

The Wound Healing Effect of Allogeneic Freeze-Dried Platelet-Rich Plasma in a Full-Thickness Wound Animal Model

Abstract

Background: Epithelialization is an indicator of wound healing. Platelet-rich plasma (PRP) may accelerate the epithelialization of the wound due to high amount of growth factors. Generally, allogeneic PRP provides a smaller immunological effect than autologous PRP. By a freeze-drying method, it is assumed that allogeneic PRP has lower allergenic activity. **Aims and Objectives:** The aim of this study was to investigate the effect of allogeneic freeze-dried PRP on wound healing of a full-thickness wound in New Zealand rabbits. About 2×2 cm² full-thickness wounds were created on rabbits using a template on both sides of the dorsum and divided into treatment group and control group. The treatment group was treated with allogeneic freeze-dried PRP and the control group was treated with moist dressing. Acceleration of wound healing was shown by the epithelialization and measured on day 7 using digital Visitrak. Nine New Zealand rabbits were used in this study. **Results:** In the treatment group, the epithelialization was significantly higher 3.00 ± 0.96 cm² than the control group 1.35 ± 0.85 cm² ($P < 0.000$) by independent t test. Another finding of our study was the allergic reaction was not observed after the administration of allogeneic freeze-dried PRP in rabbits. **Conclusion:** Our results indicate that allogeneic freeze-dried PRP accelerates epithelialization compared to the control group and does not cause an allergic reaction in full-thickness wounds in rabbits. The effect of allogeneic PRP, prepared with a freeze-drying method, on the process of wound healing is reported for the first time in this article.

Keywords: *Allogeneic, epithelialization, freeze-dried PRP, full-thickness wound*

Introduction

The role of platelets in blood coagulation has been known for a long time. In addition, platelets are also a source of various growth factors that play an important role in the process of wound healing, acute tissue response to trauma, and involved in several cellular physiological processes, such as growth, differentiation, and cell replication.^[1] Platelet-rich plasma (PRP) is a plasma fraction with platelet concentrations three to five times above normal value than in whole blood.^[2] In dermatology, PRP has been used for wound healing on the skin surface and soft tissue. An *in vitro* study states that there is a relationship between platelet concentration and mesenchymal stem cell (MSC) proliferation, fibroblast proliferation, and collagen type I production. It supports the role of PRP to accelerate wound healing.^[3]

Wound healing is a complex process involving various cells and biochemical mediators, one of them is platelets. In response to damaged tissue, activated platelets will form platelet clots and

secrete a variety of growth factors. They play an important role at various stages of wound healing (inflammatory until the remodeling phase).^[4] PRP is a blood product derived from whole blood containing a high concentration of thrombocytes with anti-inflammatory and pro-regenerative properties to repair wound healing more efficiently.^[5] PRP is an agonist of growth factor that has mitogenic and chemotactic properties, such as blood clots and growth factors. In the process of wound healing, growth factors such as platelet-derived angiogenesis factor (PDGF) and total growth factor (TGF) will encourage fibroblasts to proliferate, migrate, and increase the formation of the extracellular matrix and stimulate endothelial cells to form new blood vessels.^[3] Most of the studies reported the beneficial effects of autologous PRP in wound healing. Unfortunately, it is not applicable in patients with a hematological disease or who use antiplatelet drugs, such as aspirin, and older with many underlying or chronic diseases as well as patients refused to draw their blood for any reason. It causes inconsistent amount of growth factors in autologous PRP and leads to the variation of wound healing process.^[6]

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Another limitation of autologous PRP is the need for at least -20°C to preserve the product's characteristics. A study by Gandhi *et al.*^[7] stated that the administration of PRP percutaneously accelerated fracture healing in the diabetic rat model. However, autologous PRP may not be appropriate for that case, because the level of PDGF was lower in diabetic animals compared to the non-diabetic animals model.^[8] In addition, autologous PRP requires additional equipment, time-consuming, and needs a large amount of blood.^[9] Consequently, autologous PRP is difficult to be used as commercial products. Therefore, an alternative to autologous PRP may be required, and allogeneic PRP is the best option to accelerate wound healing.

Allogeneic PRP can be well preserved by a freeze-drying method. The advantage of the freeze-drying method is a drying system that does not use high temperature, so it is suitable for heat-labile substances. In addition, the freeze-dried allogeneic PRP creates a more stable and more consistent product, and it can be stored at room temperature. It may be assumed that allogeneic PRP has the same effect on cell function as autologous PRP and hence can be used as an alternative source of PRP. Therefore, the purpose of this study was to evaluate the effect of allogeneic freeze-dried PRP on the process of wound healing (epithelialization) of full-thickness wounds in rabbits as an experimental animal.

Materials and Methods

Animals

This study was an animal experimental design. This study was approved by the Ethics Committee of Dr. Soetomo Hospital, Surabaya with registration number 531/Panke.KKE/IX/2016. Nine New Zealand male white rabbits were used in this study with the inclusion criteria: aged 3–4 months and weight 3.0–4.0 kg. The animals were housed in individual cages in the Institute of Tropical Disease, Airlangga University under standard laboratory conditions (temperature $20\text{--}22^{\circ}\text{C}$, natural day/night cycle) and they had free access to commercial chow food and water. The animals were acclimatized for approaching

and handling for a period of 15–20 days before the start of the study.

Wound excision

The animals were anesthetized by injecting ketamine (1 mg/kg) and xylazine. Ketamine provides analgesic effects whereas xylazine causes good muscular relaxation. The use of the combination of ketamine–xylazine as general anesthesia also has many advantages, including easy administration, economical, fast induction as well as its recovery, has a good relaxation effect, and rarely causes clinical complications.^[10]

The dorsal surface of each animal was shaved and scrubbed for aseptic surgery using 0.1% chlorhexidine solution three times. The site was painted with 5% povidone-iodine before surgery. A rectangular, full-thickness excisional skin wounds measuring $2 \times 2 \text{ cm}^2$ were created on the left side and on the right side of the dorsum, about 2 cm away from the midline in each animal, using a sharp surgical knife.

Full-thickness wounds are wounds that extend into the two layers of skin (dermis and epidermis) and further into the subcutaneous tissue fat, not muscle. The full thickness wound was compressed with adrenalin in normal saline solution (NaCl 0.95%) with a concentration ratio 1:200,000 U for 2 min if any bleeding occurs. A gap of 2 cm was maintained between the two wounds on each side as shown in Figure 1B. Thus, a total of two wounds were created for each animal. The two wounds in each animal were designated as control and treatment groups. The right wounds were treated by topical application of moist wound dressing (control) and the left wounds were treated with freeze-dried PRP, as shown in Figure 1C. Both wounds were covered with a transparent dressing and wrapped with elastomull. All the topical agents were applied to the wounds of their respective groups using sterile gauze pieces from day 0 until 14 and wound healing was complete.

Preparation of allogeneic freeze-dried PRP

Allogeneic freeze-dried PRP was prepared in the tissue bank of the Dr. Soetomo Hospital, Surabaya. PRP was prepared from

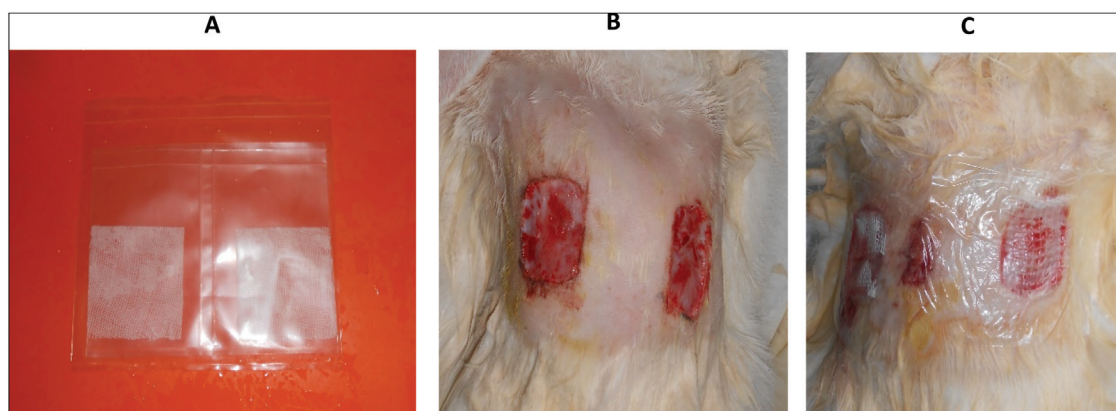


Figure 1: (A) Allogeneic freeze-dried PRP, (B) full-thickness wounds, and (C) wound treated with allogeneic freeze-dried PRP (left) and with moist dressing (right)

the blood of five healthy rabbits. Whole blood was drawn as much as 10 mL by 18 gauge needle into 15 mL falcon tubes containing the anticoagulant heparin. Blood was homogenized and centrifuged for 10 min at a speed of 4000 rpm. The centrifugation will result in the formation of two layers. The top layer is the plasma and the bottom is red blood cells. Plasma was taken and recentrifuged for 10 min at a speed of 4000 rpm. The second centrifugation will produce PRP, accumulated at the bottom of the tube. The PRP was placed on the sterile gauze and stored at -83°C for 24 h and then was lyophilized by freeze-dryer for 12 h. The freeze-dried PRP was placed on a sterile gauze and sterilized by ultraviolet light. As a result of the process of freezing and drying, allogenic freeze-dried PRP was prepared as shown in Figure 1A.

Assessment of epithelialization of full-thickness wounds

The next stage of this study was to assess the effect of freeze-dried allogeneic PRP on the acceleration of epithelialization of full-thickness wounds in rabbits. The measurement of epithelialization was carried out on day 7 by digital Visitrak as shown in Figure 2.



Figure 2: Epithelialization measurement by digital Visitrak

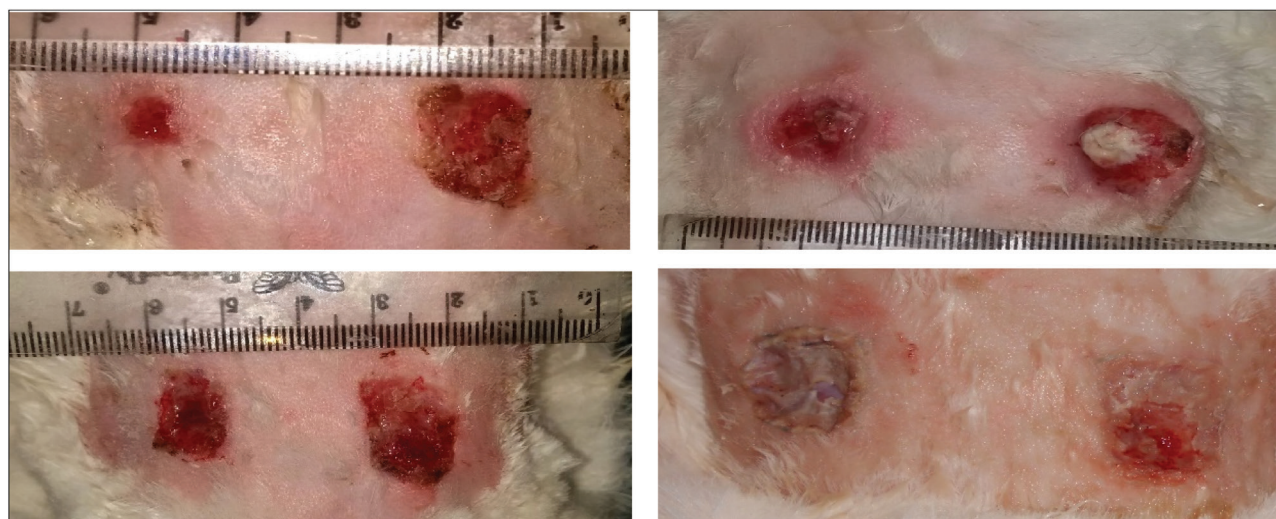


Figure 3: Observation of clinical wound on the seventh day: the left side is the group treated with allogeneic freeze-dried PRP and the right side is the control group treated with a moist dressing

Statistical analysis

The Kolmogorov–Smirnov test was used to assess the normality of the distribution of the variables. The independent *t*-test was performed to assess the differences between the two groups. The data were reported as mean \pm standard deviation and analyzed by Statistical Package for Social Sciences, version 20.0 (SPSS, Chicago, Illinois). Statistical significance was set at $P < 0.05$.

Results

Observation of the wounds showed that epithelialization occurred both in the treatment and the control groups on the seventh day. The epithelialization was significantly higher in the group treated with allogeneic freeze-dried PRP than that of treated with moist dressing as shown in Figure 3. In both treated wounds, we found no signs of infection or allergic reactions. Epithelialization measurement was carried out by digital Visitrak to see how wide the wound is covered by the epithelium. The data were normally distributed by Kolmogorov–Smirnov ($P = 0.551$) and then analyzed by independent *t* test between variables of the two groups. There was significant differences in the epithelialization between the treatment group and the control group on the seventh day. Epithelialization in the treatment group was significantly higher ($3.00 \pm 0.69 \text{ cm}^2$) than the control group ($1.35 \pm 0.85 \text{ cm}^2$; $P < 0.000$) as shown in Figure 4.

The wounds were left open, and the animals were housed in individual cages. Wound healing was monitored by taking digital photographs and blindly measuring the wound area by tracing the wound perimeter with a thin-tipped marker onto sterile Visitrak Grid. Tracings were then placed onto the wound measurement device and copied with an electronic stylus to obtain a digital reading of the wound area.

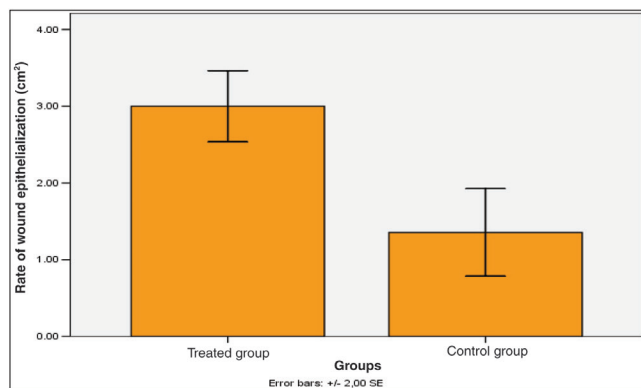


Figure 4: Mean (\pm SD) of the epithelialization in both groups on the seventh day ($P = 0.000$)

Discussion

The objective of this study was to determine the effect of freeze-dried allogeneic PRP on wound healing. From this study, it was found that on the seventh day, the wound healing process was higher in the treatment group compared to the control group, based on how wide the epithelium covered the wound. PRP functions as a tissue sealant and drug delivery system, to initiate wound repair by releasing locally acting growth factors via the degranulation of α -granules.^[11] A study by Marx^[12] showed that MSCs, fibroblasts, endothelial cells, osteoblasts, and epidermal cells express cell membrane receptors for growth factors in PRP. Transmembrane receptors induce activation of endogenous internal signaling proteins, causing expression of normal gene sequences in cells, for example, matrix formation, collagen synthesis, and cell proliferation. Growth factors in PRP do not enter the cell or its nucleus and hence PRP is not mutagenic and stimulates wound healing faster.

PRP suppresses the release of cytokines and limits inflammation, interacts with macrophages to improve tissue healing and regeneration, triggers new capillary growth, and accelerates the growth of new capillaries and epithelialization in chronic wounds.^[13] Thrombocytes contained in PRP play a role in the host defense mechanism in the injured area by producing signaling proteins that attract macrophages. Previous studies showed that PRP has antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, including methicillin-resistant *S. aureus*, *Candida albicans*, and *Cryptococcus neoformans*.^[11] After the initial release of growth factors contained in PRP, platelets will synthesize and secrete other growth factors during 7 days of life.

Because most individuals have a baseline platelet count of 200,000 per microliter, the average platelet count of more than 1,000,000 per microliter measured in 6 mL of total plasma can be a benchmark for “therapeutic” PRPs.^[14] Fibroblast proliferation and type I collagen production increased with increasing platelet levels and most of these responses was pH-dependent and the best response occurred at a more acidic pH.^[13] It was supposed that the concentration of platelet count in PRP was several folds higher than in whole blood. A study by

Jo *et al.*^[15] reported that the average platelet count in allogeneic PRP was significantly increased compared to whole blood, from 256.71 ($\times 10^3$) to 918.14, also the level of red and white blood cells was significantly low in allogeneic PRP. In clinical outcomes including pain, range of motion, muscle strength, and functional scores, there were no significant differences after a 2-year follow-up between autologous and allogeneic PRP groups in patients who underwent rotator cuff repair. Another study by Zhang *et al.*^[16] also reported that the platelet concentrations, the level of TGF- β 1, PDGF, epidermal growth factor, and vascular endothelial growth factor are significantly much higher than whole blood.

The study by Liao *et al.*^[17] showed that the platelet count in PRP was significantly four to seven times higher than in whole blood ($P < 0.05$) and the healing rate of a chronic wound after 3 weeks of the administration of allogeneic PRP was more than 85% compared to the control group, which is less than 55%. No rejection or adverse reaction was observed in this study. A meta-analysis study reported that the administration of PRP in patients with rotator cuff repair improved healing rates, reduced pain levels, and increased functional outcomes compared to platelet-rich fibrin.^[18] The study by Garbin *et al.*^[19] showed that the allogeneic freeze-dried plasma lysate has similar effects to frozen autologous plasma lysate.

A previous study reported that the level of inflammatory markers was lower (IL-1 β and TNF- α) in PRP injection in patients with osteoarthritic knees *in vivo* by ELISA technique.^[20] A comparative study measuring the level of TGF- β in PRP before and after the freeze-drying method showed that the highest level of TGF- β in PRP before freeze-drying is 232 pg/mL, while the highest level of TGF- β in PRP after freeze-drying was 192 pg/mL. The level of TGF- β before and after freeze-drying was not statistically significant ($P = 0.081$). It indicated that the freeze-drying method did not disturb the level of growth factors in PRP.^[21]

Our finding was similar to a meta-analysis study which reported that the application of PRP has positive effects on full-thickness skin wounds in animals compared to the control or placebo groups. PRP reduces the size of the open wound area significantly and generally effective to stimulate the healing process. The concentration of platelets more than 1,106/ μ L in PRP products seems to be more effective than those with lower concentration.^[22] In particular, the pretreatment of skin biopsies showed cellular debris and infiltrate of inflammatory cells. After administration of the combination of PRP and hyaluronic acid, reactive epidermal proliferation and newly formed dermal tissue occurred as well as deposition of collagen and newly formed vessels. The combination of PRP and hyaluronic acid caused significantly complete re-epithelialization (96.8%) compared to the control group (87.8%) within 80 days.^[23]

The properties of wound healing in PRP were also proposed due to the high affinity of vascularization. A previous study reported that the combination of PRP and MSC induced higher vascularization of the wounds than the administration of MSC

or PRP alone. It is because PRP stimulated the expression of transcription of proangiogenic factors in engrafted MSC. These findings suggest that PRP could stimulate the proangiogenic potential of engrafted MSC through activation of their secretome.^[24]

A study by Rajabi *et al.*^[25] showed that as a result of the administration of PRP injection together with aquatic activity in Achilles tendon injury in rats, there was a significant increase in the number of fibroblasts and cellular density and also there is an increase in collagen deposition compared to without the administration of PRP. A study by Semenic *et al.*^[26] showed that the healing of chronic lower leg ulcers with allogeneic platelet gel was significantly more effective compared to the treatment with hydrogel. In allogeneic PRP, the value of laboratory blood and the inflammatory parameters is within the normal limits and did not significantly differ before and after formulated in gel preparation.

In this study, we also found no allergic reactions due to the use of allogeneic PRP in rabbits. This is because the freeze-drying method was able to damage the antigen molecules in platelet membranes without damaging the existing content in the platelet cells.^[27] The freeze-drying is a cryopreservation method which consists of three stages: freezing of tissues (freezing) in the temperature of -83°C , primary drying with the sublimation of the frozen tissue (primary drying), and secondary drying to remove the remnants of the water contained in the heating technique (secondary drying).^[28] The freezing process to -83°C is expected to damage the structure of the molecules of antigen contained in the membrane surface of the platelet.^[27]

The low immunogenicity of allogeneic PRP was explained by the fact that allogeneic PRP was designed in the form of a gel, so it did not enter the circulation system and encapsulated the human leukocyte antigen (HLA) inside the gel and thereby decreases the antibody and lymphocyte response. The main function of HLA molecules is to recognize foreign proteins from pathogenic germs (called peptides) that enter the body. Immune reactions occur when there is a reaction between the two molecules. When this interaction occurs, a protein complex will be brought to the cell surface to be recognized by T cells, resulting in the emergence of an immune response. Furthermore, the activation of platelets in the final step of the allogenic PRP preparation may change the structure and the level of expression of surface immune antigens and thereby lowering their immunogenicity. Moreover, PRP was completely degraded and absorbed within a few weeks and finally reduces the possibility of over-responsive immunity.^[29] Therefore, due to the low immunogenicity, allogeneic PRP can be applied for clinical settings.

There is no adverse event related to immune responses as well as erythema and swelling at the wound site after the administration of allogeneic PRP.^[15] Furthermore, the application of PRP injection intramuscularly only induce a mild immune response, characterized by little change in CD4⁺ and CD8⁺ cell subpopulations even statistically nonsignificant. Besides that, allogeneic PRP did not show fibrous tissue and the infiltration of IL-2 as an inflammatory mediator was spotted at week 1.^[16]

A study by Rachmawati *et al.*^[21] reported that the administration of allogeneic freeze-dried PRP did not induce inflammatory responses as well as humoral immune response as primary antibodies. There were no differences in body temperature and level of IgM after the administration of allogeneic freeze-dried PRP and autologous PRP intramuscularly in rabbits. A case report by Latalski *et al.*^[30] showed that after the administration of injection of autologous PRP, within the first 24 h, the skin rash appeared with the elevated total IgE serum level 171 kU/L (normal: 22–85). This report indicated that the safety of the use of autologous PRP is not absolutely without risks. The pure autologous PRP is generally safe, but the preparation for its use can significantly decrease the safety. The limitation of our study was that the level of platelet concentration of freeze-dried PRP and the collagen density in the proliferation phase and wound maturation phase were not measured. Further study was needed to clarify the effects of allogeneic freeze-dried PRP in other wounds, such as chronic wounds.

Conclusion

The freeze-dried allogeneic PRP significantly accelerates the epithelialization in full-thickness wounds and did not cause any local or general adverse reaction.

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Patient consent statement

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

All authors declare that there is no actual or potential conflict of interest in our study.

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