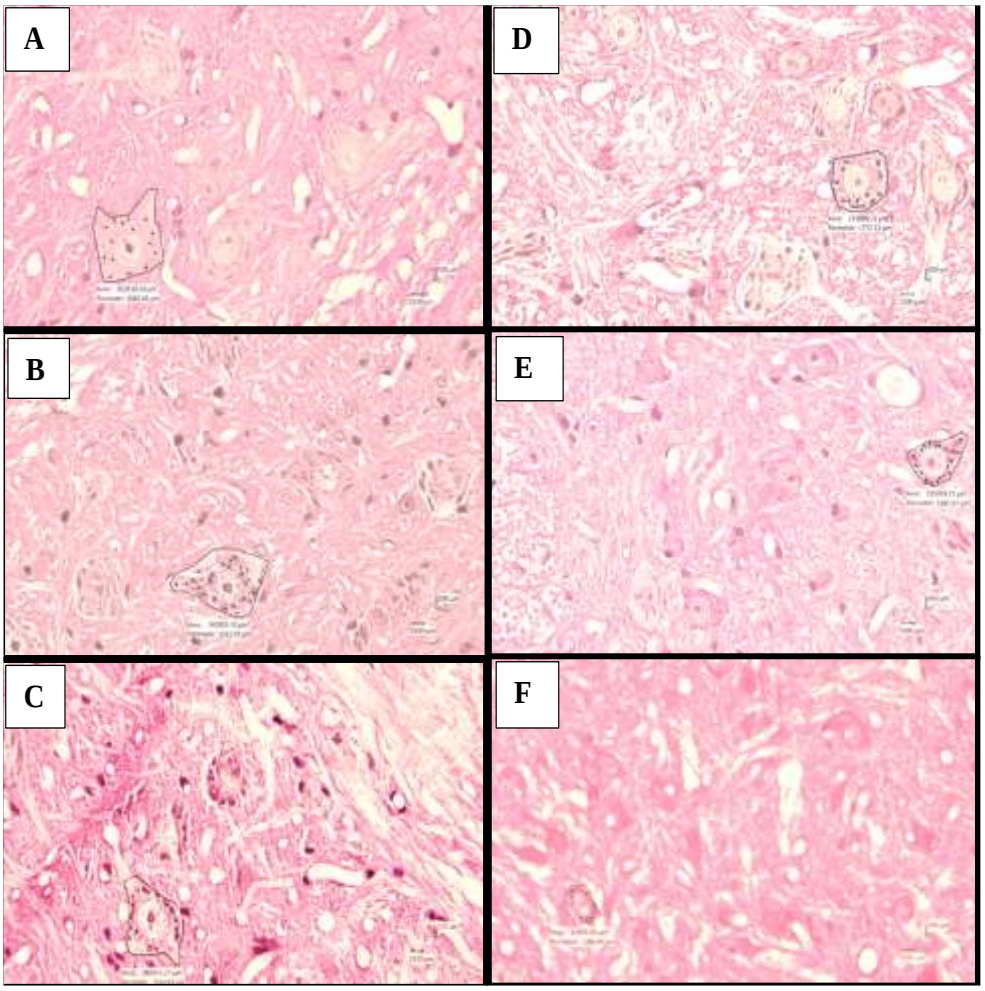

Figure 2. An illustration of the Nissl body structure in α motor neurons located in the anterior horn of the spinal cord using histology. The H-42 sciatica group (picture E) and the H-7 sciatica group (image B) show that the Nissl body is smaller and looks to be dissolving (dust-like)

Expression of HSP 70 in the Anterior Horn of the Spinal Cord

The results of assessing HSP 70 expression in the anterior horn of the spinal cord between the day 7 and 42 termination groups showed different results. Based on the ANOVA test, the day 7 termination group showed results that were not significantly different ($p>0.05$) between the three groups (control, sciatica, sciatica + PRP). In the day 7 termination group, there was an increase in the H-score from the results of the HSP 70 expression assessment in the sciatica group compared to the control group and there was a decrease in the H-score value in the sciatica + PRP group compared to the sciatica group, but based on statistical tests this difference was not significant. Meanwhile, in the day 42 termination group there was a significant difference ($p<0.05$) in the mean H-score values in the three treatment groups. The results were significantly different between the control group and sciatica (LSD-test P= 0.004) and between the sciatica group and sciatica + PRP (LSD-test P= 0.02), but between the control group and sciatica + PRP there were no different results (LSD-test P= 0.405). So, it can be concluded that the treatment group given PRP had a lower H-score of HSP 70 expression compared to the treatment group without PRP on day 42, and had an H-score value that was close to the control group.

Based on statistical tests using the Independent T-test, the comparison of termination days between day 7 and day 42 revealed substantially different results ($p<0.05$) in the day 7 sciatica group compared to the day 42 sciatica group. In contrast, there was no significant difference ($p>0.05$) between day 7 and day 42 in the sciatica + PRP group. Thus, it can be said that the H-score of HSP 70 expression had been declining in the PRP-treated treatment group from the seventh day following

surgery.

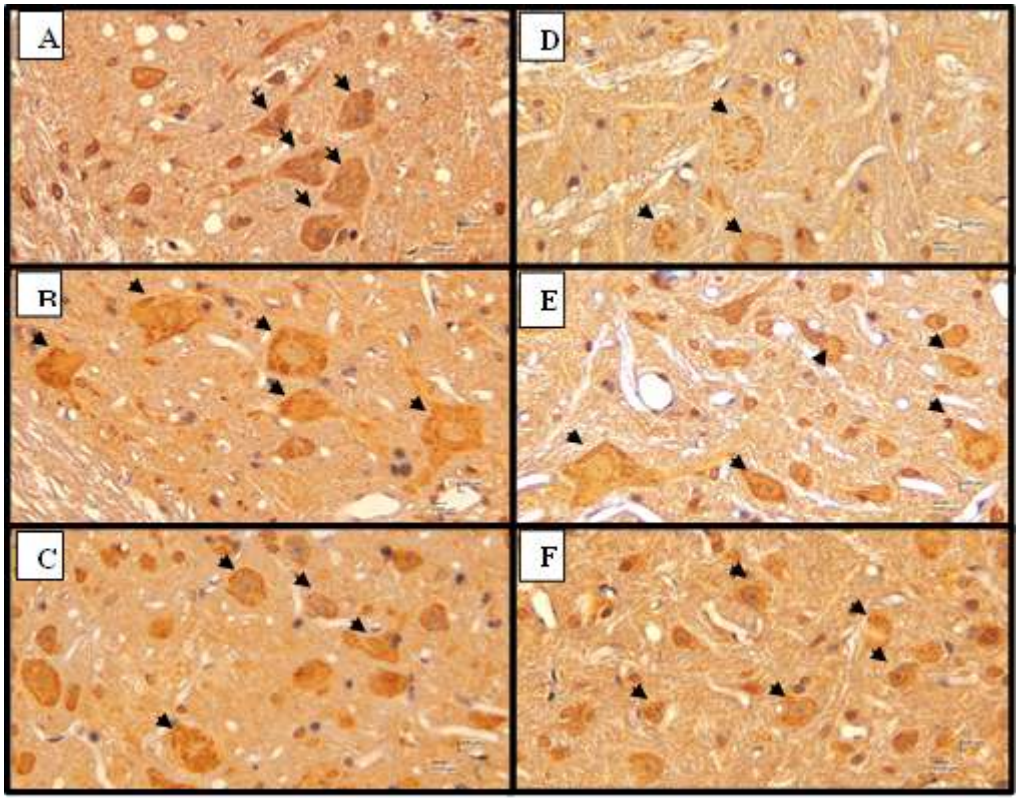

Figure 3. HSP 70 expression in α motor neurons in the spinal cord's anterior horn. HSP 70 expression appears to be present in the cytoplasm of neurons at three different intensities: Sciatica group H-7 in picture B and H-422 in image E yielded the greatest H-Score scores.

The peripheral nervous system possesses a remarkable capacity for spontaneous regeneration following injury, attributed to its inherent plasticity. However, the recovery of motor nerve function post-injury often faces several impediments. Key challenges include the formation of fibrotic tissue and perineural scars, which can lead to neuroma development and hinder axonal regeneration by disrupting electrical signal transmission. These obstacles underscore the need for adjunctive therapies, such as Platelet-Rich Plasma (PRP), which has shown promise in enhancing nerve regeneration. PRP contains a rich array of growth factors, including PDGF, VEGF, BDNF, and NGF, which are crucial for initiating and sustaining the regeneration process. The use of Pure-PRP in this study, as opposed to Leukocyte-rich PRP (L-PRP), was based on its favorable profile in reducing inflammation and apoptosis, thereby supporting a more conducive environment for nerve healing. Our findings indicate that PRP administration significantly increases neuron density and Nissl body presence, particularly evident on day 7 post-injury. This suggests an accelerated initiation of the regenerative process, potentially due to the early activation of neurotrophic pathways. Notably, NGF, a major component of the PRP used, binds to TrkA and p75NTR receptors, which are upregulated during nerve injury. This binding promotes axonal growth and neuronal survival, key factors in effective nerve regeneration. The study also observed a lower HSP 70 expression in the PRP-treated group, which may indicate a reduced need for cellular stress response mechanisms as regeneration progresses. While HSP 70 typically serves as a protective chaperone protein during injury, its decreased expression could reflect a stabilized cellular environment as the repair processes take hold.

Limitations and Future Directions

The study’s design included termination points only at days 7 and 42, limiting the ability to fully understand the dynamics of the regeneration process over time. Future research should incorporate additional time points to capture the progression from acute to chronic phases of recovery. Moreover,

the study did not explore varying concentrations of PRP, which could provide insights into optimizing treatment efficacy. These limitations suggest avenues for further investigation, including exploring the differential impacts of PRP concentrations and extending the timeline for assessment to better understand the long-term outcomes of PRP therapy in nerve regeneration.

The ability of peripheral nerves to heal spontaneously following nerve damage is one way in which the peripheral nervous system exhibits good adaptability upon injury. On the other hand, it is stated that barriers are frequently encountered during the process of healing peripheral nerves following injury, particularly when it comes to enhancing motor nerve function. These obstacles may include reduced walking ability, neurological deficiencies, muscle atrophy, and joint contractures, among other things, which may result in limitations to the healing process. This can occur due to the development of fibrotic tissue which triggers the emergence of perineural scars, causing adhesion of neurons with the surrounding connective tissue and ultimately can cause the formation of neuromas which will inhibit electrical impulses in the axons and inhibit the axonal regeneration process towards the target organ. Based on the potential consequences following peripheral nerve injury, adjuvant therapy—specifically, the administration of Platelet-Rich Plasma (PRP)—is required to halt this process and accelerate the process of nerve regeneration.14,15

When platelets are applied to an injured location, tissue fibrinolysis takes place. This breaks down fibrin and releases signalling molecules like PDGF, VEGF, BDNF, and NGF. These factors then attach to their respective receptors on neurons to initiate the process. recuperation. The type of PRP used in this research is Pure-PRP, this type of PRP has advantages in terms of safety against inflammation and apoptosis in the regeneration process, when compared to L-PRP. According to other reports, PRP concentration has a significant impact on how well it supports the process of nerve regeneration. NGF, at 17.039 pg/ml, and IL-6, at 182.16 pg/ml, were the two main components of the PRP content in this investigation. Studies by Su-Long Wang et al. demonstrated that PRP concentrations whether low, medium, or high did not distinguish between PRP's capacity to produce different growth factors that aid in the process of nerve regeneration. Other research conducted by Sowa et al, showed that administration of low concentrations of P-PRP was able to induce proliferation, migration of endogenous Scwhann cells and induce Scwhann cells to increase the secretion of neurotrophic factors. The highest component in PRP in this study was NGF.16,17 NGF is a neurotrophic factor that can stimulate axonal growth and maintain the survival of neurons. NGF will bind to its receptors, namely TrkA and p75NTR, where in the process of nerve injury the expression of the TrkA and p75 receptors will increase, so that by administering PRP, the NGF components in it can bind to more of these receptors to induce the regeneration process. In addition, IL-6 is an inflammatory response regulator that has both pro- and anti-inflammatory properties and has neuroprotective properties in the nervous system. The IL-6 component contained in PRP in this study, where IL-6 can also induce the regeneration process through activating the expression of regeneration associated genes (RAG) via the JAK-STAT signaling pathway, where the STAT transcription factor in the cytoplasm will undergo phosphorylation and dimerization then translocates the STAT transcription factor to the nucleus to then bind to a specific RAG gene sequence.18,19.20

From the results of this study, in the group given PRP there was an increase in neuron density values when compared with the injured group without PRP administration. Growth factors generated by the platelet component of PRP played a role in inducing this increase in motor neuron cell bodies in the anterior horn of multiple sclerosis (MS). This increase was caused by inducing neuron mitosis. Furthermore, no statistically significant changes were observed between day 7 and 42 in the sciatica group administered with PRP when termination times were compared. This suggests that the neuron density increased more quickly in the PRP administration group, specifically on day 7. These results are supported by an increase in the density of Nissl bodies in α motor neurons which was higher in the group given PRP, indicating that there was a higher protein synthesis process to support the regeneration process. Giving PRP has the effect of increasing the expression of several genes that generate proteins needed for the regeneration process. According to the findings of several studies,

the group receiving PRP produced over 50 different proteins following peripheral nerve injury, including actin cytoskeleton, extracellular exosomes, intracellular transport proteins, and calcium ion binding. Apart from that, the positive effect of giving PRP was also seen in the analysis of HSP 70 expression, where the H-score of HSP 70 was found to be lower in the injured group given PRP compared to the injured group without PRP, indicating that the protective function of HSP 70 on neurons has decreased because the regeneration process has begun.8,21,22,23

It is known that HSP 70 is a chaperone protein that will be expressed in response to injury to the peripheral nerves. The increase in HSP 70 expression aims to ensure that the process of protein synthesis and protein modification runs well and normally, meaning that this protein can prevent protein modification disorders such as the process of folding abnormal polypeptide structures. Apart from that, HSP 70 can also recognize various abnormal proteins and repair these structures, thereby preventing protein aggregation and stress on the endoplasmic reticulum. This of course can prevent further apoptosis processes due to stress on the nervous tissue. There are limitations to this research, such as the termination time which is only carried out at day 7 and 42 so that analysis of molecular and microscopic results is carried out at both times, it is best to also carry out examinations between day 7 and 42 and several weeks after day 42. to be able to compare acute and chronic post-injury conditions through molecular and cellular assessments. Due to the limits of the materials utilized to create PRP in experimental animals, another drawback of this study is that we are unable to compare different PRP concentrations.

4. Conclusion

The study's findings indicate that Pure-PRP significantly enhances peripheral nerve regeneration, as evidenced by increased neuron density and Nissl body presence, alongside a reduction in HSP 70 expression. These changes occur more rapidly compared to untreated nerve injuries, suggesting that Pure-PRP may expedite the recovery process. While the regenerative effects observed are promising, further research is necessary to optimize dosing, understand long-term outcomes, and assess clinical applicability. These results contribute to the growing body of evidence supporting PRP as a viable therapeutic option for peripheral nerve injuries, warranting additional investigation in human clinical trials.Based on the improvement in the results of HSP 70 expression examinations, neuron density, and Nissl bodies, that happen faster after nerve injury, it can be concluded that Pure-PRP has a fairly good regenerative effect on peripheral nerve injuries.

Conflict of Interest

This study does not have any conflicts of interest because the researcher's publication or research would not be influenced by any financial or personal interests.

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