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Research Article

THE AUGMENTATION OF PLATELET-RICH FIBRIN TO OSTEOCALCIN AND HISTOPATHOLOGICAL SCORE OF BONE HEALING IN BONE DEFECT MODEL RATS SUPPLEMENTED WITH BONE AUTOGRAFT

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ABSTRACT: Platelet-rich fibrin (PRF), is a biomaterial that promotes soft tissue and bone healing. In a long bone defect rat model, the current study compares the effectiveness of bone autograft with PRF augmentation versus a standard bone graft procedure. There are two groups in this study, and the control group is limited to the post-test. There were 7 rats in each control (CG) and intervention group (IG). After the making of the autologous PRF, the intervention group received an autologous bone graft and PRF, as the control group only received a bone graft from the iliac crest. The graft was maintained for 4 weeks. Selected bone was chosen for immunohistochemistry of osteocalcin and histopathological measurement of bone healing. There was a statistically significant increase in osteocalcin expression compared to control in the cell cytoplasmic (p=0.040) and the osteoid matrix (p=0.025). Compared to the control group, there were no statistically significant increases in the nucleus's expression of osteocalcin in the intervention group (p=0.067). The intervention group had a clinically significant better histopathological score of bone healing (p=0.015). This study shows the effect of PRF as a biomaterial that can enhance the new bone formation process in the bone defect. This effect could be a result due to the fibrin mesh of PRF could protect the GF from proteolysis. The application of PRF combined with autologous bone graft could increase the expression of osteocalcin and increase the formation of new bone tissue in rats with a long bone defect model.

Keywords: PRF; Osteocalcin; Immunohistochemistry; Bone defect.

INTRODUCTION

Trauma, congenital defect, infection, or implant failure can cause bone defects that cannot heal as well as healthy bone. According to RISKESDAS (2013), the prevalence of injury to the population of all ages in Indonesia is 8.2 percent, with the highest incidence observed in South Sulawesi (12.8 percent) and the lowest in Jambi (4.5 percent) [1]. Bone graft is defined as a material implanted to help enhance the bone healing process either alone or in combination with other materials through osteogenesis, osteoinduction, and osteoconduction [2]. Rat is commonly used as a model for research on bone regeneration and other related effects [3,4,5]. Platelet-rich Fibrin can be used as a single biomaterial or in conjunction with grafting material to accelerate bone regeneration [6]. In addition to promoting angiogenesis and soft tissue maturation, platelet-rich fibrin also stimulates immunity and epithelialization [7]. This osteogenesis process can be described by osteocalcin as a biochemical marker of osteogenesis that describes the number and activity of osteoblasts. Osteocalcin is a protein-carboxyglutamic

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acid consisting of 46-50 amino acids with a molecular weight of 5.6 kDa, produced mainly by osteoblasts during bone formation [8].

This study tried to prove and analyze the effect of platelet-rich fibrin and autografts in increasing osteogenesis activity as seen from the expression of biochemical markers osteocalcin and histopathology as a description of osteoblast activity in the process of osteogenesis from bone defects.

MATERIALS AND METHODS

Experimental design

This study used an experimental design with a control group which used a post-test-only model. From November 2021 to February 2022, this study was carried out on the Gadjah Mada University medical faculty. In this study, the sample was divided into control and experimental groups with a total of 7 Wistar rats for each group.

Bone defects given bone graft and platelet-rich fibrin (PRF)

In this study, defects were made with a diameter of 2 mm and a depth of 3 mm. The defect was created using a burr accompanied by washing with saline to reduce osteonecrosis. The defect was then filled with autologous cancellous bone taken from the iliac crest and additional PRF according to the division of the experimental animal group. The cancellous bone was removed by separating the incision 1 cm from the top of the iliac crest. The cancellous bone is removed using a curette between the layers of cortical bone. The wound was irrigated with saline, and the skin was closed using 1 absorbable metric suture. All procedures are carried out by people who are experts in their fields.

Platelet-rich fibrin (PRF) is a platelet concentrate obtained from the centrifugation process of venous blood puncture samples without the addition of anticoagulants. Mice were presented with 2 milliliters of blood, which were centrifuged for 10 minutes at 3000 rpm. During the centrifugation process, the phenomenon of hemostasis divides the blood sample into parts, one of which is PRF, a fibrin layer consisting of platelets and plasma [9]. In this study, PRF was administered concurrently with bone graft for the experimental group, and bone graft only for the control group.

Antiperoxidase immunohistochemical analysis was carried out afterward by giving osteocalcin antibody using the streptavidin-biotin immunoperoxidase method. The expression of osteocalcin was observed with a light microscope with a magnification of 400x where a positive value is indicated by brown color. Results were assessed semi-quantitatively using percentages [10,11].

Statistical analysis

Statistical Product and Service Solutions (SPSS) for Microsoft Windows release 25.0 was used to statistically analyze the data, and an unpaired t-test was used for the statistical analysis. In cases where the data are not normally distributed, the Mann-Whitney test is employed.

RESULT AND DISCUSSION

The administration of platelet-rich fibrin had a significant effect on increasing the expression of osteocalcin in the process of new bone formation for long bone defects in rats using the bone autograft method at the cell cytoplasm and osteoid matrix with p values of 0.040 and 0.025, respectively.

As for the microscopic results, there were 5 samples (71.4%) in the control group scored 2, while in the experimental group, there were 4 samples (57.1%) scored 3 for histopathological results. Meanwhile, the statistical test results group obtained a value of p=0.015, which means that the administration of platelet-rich fibrin has a significant effect in increasing the formation of new bone tissue faster in histopathology in long bone defects in rats with bone autograft method (Table 1 and Table 2).

This study aims to determine the effect of platelet-rich fibrin augmentation on the histopathology of new bone formation of rat model with defects in long bone using the autograft bone graft method. The results of this study showed that PRF increased the expression of osteocalcin. This was indicated by clinically increasing osteocalcin reaction in the histopathological cross-section of the treated rat group accompanied by statistically significant results.

Osteocalcin is a protein that constitutes 1% - 2% of bone mineralization in the bone matrix as a significant macromolecule. This bone-specific protein, synthesized by osteoblasts, serves as a specific marker for late-stage osteogenic differentiation. [12]. This work is consistent with a study by To (2019), wherein at 14 days postoperatively, the group receiving PRF with histological examination showed newly formed bone filling the defect to the middle of the medulla. Additionally, after 30 days, the study showed that thick, regularly arranged bone trabeculae formed in porous bone. In the PRF group, there was higher expression of osteopontin and osteocalcin in newly formed bone. PRF more rapidly induces bone formation

Table 1. The effect of administration of platelet-rich fibrin to the expression of osteocalcin in rats with long bone defects by bone autograft method.

Osteocalcin Expression	Group		Z stats	p-value
	Bone graft (n=7)	Bone graft and platelet-rich fibrin (n=7)		
Cell nucleus				
0: Negative	5 (71.4%)	2 (28.6%)		
1: Mild (>25%)	2 (28.6%)	2 (28.6%)	1.875	0.067
2: Moderate (>50%)	0 (0.0%)	3 (42.9%)		
Cell Cytoplasm				
0: Negative	3 (42.9%)	0 (0.0%)		
1: Mild (>25%)	3 (42.9%)	3 (42.9%)	2.054	0.040*
2: Moderate (>50%)	1 (14.3%)	4 (57.1%)		
Osteoid Matrix				
0: Negative	7 (100.0%)	3 (42.9%)		
1: Mild (>25%)	0 (0.0%)	2 (28.6%)	2.248	0.025*
2: Moderate (>50%)	0 (0.0%)	2 (28.6%)		

*In Mann Whitney test, * Significant at p<0.05 or Z>1.9.

Table 2. The effect of administration of platelet-rich fibrin to the histophatology of new bone formation in rats with long bone defects by bone autograft method.

Histophatology	Group		Z stats	p-value
	Bone graft (n=7)	Bone graft and platelet-rich fibrin (n=7)		
Score 1	2 (28.6%)	0 (0.0%)		
Score 2	5 (71.4%)	3 (42.9%)	2,442	0.015
Score 3	0 (0.0%)	4 (57.1%)		
Score 4	0 (0.0%)	0 (0.0%)		

*In Mann Whitney test, * Significant at p<0.05 or Z>1.9.

(Assessment criteria: Score 1 = there are still many bone fragments that have not been connected, connective tissue components are still found, infiltration of inflammatory cells, PMN leukocytes and lymphocyte cells are still abundant. Score 2 = there are still many bone fragments that have not been connected, connective tissue components are still found, infiltration of inflammatory cells, PMN leukocytes and lymphocyte cells are still abundant. However, components of hyaline cartilage began to be found. Score 3 = more bone fragments are connected, lamellar bone structure is found, osteoblastic rimming is abundant, the distribution of inflammatory cells is reduced. Score 4 = bone fragments are connected, connective tissue is much reduced, lamellar structure of bone is dominant, the callus is completely formed) [20].

by increasing osteoblast activity which increases osteocalcin expression [12,13]. This study is also in accordance with research by Nugraha (2017) which showed that an increase in osteocalcin expression occurred on day 21 after treatment with PRF [14].

This study showed that PRF increased the formation of new bone tissue faster histopathologically. This could occur given that PRF is a platelet and immune concentrate that comes in a single fibrin membrane and contains every component of a blood sample needed to promote immunity and healing. Platelet-rich fibrin (PRF) contains TGF-1, PDGF, VEGF, EGF and PRF. TGF-1 is a crucial factor in promoting fibrosis, IGF delays premature apoptosis, VEGF promotes

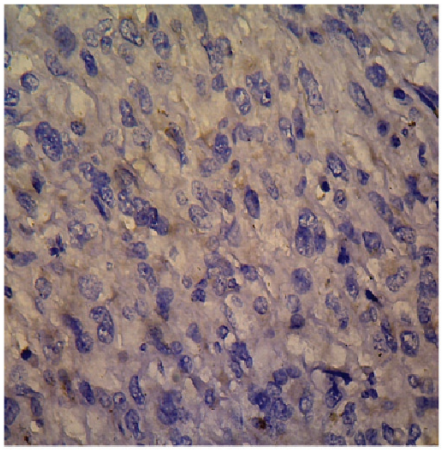

Fig. 1. 400X magnification. A negative control pre-analytical tissue.

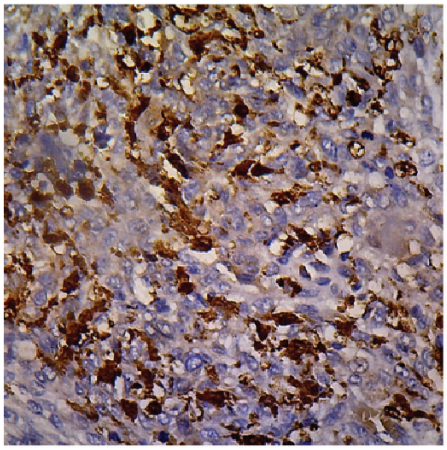

Fig. 2. 400X magnification. A positive control (right) pre-analytical tissue, there were colour change to brown at the extracellular matrix.

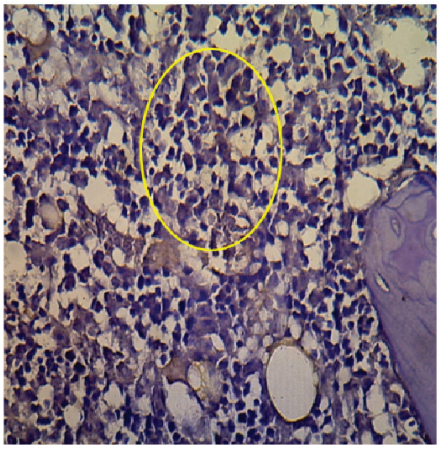

Fig. 3. 400X magnification. A score of 0, scored on the cell nucleus and cytoplasm

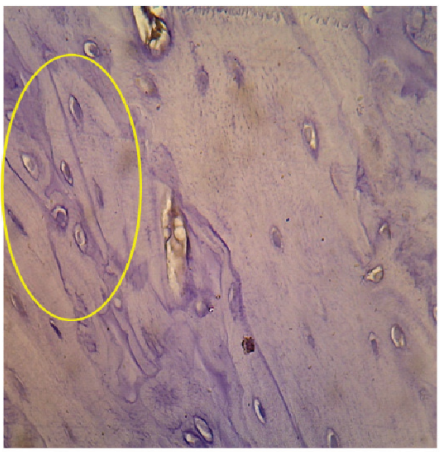

Fig. 4. 400X magnification. A score of 0, the cell nucleus, cytoplasm with osteoid matrix (right).

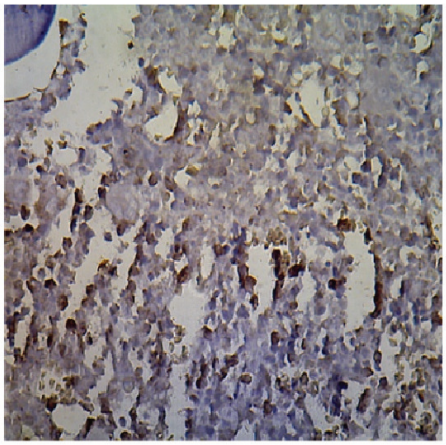

Fig. 5. 400X magnification. Score 1, on the cell nucleus.

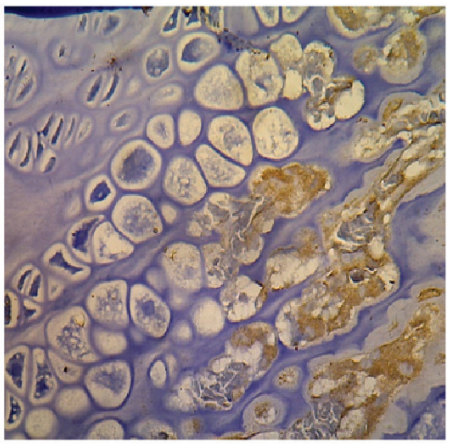

Fig. 6. 400X magnification. Score 1, on the cytoplasm and osteoid matrix.

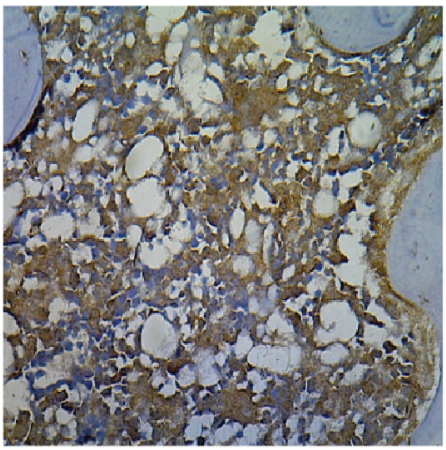

Fig. 7. 400X magnification. Score 2, on the cell nucleus.

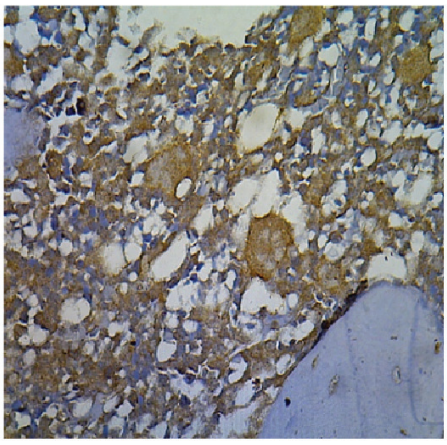

Fig. 8. 400X magnification. Score 2, on the cell cytoplasm.

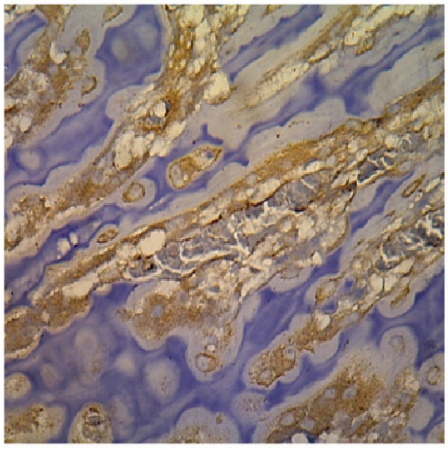

Fig. 9. 400X magnification. Score 2, on the osteoid matrix.

angiogenesis and vasculogenesis, and PDGF aids in progenitor cell migration and survival. During cell division and proliferation, EGF is active. PRF may be useful in promoting the creation of new bone since it can increase the expression of osteoprotegerin and protein kinase in osteoblasts, which in turn promotes osteoblast proliferation (Fig. 1 to Fig. 9). Furthermore, PRF can stimulate mesenchymal stem cells (MSCs) to differentiate into osteoblasts in vitro, including MSCs generated from bone marrow [14].

Bone biomarkers resulting from the bone remodeling process include bone formation biomarkers, bone resorption biomarkers, and bone turnover regulators [15]. Research in recent years has investigated the effect of growth factors on bone healing dynamics [16].

The growth factors within PRF have demonstrated the ability to expedite bone repair and enhance the proliferation of fibroblasts. Moreover, these elements support mesenchymal stem cells, endothelial cells, and osteoblasts during mitosis and enhance tissue vascularity and collagen production. Several studies have demonstrated that the fibrin matrix in PRF provides mesenchymal stem cells with the best possible support, assisting in the repair of damaged bones and other tissues [17,18].

Titirinli (2017) evaluated osteoblast proliferation after the application of PRF. There is an increase in OPG with stimulation by PRF. PRF does not change the RANKL value. Studies have shown that new bone formation is stimulated by p-ERK and OPG, which are activated by the use of PRF. It has been shown that new bone is formed through molecular signals mediated by cytokines and growth factors. With PRF there are a large number of monocytes trapped in fibrin due to a decrease in the rate and duration of centrifugation, which leads to accelerated transformation of monocytes into macrophages, resulting in stimulation of bone tissue [16]

The use of single-stage centrifugation and the lack of bovine thrombin are two important benefits in the manufacture of PRF. PRF offers several benefits, such as: (i) avoiding the use of anticoagulants; (ii) slowing down natural polymerization; (iii) forming a 3D fibrin network that produces a matrix that promotes long-term cytokine retention; (iv) creating an elastic and flexible PRF membrane; and (v) being easy to use and reasonably priced. The limitations of PRF include (1) since PRF is an autologous product, the availability of this biomaterial in greater quantities is a concern. Therefore, its use in surgical procedures should be closely monitored. (2) PRF has circulating immune cells and antigenic molecules that prevent its use as an allogeneic agent. Also, there is an increased risk of transmission of infectious agents [19].

The limitation of this study was only investigating the effect of PRF on the bone defect. Further study could include other interventions related to bone healing like an acute fracture or bone tumour and broader aspects of the variable observed like the clinical outcome and radiological union.

CONCLUSION

Platelet-rich fibrin (PRF), by using the bone autograft technique, can enhance the expression of osteocalcin and the formation of new bone tissue in rats with long bone defects. The next study may build on the findings of this one.

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