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by Dwikora Novembri

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Implantation of platelet rich fibrin and allogenic mesenchymal stem cells facilitate the healing of muscle injury: An experimental study on animalDwikora N. Utomo^{a,*}, Ferdiansyah Mahyudin^a, Kukuh D. Hernugrahanto^a, Heri Suroto^a, Muhammad Zaim Chilmi^a, Fedik Abdul Rantam^b^a Orthopedic and Traumatology Department, Medical Faculty of Universitas Airlangga/Dr. Soetomo Hospital, Surabaya, East Java, Indonesia^b Stem Cell Research and Development Centre, Universitas Airlangga, Surabaya, East Java, Indonesia12
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Introduction: Muscle injury has caused adverse impacts on athletes' performance. Muscle injury treatments are based on the degree of severity. Unfortunately, in extensive injuries, surgical treatments are often unsatisfactory especially in athletes with high functional demand. More effort is needed to achieve a better result in muscle injury healing. The use of platelet-rich fibrin (PRF) and mesenchymal stem cell (MSC) would provide all the necessary factors to achieve good tissue healing: cells, growth factors, and scaffold. The study aims to evaluate the role of PRF and MSC in facilitating the healing of muscle injury on animal models.

Methods: A model defect was created in the gastrocnemius muscle of each hind leg of twenty New Zealand white rabbits. All legs were randomly divided into four groups: (1) control; (2) PRF-only; (3) MSC-only, and (4) PRF-and-MSC group. After two and four weeks, the muscle was retrieved and sent for immunohistochemistry examination to evaluate the expression of *Pax7* and *MyoD* protein.

Results: The mean score of all treated group was higher compared to the control group. The group that received both PRF and MSC showed the highest score.

Conclusion: Considering the promising result, application of PRF and MSC could be an option for the treatment of muscle injury as this would provide all necessary elements of tissue engineering to facilitate the healing process of muscle: the cells, the scaffold, and the growth factors.

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1. Introduction

Muscle injuries are very common in sports whether recreational or professional one. The incidence of muscle injuries in professional sportsmen varies from 23% to 46% [1]. These injuries put on a challenge due to the slow recovery which keeps the athletes away from training and competition. Not only do these injuries cause loss of minute-play to the athletes but also a financial burden to the teams [2].

The healing process of muscle after injuries are well defined. The process begins with initial hematoma formation followed by inflammatory response phase that will activate the subsequent process of satellite cell proliferation and differentiation. This process

ends with remodeling phase. As the cells proliferate and differentiate, several proteins will be expressed. For examples, *Pax7* and *MyoD* will be expressed during satellite cells proliferation and differentiation respectively. Unfortunately, despite the new formation of muscle tissue to replace the injured ones, there is also a high occurrence of fibrosis formation to replace the healthy muscle tissue. These fibrotic scars will eventually alter the original capacity and contractility of the muscle that result in decrease of strength, increase the risk for repeat injuries and limit the ability to return to a baseline or pre-injury level of function, especially in professional athletes who demand high performance [3,4].

Muscle injury treatments are based on the degree of the severity of the injuries. For muscle injuries of lesser severity, non-surgical treatment results in good functional outcomes. For more severe injuries, surgical repair of the injuries is often needed. Unfortunately, in cases with extensive injuries, surgical treatments are often unsatisfactory, especially in athletes with high functional demand [5].

* Corresponding author. Jl. Manyar Tirtosari IV/7, Surabaya, East Java, Indonesia.
E-mail addresses: dwikora-novembri-u@fk.unair.ac.id (D.N. Utomo), ferdiortho@yahoo.com (F. Mahyudin), kukuhdh@gmail.com (K.D. Hernugrahanto), hsuroto@yahoo.com (H. Suroto), chilmiortho@yahoo.co.id (M.Z. Chilmi), fedik.ar@gmail.com (F.A. Rantam).

10 Growth factors play major roles in healing process. Several growth factors (GFs) such as Insulin-like Growth Factor (IGF), Vascular Endothelial Growth Factor (VEGF) and Platelet-derived Growth Factor (PDGF) have been studied in-vitro and in-vivo to enhance the healing process. One of the sources of GFs that has been studied extensively is platelet-rich plasma (PRP). PRP is known to facilitate the healing of many tissues including bone, cartilage, tendon, and ligament [6]. Despite its beneficial effect, PRP has some drawbacks. Firstly, to produce PRP, anticoagulant and bovine-derived thrombin are required. The addition of these foreign agents makes PRP not fully autologous. This might cause an adverse effect of antibody formation in the host that may lead to immunologic response and coagulopathy [7]. Secondly, the nature of PRP is in liquid form. When injected in muscle defect, PRP might spread away from the defect site and become ineffective. Therefore, to be effective, PRP requires the addition of another medium as a carrier to keep the content in-situ at the defect site [8].

To address those issues, platelet-rich fibrin (PRF), a new generation of platelet-derived product is introduced. The production of PRF does not require the use of anticoagulant and bovine-derived thrombin. This will keep the content of PRF purely autologous and therefore eliminate the risk of adverse immunological reaction in the host. In addition, the nature of PRF is gel form. This gel form will give advantages as it will be easier for application in the defect site, thus, preventing the content to spread away. Moreover, this gel form will also act as a scaffold to fill in the defect of the muscle after injuries [9]. The study of PRF on muscle is still limited. However, studies of PRF in other fields have shown a promising result. For example, Giannesini et al. reported the ability of suturable platelet-rich plasma membrane to promote peripheral nerve regeneration after neurotmesis and neurotomy [10]. In bone healing, several studies demonstrated the capability of PRF to enhance bone regeneration [11,12].

Precursor cells are also important in the healing process to provide new cells for regeneration. Bone marrow mesenchymal stem cells (BM-MSCs) are multipotent adult stem cells and have become an important source of cells for tissue repair. Despite its beneficial potential, studies on stem cell therapy for skeletal muscle injuries are still limited [13]. Both GFs and MSCs can stimulate cell proliferation and differentiation of satellite cells to promote healing. In this study, rabbits were used because the gastrocnemius muscles were large enough and histologically similar to human muscle to be used as a model. The purpose of the current study was to evaluate whether the application of PRF alone, BM-MSCs alone and the combination of both would facilitate and promote the healing of skeletal muscle injury in rabbits. As mentioned earlier, proliferation and differentiation of satellite cells play a very important role in the healing process. Therefore, to evaluate the activity of satellite cells, immunohistochemistry evaluation of protein Pax7 and MyoD expression were used to examine the outcomes.

2. Methods

2.1. Study design

A controlled animal laboratory study was performed. Using the formula for calculating experimental samples, twenty male New Zealand white rabbits (*Oryctolagus cuniculus*), weighted 2000 g ± 100 g, were used in this study [14]. This study is fully compliant with ARRIVE criteria [15]. The study protocol was approved by the Animal Care and Use Committee, Airlangga University, Indonesia (certificate number: 682-KE). All rabbits were housed in the animal care laboratory and were well-taken care according to the standards of the National Institute of Health. The

rabbits were housed individually in a separate cage (100 × 60 × 75 cm) with environmental conditions: temperature of 21 °C ± 2 °C, humidity of 60% ± 10%, lighting of 350 lux with a dark:light cycle of 12:12. All rabbits were given access to regular and scheduled feeding and water *ad libitum*. All rabbits were randomly grouped into four groups: (1) control group; (2) PRF-only group; (3) MSC-only group, and (4) PRF-and-MSC group. Each group consisted of 5 samples of gastrocnemius muscles. In each rabbit, a model injury was created in both hind legs. During housing, animals were monitored three times daily for health status. No adverse events were observed.

2.2. Platelet-rich fibrin preparation

Five milliliters of venous blood was withdrawn from rabbits' ears into a sterile vacuum tube without the addition of anticoagulant. The tubes were centrifuged at speed of 2700 rotation per minute for 12 min [16]. After the process, three layers of separate content are formed (Fig. 1a). The bottom layer consisted of red blood cells, the top layer was formed by cellular plasma, and the middle layer was the fibrin content. The top layer was removed and then the middle layer was extracted until 2 mm below the separating line between the middle and the bottom layer (Fig. 1b).

2.3. Isolation, culture, and implantation of bone-marrow stem cells

Whole bone marrow was harvested from the pelvic bone of other rabbits. The marrow was then mixed and coated with Ficollophosphate-buffered saline (Ficoll-PBS) 0.077 density (Takara Bio) before centrifuged at 1600 rpm for 15 min. Using a Pasteur pipette, the "buffy coat" located on Ficoll-PBS was collected. Then, the retrieved cells were placed on 5 cm² plates and put in incubation at 37 °C with a humidity of 5% CO₂ for 24 h. The plates were examined daily under a microscope. Every three days the cells were washed with 10 mL PBS and 10 mL complete culture medium (CCM) was added until the cells became 60%–80% confluent. In the fourth passage of MSC, the phenotype was confirmed by immunocytochemistry: 89.1% of the cells showed positive expression of CD105 and were negative for hematopoietic surface markers of CD45. For the application, 2 × 10⁶ BM-MSCs were used. In MSC-only group, the stem cell solution was injected into the injury site. In PRF-and-MSC group, the stem cell solution was injected and mixed into the PRF before being implanted to the injury site. A staining of PKH-26 Fluorescent Cell Linker Kits (Sigma-Aldrich) was used to mark and trace BM-MSCs in the injury site. The presence of stained BM-MSCs was confirmed by identifying the fluorescence under the microscope (Fig. 2).

2.4. Surgical procedure and sample preparation

The animals were anesthetized with ketamine (40 mg/kg) and xylazine (5 mg/kg) intramuscularly and placed prone on a warm pad. Both hind legs were disinfected and draped in an aseptic manner. Skin incision (3 cm) was made on the posterior side of the hind leg, on the muscle belly area of the gastrocnemius muscle. About 1 cm proximal to the *tendomusculo* junction of the gastrocnemius, muscle belly defect was created with the size of 0.5 cm × 1 cm and depth of 0.5 cm (Fig. 3a). Each model defect was marked by placing non-absorbable sutures at 0.5 cm proximally and distally to the defect site in order to identify the site at the time of sample retrieval. In control group, nothing was implanted at the defect site. In three experimental groups, PRF, BM-MSCs and the combination of PRF and BM-MSCs were implanted respectively at the defect site (Fig. 3b). All groups were observed at two different times: 2 and 4 weeks. The reason is that the inflammation and

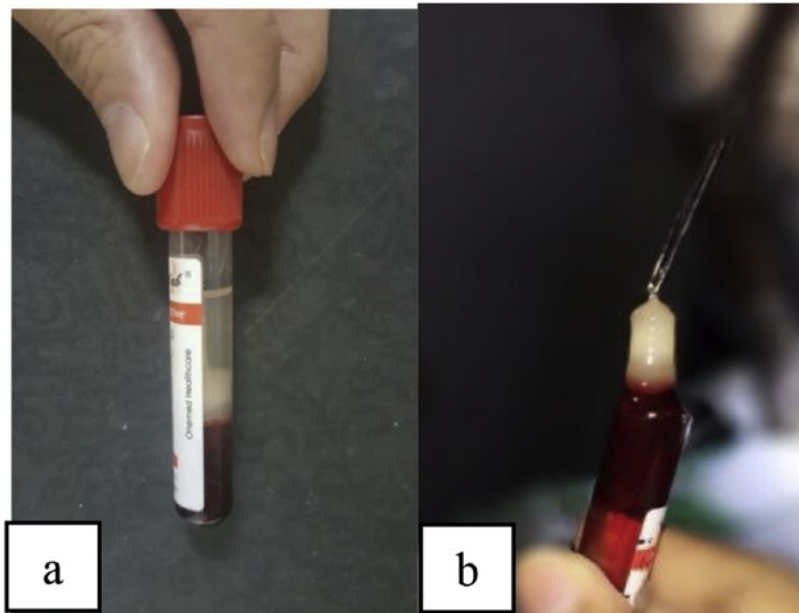


Fig. 1. (a) Three separate layers will be formed. The fibrin content was in the middle layer. (b) The middle layer was extracted after removing the top layer.

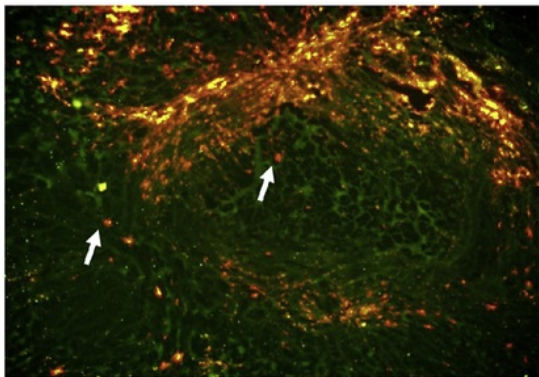


Fig. 2. PKH-26-labelled BM-MSCs in PRF examined under fluorescence microscope showed positive staining of dark-orange color (arrow).

proliferation phase in muscle healing of rabbits are expected to be completed after two and four weeks respectively. After the designated time, the samples were collected from the hind legs and sent for immunohistochemistry examination (see Fig. 4).

2.5. Immunohistochemistry evaluation

Each sample was evaluated in a blinded manner from two different observers. Immunostaining was performed using Pax7 and MyoD monoclonal rabbit antibody (Rockland Immunochemicals). The expression of Pax7 and MyoD was examined by using a light microscope (Nikon H600L with digital camera DS Fi2 200 mp and Nikon Image System software). Satellite cell with positive expression of either Pax7 or MyoD produced brown-colored cytosol [17,18].

The evaluation of the expression was carried out according to the immunoreactive score (IRS) by Remmele and Stegner. The IRS

evaluation was based on a modification that evaluated not only the visualized grade of color intensity (staining) but also adding the fraction of cells in each intensity category. The score was obtained from multiplication between the percentage of positive cells and color intensity (see Table 1) [19].

2.6. Statistical analysis

The data collected were analyzed using the Shapiro-Wilk test which verified the normal distribution of the data sets. After that, comparison among groups was performed using ANOVA test. All significant result from ANOVA ($p < 0.05$) was analyzed further using Tukey posthoc test for comparison between groups. All analyses were performed using the statistical software package SPSS version 21. An alpha value of 5% was considered significant, all data are presented as mean \pm SD with 95% confidence intervals.

3. Results

The normal distribution of the data was verified using Shapiro-Wilk test. The result of the study showed that in both expressions of Pax7 and MyoD, either in two or four weeks, the mean scores of the PRF-only group, MSC-only group, and PRF-and-MSC group were significantly higher than the control group (Table 2). This result was also further confirmed using Tukey posthoc test in Pax7 and MyoD expression between control group vs PRF ($p = 0.036$ and 0.009), control vs MSC ($p = 0.046$ and 0.028), and control vs PRF-and-MSC group ($p = 0.009$ and 0.009).

Among the experimental groups, after two and four weeks, the PRF-and-MSC group showed the highest mean score in both Pax7 ($p = 0.009$ and 0.017 respectively) and MyoD expression ($p = 0.005$ and 0.022 respectively). Interestingly, between PRF-only and MSC-only groups, there was difference result between 2 and 4 weeks. In 2 weeks, PRF-only group scored higher both in Pax7 and MyoD expression compared to the MSC-only group (6.69 ± 0.38 vs 6.64 ± 0.56 , $p = 0.009$ and 8.01 ± 0.82 vs 7.89 ± 0.95 , $p = 0.005$

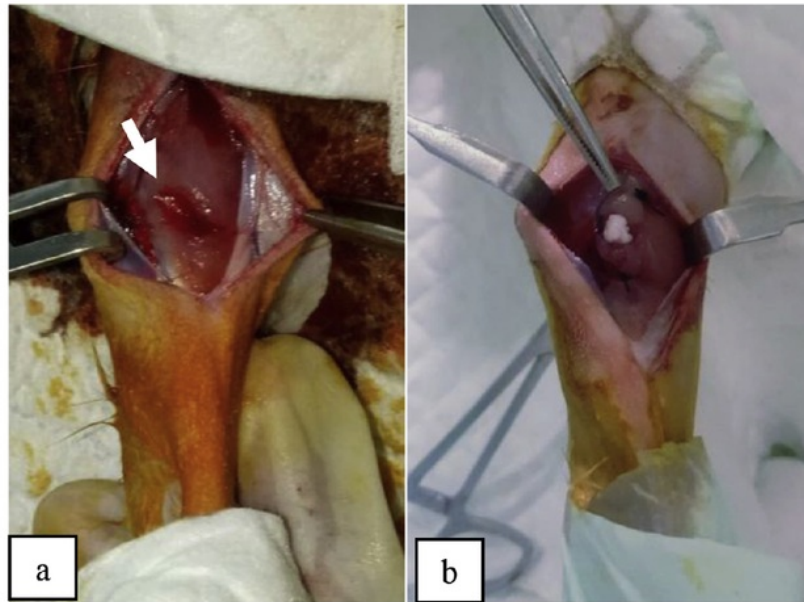


Fig. 3. (a) Effect was created at the muscle belly of gastrocnemius muscle (arrow), (b) PRF was implanted at the defect.

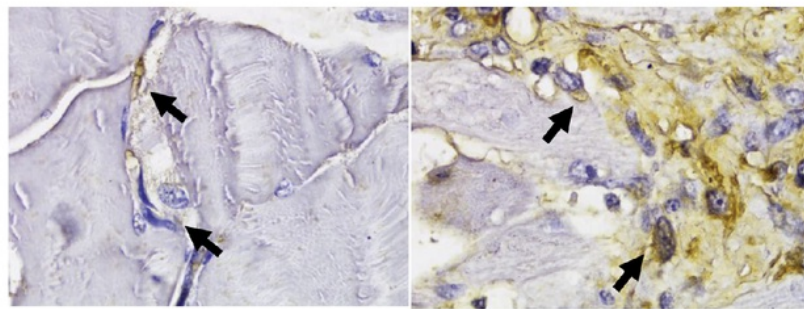


Fig. 4. Positive expression of Pax7 & (left) and MyoD (right) shown by brown-colored cytosol of satellite cell (arrow).

Table 1
Semi-quantitative immunoreactive score (IRS) is the result of multiplication between percentage of positive cells (A) and color intensity (B).

A	B
Score 0: No positive cell	Score 0: No color observed
Score 1: Positive cells <10%	Score 1: Low color intensity
Score 2: Positive cells 11%–50%	Score 2: Intermediate color intensity
Score 3: Positive cells 51%–80%	Score 3: Strong color intensity
Score 4: Positive cells > 80%	

Table 2
Expression of Pax7 and MyoD after two and four weeks.

		Control (n = 5)	PRF (n = 5)	MSC (n = 5)	PRF + MSC (n = 5)	p
Pax7	2 week	5.6 ± 0.48	6.69 ± 0.38	6.64 ± 0.56	6.94 ± 0.77	0.009
	4 week	7.62 ± 0.94	8.97 ± 0.61	8.99 ± 0.51	9.39 ± 1.02	0.017
MyoD	2 week	6.07 ± 0.89	8.01 ± 0.82	7.89 ± 0.95	8.21 ± 0.97	0.005
	4 week	9.07 ± 0.26	9.79 ± 0.71	9.83 ± 0.41	10.13 ± 0.42	0.022

respectively). In 4 weeks, the MSC-only group showed the other way around compared to the PRF-only group (8.99 ± 0.51 vs 8.97 ± 0.61 , $p = 0.017$ and 9.83 ± 0.41 vs 9.79 ± 0.71 , $p = 0.022$).

4. Discussions

Growth factors (GFs) and mesenchymal stem cells (MSC) played important role in muscle healing in this study. The result showed that the addition of PRF as a source of GFs or/and allogenic MSCs showed better score compared to the control group which meant better satellite cells proliferation and differentiation. This result was supported by the previous articles by Hamid and Wong which also stated the beneficial role of GFs in tissue healing [1,20]. Moreover, another study by Wright using PRP and Gigantic using PRF matrix also showed that adequate source of GFs would facilitate the healing process of muscle injury [21,22].

Apart from the GFs content of PRF, PRF also gave beneficial role as a scaffold. The previous study by Mira Sumarta et al. showed that PRF is composed by natural fibrin matrix which polymerizes to

form a smooth and flexible fibrin network that can be used as a scaffold to support cytokines and cell migration [23]. The nature of PRF that resembled gel made PRF capable to fill in the defect as well as to retain the content from spreading away. This importance of scaffold in muscle defect was supported by the previous article by Longo which stated that the presence of scaffold in the injured site would facilitate the emerging of bridging myotubules to fill the defect of the injured site [24].

The highest result was given by the combination of PRF and MSC. As we know, this combination of PRF and MSC would give all requirement to complete the triad of tissue engineering: cells, growth factors, and scaffold. Therefore, it was expected that this group would give highest score expression compared to other groups.

In week 2, the score of the PRF-only group was higher than MSC-only group. The reverse was shown in week 4 whereby MSC-only group showed the higher score. This might happen because of the difference of the nature of PRF and MSC. In the natural cascade of muscle healing, hemostasis was followed by inflammation, proliferation, and remodeling. Immediately after an injury occurs, hematoma formation initiated the release of inflammatory mediators. This would cause platelet aggregation, clot formation, and release of bioactive factors alpha-granules. This bioactive factors also include growth factors, chemokines, cytokines, and other proinflammatory mediators. The presence of PRF would further accelerate this initial phase of healing so that more bioactive factors, growth factors, and pro-inflammatory mediators are released and thus activating satellite cells. Therefore, this might explain the greater increase of satellite cells expression in the early week after application of PRF compared to MSC [25,26]. In week 4, the proliferation phase dominates the healing process. This phase is driven mainly by the activity of the proliferating and differentiation of satellite cells to produce myoblast and matrix to form new muscle tissue. The proliferation and differentiation of cells take time. Therefore, in the MSC-only group, higher expression was seen only in later time compared to the PRF-only group [27,28].

The promising result of this study could be used as a reference and foundation to perform a further study on muscle injury treatment, especially regarding the application of PRF as a new form of a platelet-derived concentrate in muscle healing in human. This study evaluated only the expression of Pax7 and MyoD. To further investigate the ability of PRF and MSC in facilitating better muscle healing, future study should evaluate the end-result of the treated muscle after the remodeling phase, such as comparing between the myoblast and fibroblast formation or conducting a biomechanical study. To come to final conclusion stating that PRF and MSC should be the treatment of choice, the author suggests that further studies to compare PRF, MSC and other modalities of treatment should be conducted.

5. Conclusions

Considering the promising result, application of PRF and MSC could be an option for the treatment of muscle injury as this would provide all necessary elements of tissue engineering to facilitate the healing process of muscle: the cells, the scaffold, and the growth factors.

Ethical approval

Animal ethic approval:
Animal Care and Use Committee (ACUC), Airlangga University, Surabaya, Indonesia.
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Author contribution

1. Dwikora N. Utomo, MD, Ph. D: study concept and design, data collection, data analysis, writing the paper.
2. Ferdiansyah Mahyudin, MD, Ph. D: study concept and design, data analysis, writing the paper.
3. Kuku D. Hernugrahanto, MD: study concept and design, data collection, data analysis, writing the paper.
4. Heri Suroto, MD, Ph. D: data analysis, data collection.
5. Muhammad Zaim Chilmi, MD: data analysis, data collection.
6. Fedik Abdul Rantam, Vet, Ph. D: data analysis, data collection.

Conflict of interest

The authors declared no conflicts of interest with other people or organisations.

Guarantor

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