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Research Paper

Implantation of platelet rich fibrin and allogenic mesenchymal stem cells facilitate the healing of muscle injury: An experimental study on animal

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ABSTRACT

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.

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].

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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, Giannesi et al. reported the ability of suturable platelet-rich plasma membrane to promote peripheral nerve regeneration after neurotmesis and neurorrhaphy [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 Ficoll-phosphate-buffered saline (Ficoll-PBS) 0.077 density (Takara Bio) before centrifugated 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 cm^2 plates and put in incubation at 37 °C with a humidity of 5% CO_2 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, 2×10^6 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