Osteocalcin levels evaluation of freeze-dried homologous platelet-rich plasma in periodontal treatment

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Abstract

Objective: The aim of this study was to evaluate enhanced osteocalcin levels by using freeze-dried homologous platelet-rich plasma (FD HPRP) in periodontal treatment to support the osteogenesis process. Material and Methods: FD HPRP was made by HPRP from the blood bank, then freeze-drying and for sterilization, the γ-radiation process was carried out with doses of 20 and 25 kGy. The osteoblast (Cell line MG63) was cultured to confluent and then treated with FD HPRP 20 kGy, FD HPRP 25 kGy. Osteocalcin levels were tested using enzyme-linked immunosorbent assay (ELISA) on the 7th and 14th days. The data were analyzed using a t test. Results: There was a significant difference in the osteocalcin levels between FD HPRP 20 and FD HPRP 25 (p<0.05). Osteocalcin levels increased on day 14 in each group and the highest one on the 7th and 14th days was FD HPRP 25. Conclusion: The present results showed that FD HPRP with simple preparation could support the osteogenesis process.

Keywords: Freeze-dried platelet-rich plasma, Osteocalcin, Osteogenesis, Periodontal regeneration
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Introduction

Periodontitis is an inflammatory of the supporting tissue of the teeth resulting in destruction of periodontal ligament and alveolar bone.1 Aim of periodontal treatment is regeneration.2 The combination of scaffold, cell and growth factor is important for inducing tissue regeneration.3 Platelet is an essential role in periodontal regenerative treatment. The high part of cytokines and growth factors in platelets is useful in regenerating periodontal tissue.4 Platelet Rich Plasma (PRP) has gained attention in musculoskeletal regenerative therapies as it clinically enhances neoangiogenesis, tissue repair, and regeneration.5 Several medical history, taking drugs or has a hematological disorder that can affect the results of the study.6 Platelet Rich Plasma is divided into 2 types in terms of the source of blood collection, namely autologous and homologous.7,8 Autologous PRP was obtained from the patient's own blood which had a high content of growth factor after centrifugation. The problem with autologous PRP when patient has a bad medical history, is taking drugs or has a hematological disorder that can affect the results of autologous PRP.9 Homologous PRP derived from healthy blood donors and has been screened before and has several advantages, such as ease of preparation, high platelet count rather than therapeutic ratio, and low cost.7

The release of growth factors was relatively fast in fresh PRP. To overcome this limitation, PRP is made in the form of a dry powder called freeze dried (FD-PRP). Freeze dried is a method to increase shelf life of homologous PRP and maintain the growth factor.4 According to Shiga et al.8 freeze dried PRP can promote bone fusion in a mouse PLF model after 8 weeks of storage. According to Kinoshita et al.4 PDGF in frozen dried PRP stored for 4 weeks after being freeze dried can show its pharmacological activity by inducing osteoblast proliferation. Freeze dried homologous platelet rich plasma (FD HPRP) is different from FD PRP, because the blood has been screened before and is confirmed free of disease, so it can be used by anyone.6 In the study of Murdiastuti et al.10 FD HPRP has osteogenic potential against osteoblasts by increasing of cell differentiation. And in research Olivia et al.11 FD HPRP can accelerate the migration process of osteoblast cells.

Osteocalcin is a small calcium-binding protein of bone and is the most abundant non-collagenous protein of mineralized tissue.12 Most osteocalcin is synthesized by osteoblasts, odontoblasts and hypertrophic chondrocytes. Osteoblasts begin differentiation process on the 7th day and on the 14th day the bone remineralization process begins. Osteocalcin has routinely been used as a serum marker of osteoblastic bone formation and believed to act in the bone matrix to regulate mineralization.13 Shiga et al.8 suggest that FD PRP should be prepared in a clean room using good sterilization to avoid infection. Sterilization using γ-irradiation is commonly used for terminal sterilization of freeze-dried tissues.14 In the study of Muraglia et al.15 FD PRP sterilized with gamma radiation at dose 25 KGY presented no colonies. Kusumadewi et al.16 concluded that FD HPRP with radiation doses of 20 and 25 KGY was able to increase TGF-β1 levels compared to several other radiation doses.

Based on that results, this study purpose to evaluate enhanced osteocalcin levels by using freeze-dried homologous platelet-rich plasma (FD HPRP) in periodontal treatment to support the osteogenesis process.
Table 1. Mean and standard deviation of osteocalcin levels

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Day 7 μg/mL</th>
<th>Day 14 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD HPRP 20</td>
<td>6</td>
<td>101.80±4.743</td>
<td>391.996±9.033</td>
</tr>
</tbody>
</table>

Figure 1. Mean osteocalcin level of each group by time of measurement. The asterisk symbol in the figure denotes the significant difference between two groups, P<0.05.

Material and Methods

This experimental research is approved by the Ethics Commission, Faculty of Dentistry, Universitas Gadjah Mada, Indonesia with registration number No.00520/ KKEP/FGK/-UGM/EC/2020. The research sample administering human osteoblast-like cells, MG63 cell line (ATCC - CRL 1427TM) was divided into two (FD HPRP 20 dan FD HPRP 25).

Platelet-Rich Plasma

For PRP preparation, 120 mL of type-O blood was acquired from Palang Merah Indonesia (PMI) and used as a sample source. Since the individuals with blood group O are universal donors, O blood type was a preferred choice for this study. The blood sample used was actually a waste product of PMI. The presence of hepatitis B, hepatitis C, HIV, and syphilis infection in the blood sample was tested using transfusion-transmitted infectious disease test. PRP was prepared by the centrifugation of whole blood using an RC centrifuge according to the standard PMI procedure. The resulting plasma was stored in blood bank refrigerator at 2°C–6°C. The plasma was further transferred to a cool bag at a temperature of 2°C–4°C for freeze drying. The PRP sample was incubated in a freezer at −40°C for 7 days. Following this, the sample was freeze-dried for 3 days using a freeze dryer (Modulyo, Edwards). Then was exposed dose of γ irradiation 20 and 25 Kgy in a γ-irradiator sourced from cobalt-60 (OBServo Ignsis type Gamma Irradiator).

Cell Culture

Human osteoblast cell line MG-63 (ATCC, Bethesda, USA) was cultured in a T75 Flask (TPP Switzerland) with Dulbecco’s Modified Eagle Medium (DMEM) (Gibco), FBS (Sigma), Penicillin Streptomycin (Gibco), Fungizone (Gibco) media incubated at 37°C and 5% CO2. When steoblast cells which were 80–90% confluent, the cell medium was removed. Then, DMEM (Gibco) was inserted into a flask containing osteoblasts. The cells were washed using trypsin-EDTA 0.25% (Gibco), so that the cells attached to the flask were released. The cells were placed in a 15 mL conical (Biologix) containing DMEM and then centrifuged at 1500 rpm for 5 minutes. The cells would settle at the conical base and DMEM was added and aspirated. The cells were seeded into a 96-well plate as much as 100mL. The cells were incubated overnight and treated.

Osteocalcin Level

Osteoblasts were seeded on 96-well plate with a density of 2.5 x 103 cells/well. Then treated with FD HPRP 20 and FD HPRP 25. On the 7th and 14th day, the level of osteocalcin was checked with an enzyme-linked immunosorbent assay (ELISA) using the Human Osteocalcin/Bone Gla Protein (OT/BGP) ELISA Kit (Cusabio). The experiment was performed as per the manufacturer’s instructions.

Statistical Analysis

Data were analyzed using SPSS 24. Comparing the osteocalcin levels (pg/ml) of FD HPRP 20 and FD HPRP 25 using the T-test (p < 0.05).

Results

Based on the normality and homogeneity tests of the data that have been carried out, the levels of osteocalcin in all treatments were normally distributed and homogeneous, an then continued with the T-test.

From the data, the level of osteocalcin is shown in pg/ml. The lowest mean level of osteocalcin at FD HPRP 20 in day 7 and the highest levels were at FD HPRP 25 in day 14 table 1. There was an increase of osteocalcin levels on observations day 7 to day 14 in both the FD HPRP 20 and 25 groups, although there was a greater increase in the FD HPRP 25 group.

Statistically there was a significant difference between the levels of osteocalcin on day 7 and day 14 in the two groups of FD HPRP 20 and 25 and there was a significant difference between the FD HPRP 20 and 25 groups on each day of observation both on day 7 and day 14 (p<0.05) with levels osteocalcin FD HPRP 25 was higher than FD HPRP 20 figure 1.

Discussion

Osteoblasts are specialized mesenchymal cells
that are primarily responsible for the synthesis and deposition of the mineralized, collagen-rich matrix that composes bone tissue. Osteocalcin (OCN), an important component of bone extracellular matrix, is the most abundant non-collagenous protein in bone secreted by osteoblasts. On the 7th day, osteoblast differentiation begins, which is marked by the change of osteoblasts into osteocytes and then continued to bone mineralization on day 14. In this study, osteocalcin levels increased from day 7 to day 14 on FD HPRP 20 and FD HPRP 25, this indicates that there is a bone remodeling process that occurs based on the time of observation. Osteocalcin levels were correlated with bone formation and manage mineralization process. With increasing levels of osteocalcin, this indicates that the bone mineralization process occurs with the addition of FD HPRP 20 and FD HPRP 25.

This result, according to Mubashir’s et al. research that freeze drying technique can stabilize PRP bioactivity, maintain platelet count and growth factor levels. Platelet Rich Plasma is widely used in periodontal therapy because of its growth factor. Growth factors such as PDGF-BB, TGF-β1, IGF-I, PDEGF, PDAF and PF-4, released from platelets and involved in wound healing, are considered promoters of tissue regeneration. Growth factors released by activated platelets have a stimulatory effect on the proliferation, migration and differentiation of several cell types, including osteoblasts, which play a role in the healing process of bone damage.

The results of this study showed that there was a significant difference between the FD HPRP 20 and FD HPRP 25 groups where the osteocalcin level of FD HPRP 25 was higher. In accordance with the research of Murdiastuti et al. which stated that FD HPRP 25 was more able to induce the differentiation process indicated by number of osteocytes FD HPRP 25 was higher than FD HPRP 20. One of the markers of the bone formation process is osteocalcin which is a non-collagenous protein in the bone matrix, which synthesized by osteoblasts, and secreted into the fluid of the main bone supporting tissue. Osteocalcin is the most abundant non-collagenous protein in bone and is produced by osteoblast cells.

Platelet Rich Plasma can be prepared from the patient’s own blood (autologous) or from multiple healthy blood donors (homologous)20. Ince et al.7 reported that homologous PRP had a higher platelet count than autologous, this was due to growth factor autologous PRP from 1 person only, while homologous at least from 4-5 people. Homologous PRP is prepared from healthy blood donors and has been screened which is can increase PRP7 activity. Horimizu et al.21 also reported that storage of FD PRP coated with collagen sponge at 4°C did not cause a significant loss of bioactivity.

According to Shiga et al.8 FD PRP should be prepared in a clean room using good sterilization procedures to avoid infection. Sterilization can be achieved by gamma irradiation without affecting the effectiveness of the osteoblast culture. In this study, the radiation process greatly affected the level of osteocalcin, which significantly increased the level of osteocalcin at 25 Kgy of radiation. In a previous study by Olivia et al.11 the osteoblast migration process occurred faster in FD HPRP 25. This could be due to the fact that in gamma ray 25 sterilization no bacterial colonies and bioactivity of the material would not be disturbed. Then in the study of Kusumadewi et al.16 levels of TGF-β increased at 25 Kgy radiation, TGF-β is known to play multiple functions in the regulation of various biological processes in the human body such as cell growth and differentiation, bone remodeling, angiogenesis, and maintenance of homeostasis.

Conclusion

This study concluded the highest level of osteocalcin on FD HPRP 25 at day 14. This shows that FD HPRP can support the process of osteogenesis seen from the level of osteocalcin which plays a role in the healing process of periodontal tissue and gamma irradiation also very affected bioactivity of material.

Acknowledgment

None

Conflict of Interest

The authors report no conflict of interest.

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