The Histomorphometric Analysis of Initial Bone Regeneration With Platelet -Rich- Plasma Post Implantation

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Abstract

The success of implant treatment can't be separated from the implanted biomaterials. Some surface implant modifications have been used to improve the quantity and the quality of osseointegration. This study aimed to analyze the osseointegration with the addtion PRP (Platelet Rich Plasma) at the time of the implant placement. This study used twenty four male rabbits, 4-8 months old, were divided into two groups, control and PRP group. Implant placement of groups on the distal femur of the rabbits. The examination of euthanasia was conducted on 0, 3rd, 7th and 14th days, and the histology preparation was made. The evaluation of Initial bone regeneration was analyzed histomorphologically using Friedman test and the independent t-test of repeated ANOVA. The study results indicated that significant difference was shown on day three (*P*=0.001) by the test group, while the control group did not show any difference. The mean value of *initial bone regeneration*also increased to 20% in the test group compared to the control group after 2 weeks. The Addition of Platelet Rich Plasma in the implant placement can increase the bone regeneration in the implant surface after 2 weeks of the implantation.

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Introduction

The success of a dental implant treatment focuses on a phenomenon called osseointegration which was first introduced by Branemark, the microscopic characteristic of bone formation on the surface of the implant. Characteristics of the surfaces of the implant are one of the factors that can influence the success of osseointegrasi. The quality of the surface of the implant depends on its chemical, physical, mechanical and topographic properties. Several surface implant modifications have been used to improve the quantity and quality of the bone-to-implant interface. Surface composition and roughness are parameters that may play a role in the interaction of implanted tissue and osseointegration.[1,2] Evaluation of Initial bone regeneration and Bone Area (BA) through histomorphometric analysis is the most widely used parameter for measuring osseointegration. The development of the use of dental implants to replace missing teeth coupled with the patient's request to shorten the healing period after implant installation, led researchers to make efforts to improve the quality of biomaterials and to develop dental implant surfaces with macroscopic and microscopic structures to improve osseointegration. Currently, regenerative or rejuvenation therapy has become one of the core biomedical researches. The use of multiple growth factors in peri-implant healing has resulted in improved bone-implant contact and bone formation rate. Platelet-derived growth factors (PDGFs) hormones are mitogen and chemotactic factors that prove good for mesenchymal stem cells, including periodontal ligament cells and osteoblasts. In addition, some researchers have tried to increase osteogeneration rates in peri-implant bone by using biological factors, especially PDGFs, commonly known as platelet rich plasma (PRP) or plasma rich growth factor (PRGF).[3,4.]

Platelet rich plasma (PRP) was first

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introduced as a delivery system for growth factors in 1998 by Marx et al. The clinical use of PRP has evolved into the treatment of some body tissues, albeit with varying degrees of effectiveness. PRP therapy (in various methods) has been applied to stimulate tissue regeneration in bone, cartilage, skin, ligaments, tendons, muscles, and others. This therapy usually involves taking autologous blood and centrifugation to separate and obtain platelet concentrate. Although a variety of clinical reports are available in connection with the use of additional platelet concentrates for periodontal surgical procedures, a systematic review and recent meta-analysis have concluded that platelet concentrates may have a positive additional effect when used for the treatment of infrabony defects. PRP is defined as part of an autologous blood plasma fraction that has platelet concentrations. When activated PRP releases growth factor that plays an important role in healing and bone formation such as PDGFs, Transforming Growth Factor (TGF), Vascular Endothelial Growth Factor (VEGF) and others. Platelets are formed from stem cells in the bone marrow and have a major role in many body processes. Early work showed that blood platelets were the center of the clotting process but it gradually became clear that platelets have important functions in inflammatory mediation and support for bone healing processes.[3,5,6.]

PRP has been extensively studied in the field of periodontology and oral maxillo-facial, and has a very important role in the stimulation of healing processes in the ligaments, tendons, muscle cartilage and bone regeneration. In recent years PRP has wide diffusion in the treatment of soft tissue and bone healing.[5,7.]

To find out whether PRP administration can affect bone volume formed on the surface of dental implants, the authors are interested in raising and discussing and undertaking research to saw how the effect of dental implant coatings use PRP on bone volume formed on the surface of the implant.

Materials and methods

The study was conducted in October-November 2016. The research site is in the Laboratory of the Faculty of Veterinary Medicine of Hasanuddin University. This type of research is clinical experimental.

The sample of this study was Rabbit, age 4 months, body weight 1500-2000 gram. The sample size was 24 male rabbits divided into 2 groups: group I of 12 rabbits with the procedure of implantation of titanium implants without PRP and group II of 12 rabbits with the procedure of implantation of titanium implant with PRP, implant placed on the femur bone. (In accordance with the surgical sequence procedure of Osstem).

The protocol of this study was approved by Ethic commission of Medical Faculty Hasanuddin University.

Preparation of PRP

Prepare the necessary tools and materials. A total of 5 ml of rabbit blood, put in a tube that has been given anticoagulant. Blood is centrifuged 2 times at 3000 rpm for 15 minutes (until plasma fluid is separated from blood cells). Plasma is separated and removed by paste pipette into another tube and then closed and marked, there is no moisture and temperature effect, because PRP is applied immediately.

Animal treatment

Before the surgical stage of implant surgery on rabbit bone, the rabbit is cleaned the fur on the femur, then it is degraded with povidone iodine and then the rabbit will be anesthetized using ketamine and xilacine (Figure 2). Calculation of dose of anesthesia drug ketamine (concentration = 100 mg/ml) combined with xylazine (concentration = 2%)

$$V = \frac{\text{Dose x Weight}}{\text{Concentration}}$$

PRP should be used no later than 10 minutes after activation, because after 10 minutes of activation, platelets have secreted 70% of their growth factor. PRP can be applied directly in the inactive form because it will be activated after entering the body. Furthermore, in the surgical stage of the femur bone to include implants with PRP and without PRP. The implant installation procedure is in accordance with the installation instructions of the osstem implant (∅3.0x8.5mm).

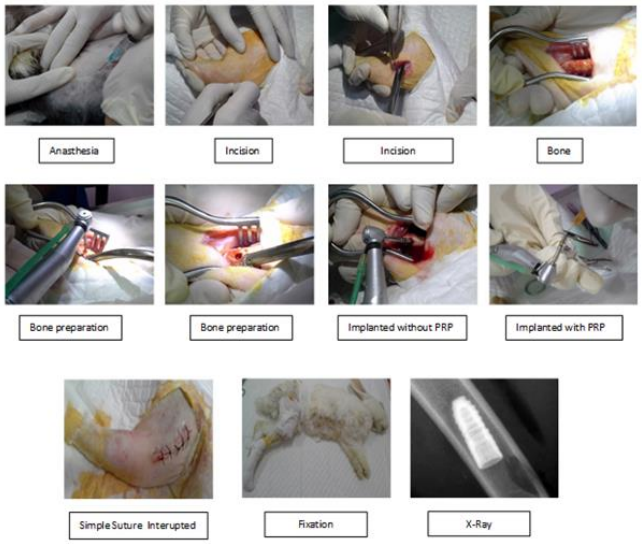

Figure 2. In the Process of Surgery the Researcher is Assisted by Veterinary Team From Hasanuddin University.

Bone histology preparation procedure

Femur specimens of rabbits that have been euthanized using xylazine 1.5 cc intra cardiac. Bone specimen in fixation in 10% formalin solution for 5 days, then rinsed with running water for 30 minutes to remove residual formaldehyde. The decalcification process begins by immersing bone specimens in a combined solution of 8% hydrochloric acid and 8% formic acid for one day (24 hours) which is repeated by replacing the new solution each day until the decalcification process is completed. The decalcification process depends on the size of the specimen. After the decalcification process is complete, the specimen is rinsed with running water then by immersion of the specimen in ammonia solution for thirty minutes to neutralize the acids from the combined solution of 8% hydrochloric acid and 8% formic acid. Rinse the specimen with running water for twenty-four hours then proceed with paraffin embedding process.

The samples were cut around the location of the implant with two vertical and vertical horizontal directions so that histopathological observation was obtained from two aspects. Subsequently the sample was immersed in a stratified alcohol solution (dehydration) starting from concentrations of 70%, 80%, 90% and 95% for one day (24 hours), followed by 100% glucose concentration (twice immersion) with the same concentration, each for one hour. The dehydrated sample is subsequently clarified in xylol (clearing) made in series (two immersions) each for 15 minutes. It soaked in paraffin. The tissue in the paraffin blocks is sliced with a thickness of 5µm using a microtome (Indoexim, India), then placed on the object glass, and stored in an incubator with a temperature of 40^{o}C for 24 hours.

The result of the incision was stained with eosin hematoxylin (HE) staining. HE staining is used to look at tissue structures that allegedly have pathological changes. Furthermore, the tissue is removed and entilen before it is covered with a glass cover (mounting). The observations were performed under a microscope with 10x and 16x subjective lens enlargements as well as 10x, 40x, and 100x objective lenses. The shooting is done using a digital camera. At 100x magnification is used emersi oil.

Data analysis

This study used the Shapiro Wilk test, because the samples below 50. The results of statistical tests $P<0.05$ means the data is not normally distributed or vice versa. Non-distributed data used the Mann Whitney Test to compare between the groups and the Friedman Test to test the changes that occur in each repetition of observations. While the data is normally distributed using t-independent test Repeated ANOVA.

Results

The calculation of the initial bone regeneration value aims to see the response of bone-forming cells in experimental animals after treatment of implants with PRP and without PRP. By using Optilab Image Raster v3, the average initial bone regeneration value is seen on days 0, 3, 7 and 14 after implantation. The results of the examination were recorded and data analysis using SPSS program version 20.0 (SPSS Inc., Chicago, IL, USA).

The result of statistical test of comparison of initial bone regeneration value in implant without PRP group from observation of third Field of View (FV), $P=0.03$ ($P<0.05$), meaning that there is a significant difference in three Field View between initial bone regeneration value days 0, 3, 7, and 14 after implantation without

PRP (Table 1). This can also be seen in the statistical test results, the comparison of initial bone regeneration value in the implants group added PRP obtained *P*=0.03 (*P*<0.05), that is, there is a significant difference in observation of the three Field of View on day 0, 3, 7 and 14 after implantation with PRP (Table 2).

Results of histologic examination at day 0, without PRP and with PRP treatment have not shown the presence of osteoblast cells and osteocytes. Results of histologic examination at day 14, without PRP and with PRP implant treatment showed osteoblast and osteocyte cells and new bone layers were developed (Figure 3). The comparison of initial bone regeneration values in FV1, FV2, and FV3 between implant groups with PRP and implants without PRP can be seen in Table 3. The p value is 0.001 (*P*<0.05), meaning that there is a significant difference between the mean initial values bone regeneration and an increase of 20% initial bone regeneration values in FV1, FV2 and FV3 between the implant groups added PRP compared with those not given PRP on days 0, 3, 7, and 14. From the bar chart shown in diagram 1, the diagram 2 and diagram 3, the increase in initial bone regeneration value is almost 5 times greater in installation with PRP than without PRP.

Discussion

This study calculated the mean value of Initial bone regeneration after implantation with PRP and implants without PRP. Initial bone regeneration measurements on the implant are standard procedures for evaluation of bone formation on the surface of the implant. Specifically, the difference in Initial bone regeneration values between the surfaces of the implant test was analyzed statistically to compare the osteogenic potential of the implant surface.

In the preparation of PRP a blood volume of 5 ml was obtained according to Marx et al. which shows that to achieve maximum effectiveness at least a minimum platelet concentration of 1.000.000/μL in 5 mL of plasma volume (Figure 1). The platelets contained in this concentrate autologous plasma release the alpha granules after the coagulation process has been locally triggered in the wound area. These alpha grains contain a mixture of growth factors that initiate proliferation, chemotaxis and cell differentiation, which are important for osteogenesis. Thus, in addition to its procoagulant effect, PRP is a source of growth factors involved in initiating and maintaining wound healing by accelerating bone repair, promoting fibroblast proliferation, and enhancing tissue vascularization.[7]

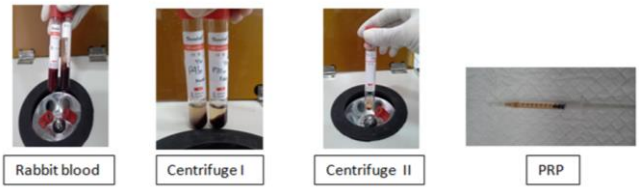

Figure 1. Preparation of PRP.

Blood sample was centrifuged as much as two times. This is in accordance with previous study by Rachita Dhurat that describes some of the techniques of making PRP. After the first round, WB (Whole Blood) will be three layers: the top layer containing most of the platelets and the WBC (White Blood Cells), an intermediate layer known as buffy coat and leukocyte-rich, and the lower layers consisting largely of red blood cells. For the production of pure PRP (P-PRP), the top layer and buffy coat are transferred to an empty sterile tube. The second round is subsequently performed, the speed for the second round should be sufficient to obtain soft pellet (erythrocytes) at the bottom of the tube. The upper part of the volume consisting mostly of PPP (Platelet Poor Plasma) is removed. The pellet is homogenized under 1/3 (5 ml of plasma) to create PRP.[8] The research used 3000 rpm speed for 15 min according to the centrifuge tool manual (80-1 Low Centrifuge, Jiangsu Zhengji Instruments Co., Ltd. Jiangsu, China).

The main effect of PRP comes from PDGFs, which has been identified as an essential protein for healing of hard tissue and soft tissue. PDGFs have been demonstrated to stimulate chemotaxis, mitogenesis and replication of stem cells at wound sites to areas of tissue injury. This leads to the formation of bone matrix and angiogenesis by stimulating increased levels of VEGF. This in turn can lead to accelerate soft tissue healing due to vascularization. PDGFs also stimulate the production of fibronectin, the cell adhesion molecule used in the cell.[9,10]

The regeneration of bone-forming cells in this study was seen in the Initial bone regeneration parameters, shown higher in the implant placement group with PRP than in the control groups in Table 1, Table 2, and Table 3

where the difference was statistically significant (*P*=0.001). This proves that the use of PRP increases the initial bone regeneration cell reaction on the surface of the implant. This finding is in accordance with the results reported by Wojtowics et al. and Fuerst et al. Contrary to, Butterfield et al., Schlegel et al., and Russy et al. different results have reported, are the use of PRP does not show significant results.[11]

The use of PRP on the surface of the implant not only enhances the healing process of bone implant compared with control, but also increases Initial bone regeneration in the statistically significant PP group (*P*=0.03) of control. This is consistent with those reported in previous studies including Ricardo et al. Investigating that histology analysis on day 15 of peri-implant placement by adding PRP to dog animal mandibles showed that most parts of the control group showed the area of peri-implant space occupied by solid connective tissue and other regions by the trabeculae of fine bones formed by the immature bone in the presence of numerous osteoblast cells. Study by Fontana et al, Kim et al and Furest et al. The researchers placed an implant on two mini pig jaw sides. In their study, PDGFs were applied to one implant while the other was installed without growth factor. They measured BIC after a period of 4 to 8 weeks and reported a 55.3% BIC value for implants with growth factor addition and a BIC score of 38.91% for control. They reported that anchorage implants could be strengthened in the jawbone by applying PDGFs. However, other studies using PDGFs for bone graft or bone replacements do not report considerable attainment in terms of bone osteogeneration or on the surface of the implant. Arpornmaeklong et al. comparing the in vitro effects of PRP and BMP-2. They reported that high concentrations of PRP were controlled by alkaline phosphatase activity, thus having no induced effects on the osteogeneration process. They claim this as the ineffective PRP in bone reconstruction. Schmitz and Hollinger also suggested the effectiveness of PRP in the presence of PDGFs in PRP would inhibit osteoblast regeneration when it continues contact with cells, which causes bone loss.[9,12,13]

The main effect of PRP comes from PDGFs, which has been identified as an essential protein for healing of hard and soft tissues. PDGFs stimulatechemotaxis, mitogenes-is and replication of stem cells at wound sites to the site of tissue injury. This results in the formation of bone matrix and angiogenesis by stimulating elevated levels of VEGF. This speeds up soft tissue healing because new vascularization is formed. PDGFs also stimulate the production of fibronectin, the cell adhesion molecule used in cellular proliferation and migration during healing, including osteoconduction and hyaluronic acid, and helps in promoting contraction and wound remodeling.[14]

Cytokines released by the alpha granule PRP are TGF-b1 and TGF-b2, both of which are involved in the repair of connective tissue and bone regeneration. Their most important role seems to be to stimulate chemotaxis of fibroblasts and the production of collagen and fibronectin by cells while inhibiting collagen degradation by reducing proteases and increasing protease inhibitors. In vitro and in vivo studies also show that TGF increases the proliferation of mesenchymal stem cells and osteoblasts, leading to bone regeneration. In particular, TGF-b2 has been shown to increase osteoblast and osteoclast activity. Increased TGF-b2 can accelerate bone regeneration by controlling the activity of osteoblasts and osteoclasts.[14]

It was reported that high concentrations of PRP were controlled by alkaline phosphatase activities, thus having no induced effects on the osteogeneration process. This is as a PRP ineffectiveness in bone reconstruction and suggests that useful clinical effects observed in the application of PRP may be due to one of two causes:[15]

a). Formation of autologous fibrin gel which may have stabilized graft and blood material thickened by adhesive strength;

b). PRP is a powerful mitogen for soft tissue cells that improve the maintenance of reconstruction due to the associated rapid repair process and reduce the likelihood of wound dehiscence.

Schmitz and Hollinger have also raised doubts about the effectiveness of PRP and maintain that PDGFs present in PRP inhibits regeneration by osteoblasts while continuing contact with these cells, leading to bone loss. They also claimed the formation of fibrin gel leading to graft material stabilization as a possible cause of the beneficial effect of PRP.[15]

Although it was found among the results obtained in this study and previously pointed to

the effectiveness of PRP-PRGF in bone healing, the following may be claimed as possible reasons for the controversial results reported elsewhere:[15]

a). Improper anticoagulation applications, differences in results obtained from one stage and two stages of centrifuge unit, and failure to accurately observe centrifugal and tariff times. Additionally, as platelets present in the PRP-PRGF secrete their growth factors only after freezing, it is, therefore, important to apply PRP-PRGF during their freezing process; otherwise they will not contribute to the wound or graft of the healing process.

b). Based on studies conducted so far, reduplication and differentiation of mesenchymal stem cells or bone marrow cells appear to be directly proportional to platelet concentrations. There is a very narrow range of platelet concentrations in PRP-PRGF (about one million in 1 ml) leading to positive results so that any value below or above this may cause inhibitory effect.

c). Most evidence of clinical potency of PRP-PRGF is only reported in case series reports or cases whose results are uncertain. Therefore, it is necessary to conduct randomized controlled clinical studies well to obtain reliable results.

The limitation of this study is the observation time of the use of PRP for only 14 days to see the initial bone regeneration and the effectiveness of the use of PRP is only measured by the initial bone regeneration parameter through bone histology examination. Further research is needed with a longer time to look at the process of bone formation up to the remodeling stage, then using more parameters indicating whether the use of PRP during implant placement improves the implant prognosis in different bone qualities and contributes to shortening the dental implant healing time.

Conclusions

The conclusions that can be drawn from this research are: There is a difference in the mean value of Initial bone regeneration after the installation of implant with and without PRP.

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Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

Histopathological examination results (day)	Field of view 1		Field of view 2		Field of view 3	
	Mean±SD	*P* Value	Mean±SD	*P* Value	Mean±SD	*P* Value
0	0.00±0.00	0.03	0.00±0.00	0.03	0.00±0.00	0.03
3	0.00±0.00		0.00±0.00		0.00±0.00	
7	21.57±0.50		20.50±0.34		21.81±0.75	
14	55.79±0.31		54.81±0.38		57.07±0.85	

Table 1. Comparison of Initial Bone Regeneration Values in the Implant Group without PRP. Test of data normality: Shapiro-Wilk test; *P*<0.05; abnormal data distribution; *Test Friedman; *P*<0.05; significant

Histopathological examination results (day)	Field of view 1		Field of view 2		Field of view 3	
	Mean±SD	*P* Value	Mean±SD	*P* Value	Mean±SD	*P* Value
0	0.00±0.00	0.03	0.00±0.00	0.03	0.00±0.00	0.03
3	98.78±0.77		83.78±0.49		89.29±0.85	
7	237.89±2.33		199.83±1.70		188.34±1.04	
14	246.14±0.49		238.09±0.91		246.14±0.49	

Table 2. The Comparison of Initial Bone Regeneration Values in the Implant Group with PRP. Test of data normality: Shapiro-Wilk test; *P*<0.05; abnormal data distribution; *Test Friedman; *P*<0.05; significant.

Histopathological examination results (day)	Field of view 1		Field of view 2		Field of view 3	
	Mean±SD	*P* Value	Mean±SD	*P* Value	Mean±SD	*P* Value
0	0.00±0.00	0.03	0.00±0.00	0.03	0.00±0.00	0.03
3	49.39±54.10		41.89±45.89		89.29±0.85	
7	129.73±118.49		110.16±98.22		188.34±1.04	
14	147.97±100.97		146.45±100.38		246.14±0.49	

Table 3. Comparison of initial bone regeneration values between implant groups with PRP and implants without PRP. Test of data normality: Shapiro-Wilk test; *P*<0.05; abnormal data distribution *Test Friedman; *P*<0.05; significant

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