

Functional Recovery of Axonal Injury Induced by Gelatin-Hydrogel Film and PRP: An Initial Study in Rats

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Abstract

In today's society, axonal regeneration is considered very important because of high incidence of nerve injuries. The injury may cause vast damage of extracellular matrix leading to the condition wherein cell lost their environment to live. In this situation, new strategies by the introduction of scaffold to function as synthetic extracellular matrix are needed. In the field of tissue engineering, a large variety of carriers and matrices have been utilized as a scaffold to direct axonal growth. In this study, a new strategy by the combined application of gelatin hydrogel film and Platelet Rich Plasma (PRP) to induce functional recovery of sciatic nerve crushed injury was investigated in Wistar rat model. Assessment of motorist and sensory functions were done to evaluate the recovery processes. It was found that the application of gelatin hydrogel film with controlled release manner combined with PRP results faster axonal regeneration. Further research by different injury model and other investigation factors are needed to confirm the effectiveness of the strategy.

Keywords

Tissue Engineering, Functional Recovery, Axonal Injury, Gelatin Hydrogel, PRP

1. Introduction

In the area of medicine and dentistry, for example, tooth extraction, implant treatment, and reconstructive surgery may cause axonal injury. When axonal injury happens, it is considered as serious health problems, leading to life-long disability or permanent impairment. Although spontaneous regeneration is rarely occurs in axon, its

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regenerative potential is basically high. Therapy to repair damaged axon has been extensively developed, but full functional recovery is still difficult to achieve [1]-[3].

Surgical reconnection, autologous graft and devices application are among therapies indicated for small axonal injury. Meanwhile, in larger defect autologous graft may be applied but it needs multiple surgeries remaining defect on donor sites [4]. This is because vast damage of extracellular matrix happens in large defect leading to the condition wherein cell lost their environment to live. In this situation, new strategies by the introduction of scaffold to function as synthetic extracellular matrix are needed [5].

In the field of tissue engineering, a large variety of carriers and matrices have been utilized as a scaffold to direct axonal growth, widely known as entubulation method, by minimizing cellular and humoral factors suppressing nerve regeneration in the environment. Some conduits made of various biomaterials have been being developed and tested in promoting regeneration [1] [2] [6]. In this context, gelatin is known to be good bio-polymer candidate that can be designed to form conduit. The advantage of the use of gelatin hydrogel, apart from the three dimensional (3D) structure provided to the cells embedded, is that material properties can be adapted including adhesiveness, stiffness, cell signaling potential, size and shape. Hydrogel film from gelatin is also easy to be prepared and its safety has been proven. Haraguchi *et al.* investigated the application of gelatin hydrogel membrane and found it promising vein graft when combined with basic-Fibroblast Growth Factor (b-FGF) [7]. A lot of research also proved that gelatin hydrogel microsphere promoted better facial nerve recovery after compressive injury when it was combined with b-FGF [2] [6]-[9].

Besides the utilization of scaffold, to date most attention has been given to growth factors including b-FGF to induce tissue regeneration. However, the half-life of the growth factors, the complexity of recombinant technique to prepare growth factors and the cost of it remain problems [4]. These issues lead to the use of Platelet Rich Plasma (PRP) which proved to promote axonal sprouting and myelin development. Since PRP is easy to be collected from patient's blood using uncomplicated technology, the feasibility of its application is better compared to recombinant growth factors. In general, PRP is defined as an autologous concentration of platelets in a small volume of plasma [10]. It is considered to be a rich sources of platelet derived growth factor (PDGF), transforming growth factor beta (TGF- β), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF) and b-FGF [10] [11].

The focus of this study is on tissue engineering approach to axonal regeneration as one among high incidence in today's society [12]. In this study, a new strategy by the combined application of gelatin hydrogel film and PRP to induce functional recovery of sciatic nerve crushed injury was investigated in Wistar rat model. Assessment of motorist and sensory functions were done to evaluate the recovery processes.

2. Materials and Methods

2.1. Gelatin-Hydrogel Preparation

Gelatin-hydrogel used in this study was prepared from 5 wt% Type B gelatin of bovine bone (Nitta Gelatin, Japan). The gelatin-hydrogel was poured into polypropylene mold ($4 \times 4 \text{ cm}^2$, Balance dish, BioBik, Japan) and kept for 4 days in 4°C refrigerator to obtain dried gelatin film. Dehydrothermal treatment to crosslink gelatin-hydrogel film was done at 140°C for 48 hours. To control the fabrication of the film, tensile strength of the film was assessed using Universal Testing Machine (Zwick, Germany) in a 10 mm/min crosshead speed on dried condition. Furthermore, the microstructure was randomly assessed by Scanning Electron Microscopy (Hitachi, Japan). Meanwhile, swelling behavior of the film was calculated from film weight before and after 24 hours embedding in phosphate buffered saline (PBS) of 7.4 pH.

2.2. Animal Model

The protocol for this study was approved by Medical and Health Research Ethics Committee of the Faculty of Medicine, Universitas Gadjah Mada (Reference Number KE/FK/55/EC dated 18/01/2012). National guidelines for the care and use of laboratory animals were applied during the study. The animals were housed in cages located at the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada. Fifty-two (52) male Wistar rats (age 3 months, weight $248.67 \pm 17.33 \text{ g}$) were randomly divided into four experimental groups: M0P0 (crush injury), M1P0 (film), M0P1 (PRP) and M1P1 (film with PRP) groups. Functional evaluation was done at Days 3, 7, 14 and 21 after surgery by 4 blinded operators (2 operators for each sensory and motor test).

2.3. Coctail of Growth Factors Preparation

Platelet Rich Plasma as an autologous growth factor was prepared from rat blood. After sedated using intra muscular 80 - 100 mg/kg weight ketamine hydrochloride injection, retro orbital veins blood was obtained using plain haematocrit tube (Micro-Haematocrit-tubes, Assistant). Peripheral blood was double-centrifugated using 1000 and 2100 G velocity. Platelet count was conducted before and after centrifugation in Clinical Pathology Department, Faculty of Medicine, Universitas Gadjah Mada. Activation using 10% CaCl₂ was done on PRP before application to result PRP gel.

2.4. Crush Injury Model

After sedated using intra muscular 80 - 100 mg/kg weight ketamine hydrochloride with xylazine 5 - 10 mg/kg weight injection, right hind limb of each subject was shaved and sprayed by 70% ethanol as antiseptic. Surgical blade was used to incise the skin and maximus gluteus muscle. Dressing forceps and retractor were then used to visualize sciatic nerve. Injury was made by crushing the sciatic nerve using non-serrated clamp at 0.20 MPa for 60 seconds in 2.5 mm length after suturing the nerve epineurium in distal part of crush injury area (8-O black nylon suture, Bear, Japan). **Table 1** shows grouping of the rats.

2.5. Scaffold and Coctails of Growth Factor Application

Application of either gelatin hydrogel film, PRP or both was conducted after suturing the nerve epineurium. Gelatin hydrogel film was prepared in 0.5 × 1 cm² dimension and sterilized using dry Ethylene Oxide Gas (EOG) method. Gelatin hydrogel film was wrapped on the crushed injury area and PRP (in 210 mL added by 30 mL CaCl₂) was applied directly upon the area. In combined application, PRP was applied after gelatin hydrogel film was placed under crushed sciatic nerve then it was wrapped to cover the area. Skin and muscle were repositioned carefully and irrigated using antibiotic solution. The skin was sutured (4-O black nylon suture, Bear, Japan) and 10% povidone iodine as an antiseptic was applied upon surgical wound (Betadine, Jakarta, Indonesia). Antibiotic and analgesia were given twice a day for three days after surgical procedure (*ad libitum*).

2.6. Functional Recovery Test

Walking Track Analysis and Nociceptive Withdrawal Response Test as functional test applied in this study used normal limb on the same rat individual as negative control. The 3R principle in the use of animal model (especially to reduce number of rats) was applied during the study by testing normal limb of the same rat individual. Functional recovery of sciatic nerve was evaluated at Days 3, 7, 14 and 21. Nociceptive withdrawal reflex test was conducted to evaluate sensory function recovery. For nociceptive withdrawal reflex test, subject was positioned to stand at one hind limb on hot plate and the other on room temperature. The position was shifted after 15 seconds recovery phase and reproduced for 5 times. Nociceptive withdrawal reflex in 4 seconds or less when tested at 56°C hot plate was considered as normal.

Walking track analysis was used to examine motor function recovery. Rat was allowed to walk along walking track analysis apparatus lane after permanent marker ink (Snowman, Japan) application on hind limb to produce

Table 1. Grouping of the rats.

Termination Day	Crush (M0P0)	Crush + Film (M1P0)	Crush + PRP (M0P1)	Crush + Film + PRP (M1P1)
7	4	4	4	4
14	4	4	4	4
21	4	4	4	4
Sub-Total by Group	12	12	12	12
Total			Rats	

*4 Normal individual rats without treatment were used as a reference for functional recovery test.

a trace upon white paper on the lane. Five traces were made from each subject on each assessment time. The injured leg was compared to the non-injured leg from the same individual as the control. Blinded observer recorded the presence or absence of inability to lift foot entirely from the ground (foot-dragging), inability to spread the toes and to measure the paw length. Quantitative measurement was conducted using Sciatic Functional Index (SFI) counted from traces measurement according to Bain-Mackinnon-Hunter formula [13]-[15]. The traces measurement includes distance from the heel to the third toe (Paw Length or PL), distance between the first and the fifth toe (Toe Spread or TS) and distance from the second to the fourth toe (Intermediary Toe Spread or ITS). Zero value of SFI showed normal function and -100 value showed total impairment.

$$SFI = -38.3 \left(\frac{EPL - NPL}{NPL} \right) + 109.5 \left(\frac{ETS - NTS}{NTS} \right) + 13.3 \left(\frac{EIT - NIT}{NIT} \right) - 8.8 \quad (1)$$

SFI: Sciatic Functional Index; EPL: Experimental Paw Length; NPL: Normal Paw Length; ETS: Experimental Toe Spread; NTS: Normal Toe Spread; EIT: Experimental Intermediary Toe Spread; NIT: Normal Intermediary Toe Spread.

2.7. Histological Examination

Axonal growth determination was conducted by calculating axonal density per $1 \mu\text{m}^2$ area on histological preparation of each rat on 7, 14 and 21 days after surgery. On the day of termination, rats were sedated using 100 mg/kg weight ketamine hydrochloric with 5 - 10 mg/kg weight intramuscularly and perfused with 4% paraformaldehyde and 1% glutaraldehyde in pH 7.4 PBS fixatives. Transected nerve was harvested and immersed in the same fixative for 72 h in 4°C followed by graded alcohol dehydrating and paraffin embedding. The $6 \mu\text{m}$ sectioned tissue was stained using 0.1% Tuloidine Blue in aqueous solution. Histological examination was conducted by two independent blinded observers with agreement among them. Distal region next to bifurcation was used to quantify axonal density.

3. Results

3.1. Properties of the Gelatin Hydrogel Film

Table 2 shows the properties of the gelatin hydrogel used in this study. Gelatin hydrogel films prepared in this study showed the percentage of 76.00 ± 2.78 water content after being embedded in PBS for 24 hours. The tensile strength of the gelatin hydrogel film was 37.18 ± 2.14 MPa. **Figure 1** shows homogenous microstructure of the gelatin hydrogelfilm prepared in the study. There was variability of the weight of the films due to variation during manual moldings. The average weight of the films applied in this study was 1.68 ± 0.13 mg (M1P0) and 1.70 ± 0.14 mg (M1P1). Film weight was known to be normally distributed as shown by the result of F-test (F calculation 1.27; $p > 0.05$) and no significant differences between groups, as shown by the result of T test (Tcalculation 0.01; $p > 0.05$).

3.2. Platelet Number

Whole blood for preparing PRP was obtained from MOP1 and M1P1 group of animal model on the day of the treatment. Whole blood derived from each rats was individually processed to be able to prepare autologous PRP. In order to confirm platelet concentration, platelet count was done before and after double centrifugations, as shown in **Table 3**. Statistical analysis on platelet number using Mann-Whitney method showed no significant

Table 2. Properties of gelatin-hydrogel film used in the study.

Properties	Value
Water content (%)	76.00 ± 2.78
Tensile strength (MPa)	37.18 ± 2.14
Weight (mg)	1.68 ± 0.13 (M1P0 Group) 1.70 ± 0.14 (M1P1 Group)

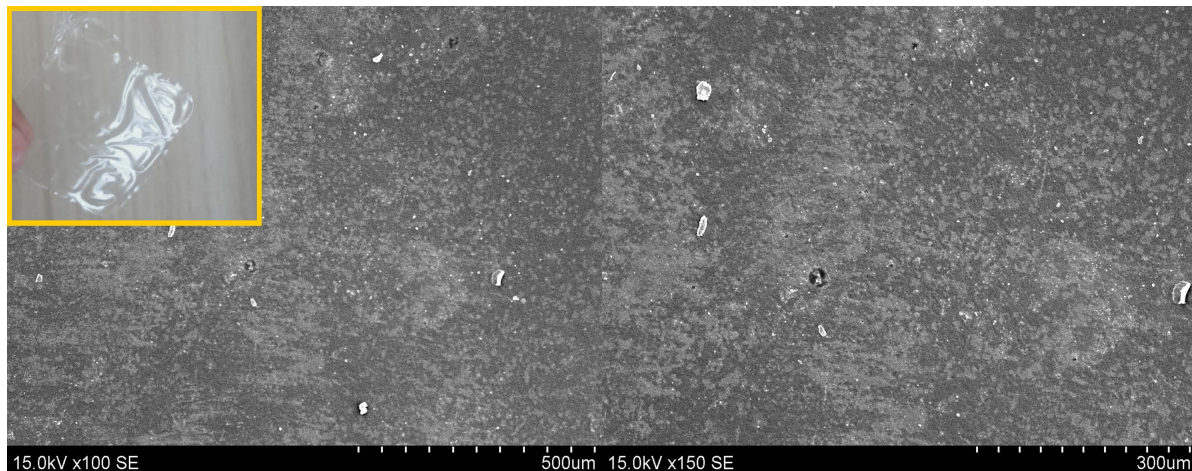


Figure 1. Macrostructure and microstructure of gelatin-hydrogel film used in the study, showing homogenous structure of gelatin hydrogel film.

Table 3. Number of platelet used in the study (per 1 μ L peripheral blood).

Group	Before	After
M0P1	630923.08 \pm 197.16	661866.78 \pm 108.06
M1P1	1553308.00 \pm 569.37	1,614,933 \pm 583.97

difference on the applied PRP between M0P1 and M1P1 groups ($p < 0.05$). Based on this result, it was assumed that platelet number of PRP applied in this study was considered well controlled.

3.3. Sensory Function Recovery

Nociceptive withdrawal reflex performed by each rat was recorded as normal and abnormal, defined by 4 s threshold. Withdrawal reflex recorded after 4 s showed abnormality of sensory function. In this study, sensory function of all rats was abnormal until 21 days of observation. **Figure 2** describes the measurement process of walking track paper and **Figure 3** describes nociceptive withdrawal reflex (NWR) during 21 days observation. **Table 4** shows normal value as a reference for NWR. The M1P1 group showed the most noticeable changes along treatment period. Kruskal-Wallis test showed that the changes was significantly different (K calculated 100.30; $p < 0.05$) and Tukey test showed that the median nociceptive withdrawal reflex test result was not significantly different from predicted order (Day 21 < 14 < 3 < 7; Z calculated 1.95).

3.4. Motor Function Recovery

Gait observation showed that no rat presented foot dragging at all examination days. Inability to spread the toes was observed on all rats at Days 3 to 14. At Day 21 all rats showed ability to spread the toes and their traces started to be measurable. Score of SFI derived from Walking Track Analysis of M0P0, M1P0, M0P1 and M1P1 is shown on **Table 5**, completed by **Table 6** for a reference in normal rats at Day 21.

3.5. Histological Structure

As shown on **Table 7**, axonal density in M0P0 group decreased from Day 7 (0.73/1000 μ m²) to 14 (0.44/1000 μ m²) and increased after Day 21 (0.53/1000 μ m²). Similar pattern was found in M1P1 group: 0.87/1000 μ m² at Day 7, 0.62/1000 μ m² at Day 14, and 0.81/1000 μ m². However, axonal density in M1P0 and M0P1 group were decreased along observation periods. At Day 21, M1P1 group showed the highest axonal density among all treatment group (K calculated -1.94, $p > 0.05$). **Figure 4** shows highest axonal density found in the M1P1 group.

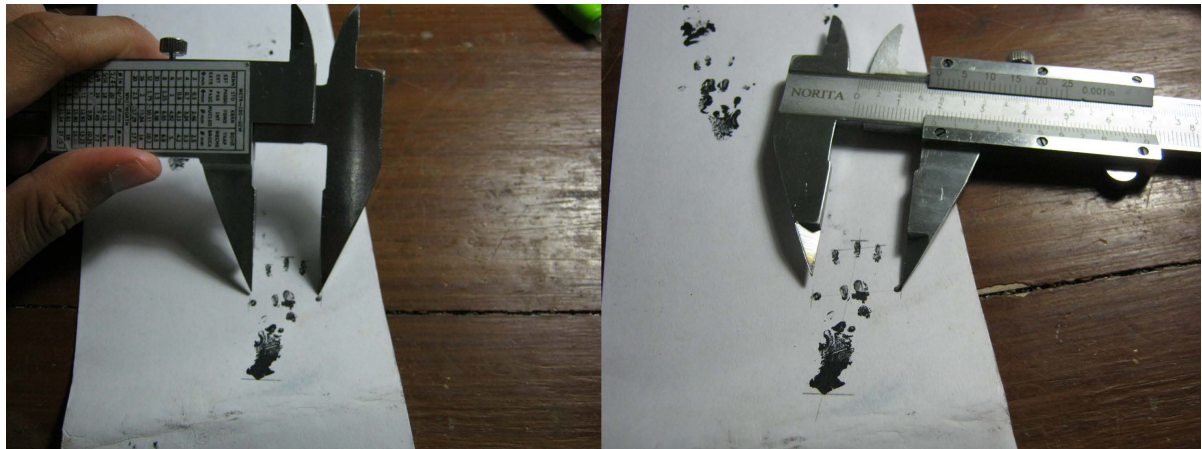


Figure 2. Measurement of walking track paper.

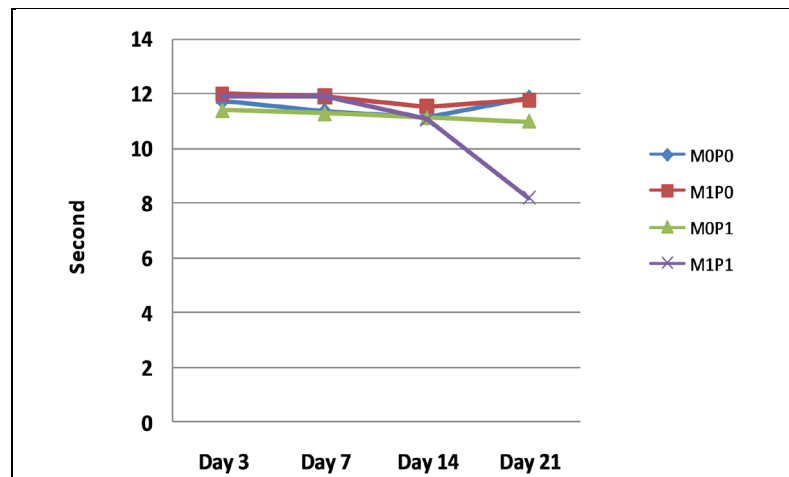


Figure 3. Nociceptive withdrawal reflect on Day 21.

Table 4. Results of NWR (Nociceptive Withdrawal Reflect) in normal rats at Day 21.

Normal Rat	NWR (in second)
Rat 1	3.6
Rat 2	2.0
Rat 3	1.8
Rat 4	4.0
Mean ± SD	2.85 ± 1.12

Table 5. Motor function recovery by means of SFI (Sciatic Function Index) from Days 3 to 21. Normal limb was applied as control.

Group	Day 3	Day 7	Day 14	Day 21*
Crush injury (M0P0)	Inability to spread toes	Inability to spread toes	Inability to spread toes	-26.29 ± 7.09
Film (M1P0)	Inability to spread toes	Inability to spread toes	Inability to spread toes	31.74 ± 12.68
PRP (M0P1)	Inability to spread toes	Inability to spread toes	Inability to spread toes	-30.74 ± 30.34
Film-PRP (M1P1)	Inability to spread toes	Inability to spread toes	Inability to spread toes	-21.29 ± 22.12

*In scoring system. Normal motor function falls at 0 score and the variation ranges from +11 to -11, meanwhile -100 means complete motor function disability [22].

Table 6. Results of SFI (Sciatic Functional Index) in normal rats at Day 21.

Normal Rat	SFI
Rat 1	-2.27
Rat 2	5.85
Rat 3	12.60
Rat 4	9.04

Table 7. Axonal density in 1000 μm^2 on Days 7, 14 and 21 in the distal part to crush injury site.

Group	Day 7	Day 14	Day 21
Crush injury (M0P0)	0.73 \pm 0.06	0.44 \pm 0.20	0.53 \pm 0.11
Film (M1P0)	0.92 \pm 0.16	0.64 \pm 0.21	0.61 \pm 0.16
PRP (M0P1)	0.86 \pm 0.13	0.73 \pm 0.19	0.67 \pm 0.24
Film-PRP (M1P1)	0.87 \pm 0.14	0.62 \pm 0.18	0.81 \pm 0.28

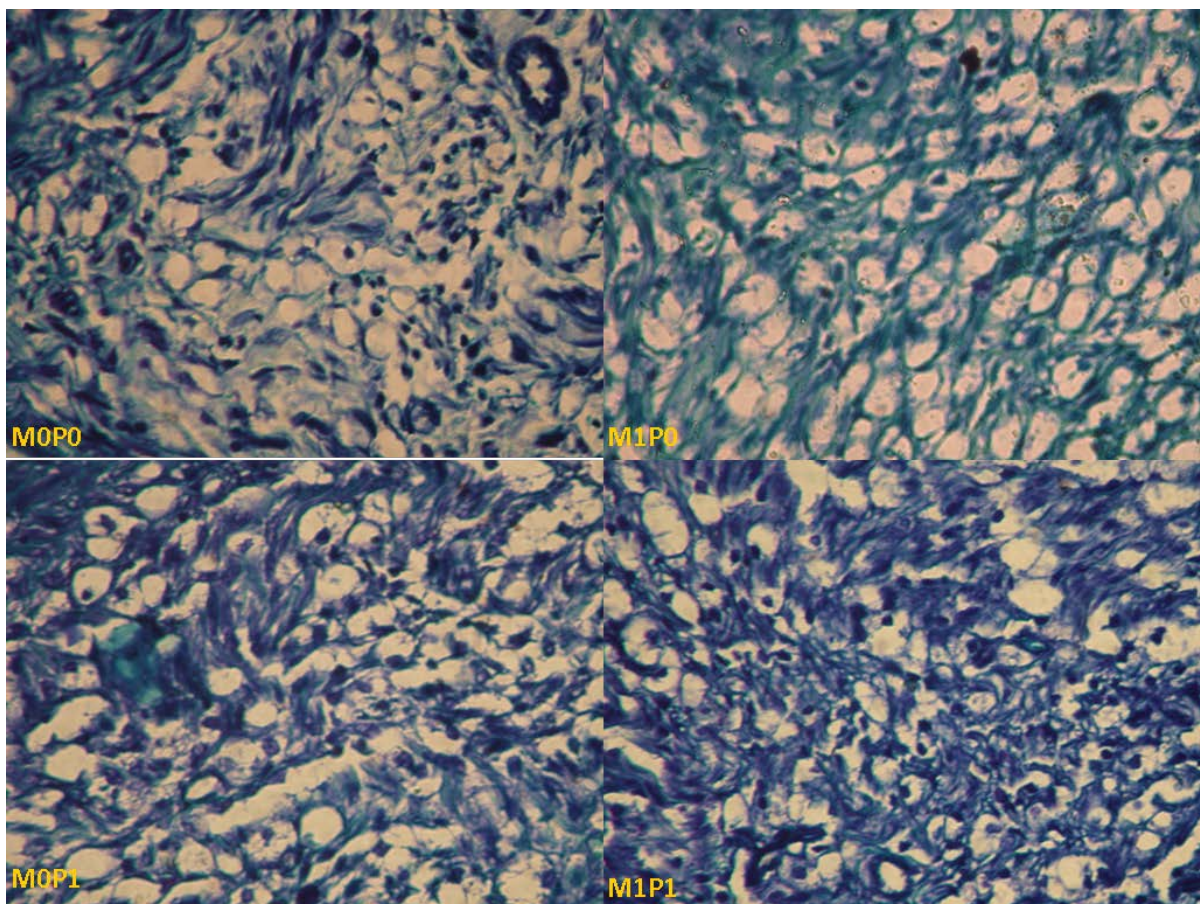


Figure 4. Axonal density after 21 days in (a) M0P0 group; (b) M1P0 group; (c) M0P1 group; and (d) M1P1 group. Highest axonal density was found in D.

4. Discussion

Platelet-Rich Plasma (PRP) has been widely known as a source of variable autologous growth factors, but these growth factors are soluble and easily diminished from the site where it is applied before showing its therapeutic effects. In this research, gelatin hydrogel films were used to maintain autologous growth factors released by PRP on crush injury area by absorbing early secreted platelet growth factors and releasing it in controlled manner. It was reported that platelets are able to secrete 70% of their stored growth factors within 10 minutes after activation [10]. It was also reported that platelets have an ability to produce additional amounts of growth factors for about 8 days until they are depleted and die [10]. Thus, the application of gelatin hydrogel film was expected to increase growth factor concentration during sciatic nerve regeneration.

Since PRP is easy to be collected from patient's blood using simple technology, the feasibility of its application is better compared to the application of recombinant growth factors. It is considered that PRP is a rich sources of platelet derived growth factor (PDGF), transforming growth factor beta (TGF- β), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), and b-FGF [10] [11].

In this research double centrifugation method as suggested by Marx was used to prepare plasma containing more than 1,000,000 platelet/ μ l or PRP [10]. Platelet number of PRP in this study was increasing up to about 250% (about 1.5 million platelet/ μ l). The increase of platelet number in this study was lower than the one reported by Messori *et al.* [16]. They found the increase of platelet number up to 390% higher (about 2.6 million platelet/ μ l) than peripheral blood using centrifugation velocity of 160 G and 400 G. Higher centrifugation power used in this study (1000 G and 1600 G) may result into the damage on platelet membrane [16]. However, referring to the study done by Plachokova *et al.* [17], lower centrifugation power (800 rpm or <160 G) to prepare PRP from rat blood did not result higher platelet number (300% higher) than that of the one did by Messori and group (390% higher) [16].

Due to variability of PRP preparation methods, comparing the effect of PRP in this study with other studies can not be precisely performed. On the other hand, studies on PRP application to enhance peripheral nerve regeneration were considered very limited. Furthermore, systematic review on peripheral nerve regeneration treatment using PRP is not available. Plachokova *et al.* [17] suggested that detailed description of the PRP preparation protocol on each study is required to perform comparison between studies, including information about cells separator used, velocity and steps of centrifugation, amount of blood collected before PRP preparation, amount of PRP and its platelet concentration, and coagulation promoters (including name, doses, mixing ratio of PRP). Thus, the PRP preparation method used in this study was considered acceptable method to result 250% higher platelet number.

Reports on PRP safety is very limited but studies showed that no clinical sign of PRP negative effect [17]. Clinical uses of PRP have been reported, such as in graft incorporation of mandibular reconstruction patient, periodontal diseases treatment, cosmetic surgery including face or neck lift and breast modification, chronic ulcer, and in enhancing surgical wound healing. However, treatment-related complication have not been reported yet [17]-[19].

In this study, functional recovery was achieved in all groups, even in the group without treatment (M0P0). The study used 0.20 MPa pressure of non-serrated clamp applied for 60 second, promoted repairable damage on sciatic nerve which is considered as axonotmesis. Axonotmesis is defined as an injury where axons are damaged but nerve sheaths remain intact. Although the pressure applied in the study was considered very high compared to other studies, it was found that the results were in agreement with the previous studies related to the phenomena of axonotmesis, as shown on **Table 8**. Axonotmesis is defined as an injury where axons are damaged but nerve sheaths remain intact. Muir [3] mentioned that functional recovery can be achieved well in axonotmesis without medical intervention. In this study also the approach by application of gelatin hydrogel film only (M1P0)

Table 8. Nerve injury model used in the previous studies.

Authors	Model	Type of Injury	Reference Number
Oliviera <i>et al.</i> (2001)	Non-serrated clamp, 500 g, 1000 g, and 1500 g, 5 mm, 10 min	Axonotmesis	[15]
Shirosaki <i>et al.</i> (2009)	Non-serrated clamp, 54 N, 30 s	Axonotmesis	[2]
Komobuchi <i>et al.</i> (2010)	Micro needle forceps, 1 mm, 15.7 Pa, 10 min	Axonotmesis	[10]

was found to be effective since the gelatin hydrogel film can act as an entubulation to provide proper micro environment for the injured nerve. Meanwhile, in the group of MOP1, localized application of PRP (even without gelatin hydrogel film) may promote faster regeneration. In view of this, proper model of nerve injury is needed to further observe whether a new strategy used in MIP1 model is effective. Crush injury may not be proper model to investigate the effectiveness of a new approach by tissue engineering, and most probably cut nerve model will be an adequate model for future observations.

Although Nociceptive Withdrawal Reflects showed abnormality up to Day 21 in all groups, but it was found that the MIP1 group showed the most noticeable changes along treatment period. Walking Track Analysis resulted into SFI also showed the same phenomena wherein MIP1 clearly showed SFI range close to normal functional recovery, compared to other groups. The result of histological study showed the same phenomena with the functional test results, where MIP1 group showed the highest axonal density among all treatment groups. However, it is considered that longer observation period is needed.

Furthermore, this study confirmed gelatin hydrogel safety when it is used for biomedical purposes as suggested by Yamamoto and Tabata [8]. Clinical trials also showed the success of gelatin hydrogel application in tissue engineering in patients with limb ischemia [20] and Sudden Sensorineural Hearing Loss [21]. Based on the study by Wall *et al.* and Zimmerman [22] [23] pain behaviour in animal following nerve injury can be shown as hyperalgesia and allodynia. In this study, hyperalgesia was not observed during the study and allodynia test was not conducted.

5. Conclusion

It was concluded that tissue engineering approach by the application of gelatin hydrogel film combined with PRP resulted faster functional recovery of axonal injury. The axonal density was found highest in the group treated with gelatin hydrogel film combined with PRP. Proper injury model is needed, e.g. with more extreme model such as cut nerve, to further observe whether a new strategy is really effective.

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