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Original article

Effect of tooth graft particle size on the healing process of femur defects in Wistar rat (*Rattus norvegicus*)

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ABSTRACT

Background: *Teeth have potential as bone graft materials because of their organic and inorganic components that can stimulate osteoinduction, osteoconduction, and osteogenesis. An important success indicator of treatment using this graft material is the formation of osteoblast and new blood vessels in the applied area.* ***Purpose:*** *To investigate the number of osteoblast, osteoclast, and new blood vessels in bone healing after the implantation of tooth-derived bone graft materials measuring 20, 40, and 60 mesh.* ***Methods:*** *Thirty-six Wistar rats with a 2 mm defect on the right femoral dextra condile were divided into four groups. P0 (n=9) was the control group, where the defect was not filled by any material. In the other groups, the defects were filled by 20-mesh (P1; n=9), 40-mesh (P2; n=9), and 60-mesh (P3; n=9) tooth graft material. The Wistar rats were sacrificed after 2 weeks, and then the preparations were hematoxylin eosin staining. The data were analyzed using one-way analysis of variance and Tukey's post hoc test.* ***Results:*** *The highest number of osteoblast was in the P3 group with a mean of 49.67, highest number of new blood vessels in the P2 group with a mean of 39.89, and highest number of osteoclast in the P1 group with a mean of 20.44. Statistical analysis showed a significant difference in the number of new blood vessels, osteoclast, and osteoblast in each group ($p=0.000$; $p<0.05$).* ***Conclusion:*** *Particle size differences in tooth graft material affect osteogenesis and angiogenesis in the bone healing process.*

Keywords: *angiogenesis; bone defect; medicine; particle size; tooth graft*
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INTRODUCTION

Tooth loss generally occurs due to caries, severe periodontal disease, and trauma. According to the World Health Organization in the Global Oral Health Status report in 2022,[1] the estimated global prevalence of total tooth loss was nearly 7% among people aged 20 years or older. For people aged 60 years or over, the estimated global prevalence was much higher at 23%. According to Riset Kesehatan Dasar (Riskesdas) / Basic Health Research in 2018, the prevalence of tooth loss in individuals aged 55–64 in Indonesia was 70.2%.[2]

Reduction of the alveolar bone wall can be interpreted as a response after tooth extraction and is usually followed by a decrease in the dimensions and volume, as well as changes in shape, of the alveolar bone and other periodontal tissues.[3] Alveolar ridge preservation is a curative procedure carried out with the aim of restoring available bone and preserving the height and thickness of the alveolar bone.[4] Ridge preservation requires appropriate treatment methods. The addition of a socket-filling material can facilitate bone formation and accelerate bone healing.[5]

There are four main biological properties for bone graft materials, namely osteointegration, osteogenesis, osteoconduction, and osteoinduction. These properties enable the new bone formation. Additional properties are required to increase the success rate of a bone graft. These include bioresorbability, sterility, structural integrity, adequate porosity for vascularization, plasticity, ease of application, cost, and compressive strength.[6]

Autogenous bone grafts have become the gold standard in bone regeneration procedures because of their osteogenic, osteoinductive, and osteoconductive properties associated with the pre-osteoblastic cells in the graft. However, it has several shortcomings, such as limited resources, need for additional surgical area, morbidity, and irregular resorption

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rate.[7] At the same time, various kinds of substitute materials have been developed as alternative options for osteoblast proliferation. Xenografts have been developed and are widely used in clinics. However, the possibility of disease transmission from other species to humans can still occur. Therefore, it is important to find donors that do not pose a risk.[8]

Tooth graft material is a patient treatment that involves filling the tooth socket using bone graft from extracted tooth material.[9] It was first introduced by the Korea Tooth Bank Research and Development Center and has been received with satisfaction by clinicians and patients owing to its equivalent ability in both osteoinduction and osteoconduction.[10]

Teeth contain organic and inorganic components such as calcium-phosphate, which have an influence on the remodeling process of bone healing and have the same potential for osteoinduction, osteoconduction, and osteogenesis as bone.[11] Teeth with high calcium content can be synthesized into hydroxyapatite (HAp) to be used as an option in bone graft material to accelerate the bone healing process.[12] An important physical aspect of bone graft selection is the particle size.[13] The particle size of bone graft is instrumental in osteogenesis and bone repair.[14]

In the wound healing process, the success indicators are the occurrence of angiogenesis and the formation of new blood vessels, osteoblast, and osteoclast. Angiogenesis supplies oxygen and nutrients to not only the wound but also stem cells that can differentiate into osteoblasts and, in the next phase, is also an ion needed in mineralization.[15,16]

Furthermore, the purpose of this study was to determine differences in the number of new blood vessels, osteoblast, and osteoclast in the bone healing of Wistar rats (*Rattus norvegicus*) after the implantation of tooth grafts measuring 20, 40, and 60 mesh, respectively.

MATERIALS AND METHODS

This research is *in vivo* with a single short case study. The research was conducted at the Integrated Oral Biology Laboratory at the Faculty of Dentistry, Universitas Brawijaya, and was approved by the Animal Care and Use Committee Universitas Brawijaya, No.1166-KEP-UB. The research sample used 36 male Wistar rats aged 3 months with a weight of 250–300 grams, which were randomally divided into four groups, with each group consisting of nine rats. The four groups were named P0, P1, P2, and P3. P0 is the control group, where a 2 mm defect was created on the right femoral dextral condyle of the rats and was not filled with any material. P1 is Treatment Group 1, where defects were filled with an implanted 20-mesh tooth graft. P2 is Treatment Group 2, where defects were filled with an implanted 40-mesh tooth graft. Finally, P3 is Treatment Group 3, where defects were filled with an implanted 60-mesh a tooth graft. Acclimatization and treatment of the experimental animals were carried out at the Integrated Oral Biology Laboratory, Faculty of Dentistry, Universitas Brawijaya. The animals were acclimatized for 7 days to adapt to the environment where the study was conducted.

The tooth graft material was made following the protocol of the Biomaterials Laboratory Centre for Isotopes and Radiation National Nuclear Energy Agency (BATAN)[17] by selecting post-extraction human teeth that did not experience caries and were not filled with restoration. Each tooth obtained was demineralized using 0.6 M hydrochloric acid solution and then lyophilized with a freeze dryer until the water content was reduced to <10% to produce demineralized, freeze-dried tooth graft. The material was then ground using a bone mill to process the teeth powder.[18]

The tooth graft in powder form was sterilized using a Co-60 source (Gamma cell 220 cobalt 60 Irradiator MDS®, Canada) with irradiation doses of 15 and 25 kGy. This process was carried out to ensure the material was safe and there was no contamination. This powder material was sieved using sieves measuring of 20, 40, and 60 mesh to produce three different powder sizes. The sizes were 840 µm (P1), 420 µm (P2), and 250 µm (P3). Sieving was followed by sterilization using gamma rays. The characterization process of the tooth grafts was carried out using scanning electron microscopy with energy dispersive X-ray spectroscopy (JEOL JSM-6510LA®, Japan) at the Science Center and Advanced Materials Technology (PSTBM), National Nuclear Energy Agency (BATAN).[18]

The experimental animals were acclimatized for 7 days. Afterward, the surgical implantation procedure was performed on Day 0 of the experimental timeline under sterile conditions. The manufacture of the defect was preceded by anesthesia with a mixture of ketamine (KETAMIL, Indonesia) and xylazine (XYLA, Holland), 0.2 mL administered intramuscularly. A longitudinal incision was made using surgical blade no. 15 (ONE MED, Indonesia) on the right femur, and the flap that was made was opened using raspatory (SCHEZHER, Germany) to reveal the bone. A low-speed bone round bur with a 2 mm diameter was drilled into the distal right femur to a depth of 2 mm. Tooth-derived graft materials were systematically implanted into each experimental group based on the standardized particle size classifications of 20, 40, and 60 mesh. Following a 14-day healing period to allow for initial tissue integration and biological response, the experimental animals were humanely sacrificed using cervical dislocation following institutional animal care guidelines. Subsequently, femoral bone tissue samples were harvested through careful dissection for histological and analytical evaluation.

The femoral bone tissues were collected and placed in a container containing 10% formalin solution, which had been labeled according to each group. Decalcification of fixed bone specimens were performed using ethylenediaminetetraacetic acid (EDTA) solution prior to paraffin wax–embedding procedures. After decalcification, the tissues were processed through graded alcohols (70%,

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80%, 95%, and 100%) for dehydration, cleared in xylene, and embedded in paraffin wax blocks. The blocks were then sectioned at 4–5 µm thickness using a microtome. The deparaffinized and rehydrated sections were then stained with hematoxylin solution (LEICA,3801570, Germany) for 5–10 minutes to visualize nuclei in blue-purple, followed by differentiation in acid alcohol, bluing in ammonia water, and counterstaining with eosin solution (LEICA,3801570, Germany) for 1–3 minutes to demonstrate cytoplasm and extracellular matrix in pink-red coloration, and then dipped in alcohol. The preparation was washed with PBS, then dipped in xylol (MERCH, 18.661.500, German). The preparation was then dripped with mounting media and covered with a cover glass (ONE LAB, CHINA). After drying, the preparation was ready to be examined under a microscope.

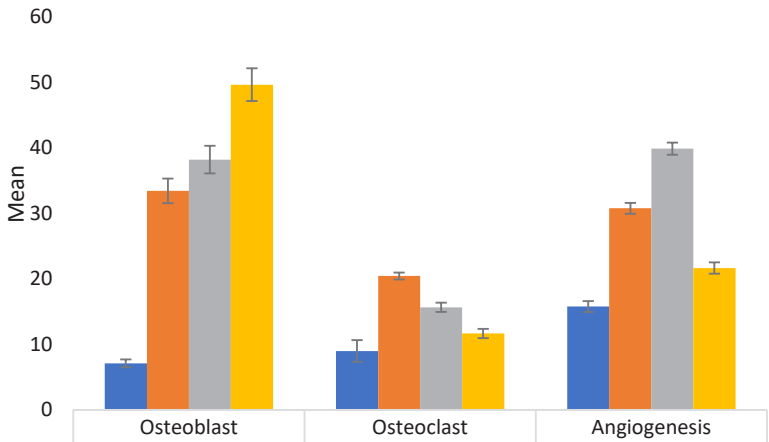
Sample preparation was carried out at the Anatomical Pathology Laboratory of the Faculty of Medicine, Universitas Brawijaya. Observation and counting of angiogenesis, osteoblast, and osteoclast from the histology of rat femurs were carried out using an OLYMPUS CX31 series microscope equipped with DP-70 digital camera and using OLYSIA software. Digital images captured at 400 magnification were imported into OLYSIA software and calibrated using stage micrometers, with the region of interest defined as the secondary spongiosa extending 1.0 mm below the growth plate. The measured quantitative parameters included osteoblast number per bone perimeter (N.Ob/B.Pm), osteoblast surface per bone surface (Ob.S/BS), osteoclast number per bone perimeter (N.Oc/B.Pm), osteoclast surface per bone surface (Oc.S/BS), vessel density (N.Ves/T.Ar), and vascular area fraction (Vas.Ar/T.Ar), with manual validation performed to ensure accuracy of automated cellular recognition algorithms.

Statistical analysis was conducted on at least three non-consecutive sections per preparation, with results expressed as mean ± SD and intra-observer reliability maintained below 8% coefficient of variation through standardized morphological identification criteria and quality control measures. Statistical tests were performed using SPSS software on all variables. The Shapiro–Wilk test was used to assess the normal distribution of the data. Factorial analysis of variance was performed using one-way analysis of variance. Tukey's post hoc test was used to identify significant differences between groups at $p<0.05$. The data are presented as the mean ± standard deviation (SD).

RESULTS

The results of the average number and SD of new blood vessels, osteoblasts, and osteoclasts are shown in Figure 1. The lowest number for osteoblasts was in the P0 group with a mean of 7.11, while P3 had the highest number with a mean of 49.67. An increase in bone graft was followed by an increase in osteoblast values. The lowest number of osteoclast was obtained in the P0 group with a mean of 9.00, while P1 had the highest number with a mean of 20.44. An increase in bone graft is inversely proportional to the osteoclast number. The lowest number of angiogenesis was obtained in the P0 group with a mean of 15.78, while P2 had the highest number with a mean of 39.89. Between P1 and P3, the difference in angiogenesis is negligible.

Figures 2–4 show the preparations resulting from hematoxylin eosin staining on 27 preparations of femur bone tissue of Wistar rats. Figure 2 shows that the formation of new blood vessels with the lowest number was in the control group (P0). The P1 group (20 mesh) showed a greater number of new blood vessels than the P3 group (60 mesh), whereas the P2 group (40 mesh) showed the highest number of new blood vessels.



	Osteoblast	Osteoclast	Angiogenesis
P0	7.11	9.00	15.78
P1	33.44	20.44	30.78
P2	38.22	15.67	39.89
P3	49.67	11.67	21.67

Figure 1. The number of osteoblast, osteoclast, and angiogenesis observed after treatment.

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Figure 3 shows that the average number of osteoclasts in P1 was higher than P0, P2, and P3. Likewise, Treatment Group 2 (P2) had a higher number of osteoclasts compared to the control group (P0) and Treatment Group 3 (P3).

In Figure 4, it can be seen that osteoblast cells are dark purple around the nucleated cells that almost fill the entire cell body. The highest number of osteoblasts was found in the P3 group, followed by the P2 group and then the P1 group, and the lowest in the control group (P0).

Figure 5 shows that there are significant differences in osteoblasts between control (P0) and treatment (P1, P2, and P3) as well as among treatment groups ($p<0.01$). In the osteoclast results from Tukey's post hoc test, there is no significant difference in P0 and P3 (sig = 0.462), but there is a significant difference between treatment groups ($p<0.01$). Meanwhile, in the angiogenesis results, differences were found between the control group (P0) and treatment groups (P1, P2, and P3; $p<0.01$), but there is no significant difference between P1 and P3.

DISCUSSION

The bone healing phase is divided into four phases: hematoma formation and inflammation, fibrocartilage callus formation, bone formation, and remodeling. Neovascularization begins

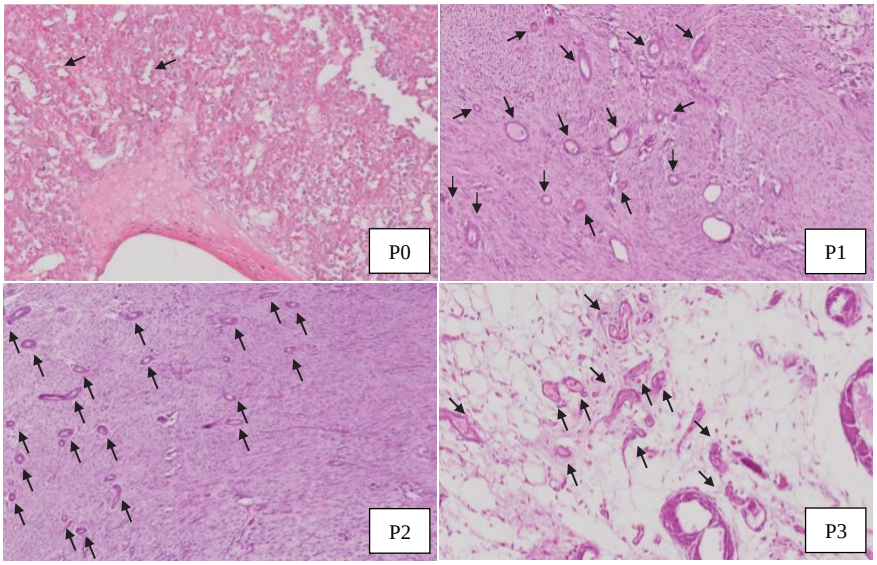

Figure 2. Histology of bone tissue preparations after hematoxylin eosin stained under 400x magnification. The cytoplasm and surrounding matrix of new blood vessels are stained pink (black arrow). The number of new blood vessels is indicated by the number of arrows. (P0) control group; (P1) 20-mesh group; (P2) 40-mesh group; and (P3) 60-mesh group.

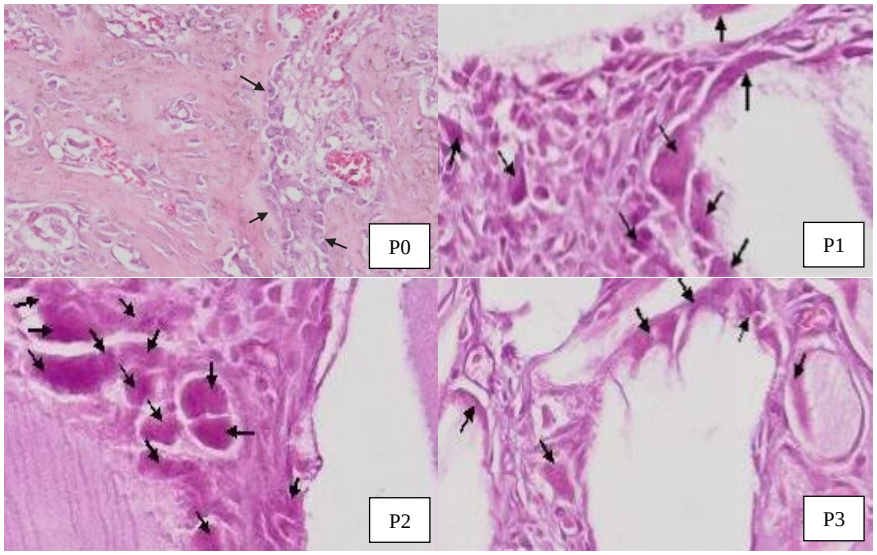

Figure 3. Histology of bone tissue preparations after hematoxylin eosin stained under 400x magnification. Osteoclast appears as a large cell with the blue nuclei and pink cytoplasm (black arrows). The number of osteoclast is indicated by the number of arrows. (P0) control group; (P1) 20-mesh group; (P2) 40-mesh group; (P3) 60-mesh group.

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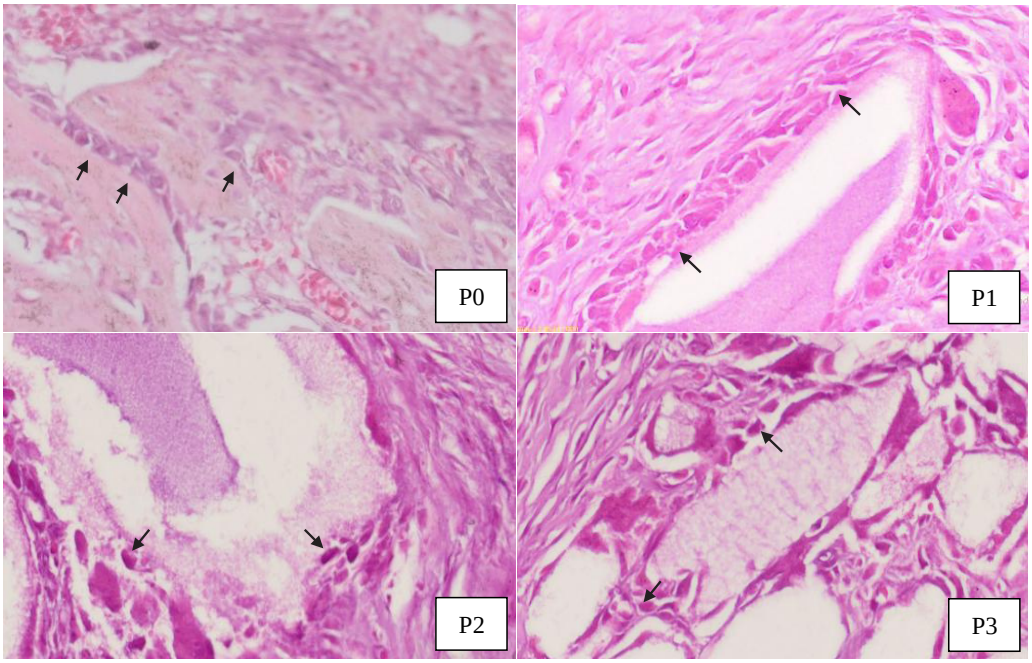

Figure 4. Histology of bone tissue preparations after hematoxylin eosin stained under 400x magnification. Osteoblasts appears as cells with dark purple around the nucleated cells and bluish red cytoplasm (black arrows). The number of osteoblast is indicated by the number of arrows. (P0) control group; (P1) 20-mesh group; (P2) 40-mesh group; (P3) 60-mesh group.

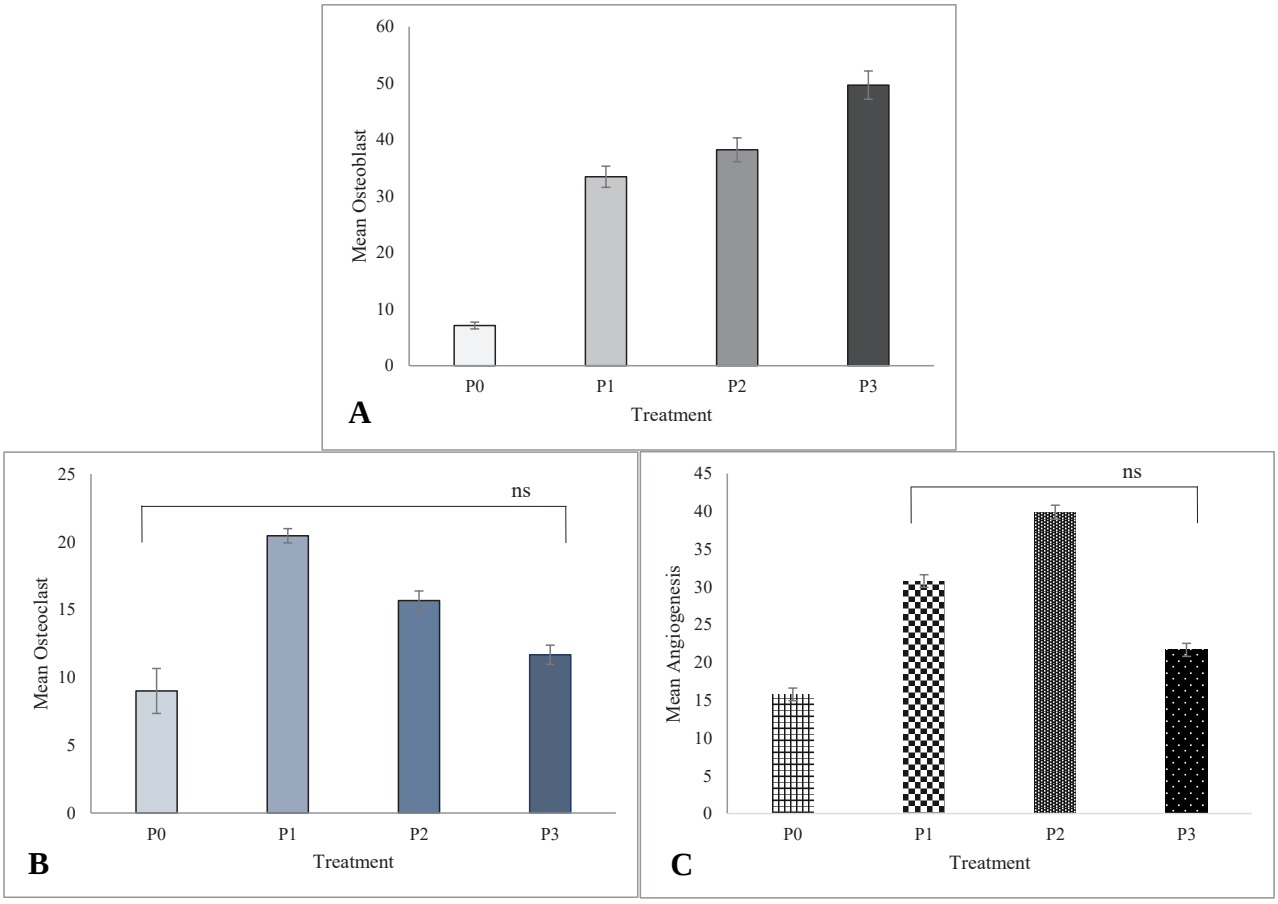

Figure 5. Post hoc results in all tretament groups. A: The differences in the number of osteoblasts in all treatment groups are significant. B: The differences in the number of osteoclasts are significant in all treatment groups except P0 vs. P3. C: The differences in the number of angiogenesis are significant in all treatment groups except P1 vs. P3.

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on the fourth day after the injury to the bone. Bleeding that occurs after an injury causes a lack of oxygen and nutrients in the body. The body responds by releasing coagulation factors to stop the bleeding (hemostasis). Endothelial cells in the wound area secrete proteolytic enzymes to stimulate the degradation of the extracellular matrix and basement membrane of blood vessels around the wound.[19] Neutrophils and macrophages that migrate to the wound area synthesize Fibroblast Growth Factor-2 (FGF-2), while endothelial cells, and chondrocyte cells synthesize vascular endothelial growth factor (VEGF). VEGF angiogenic molecule and FGF-2 bind to receptors on endothelial cells, resulting in proliferation to form new blood vessels.[20]

Treatment Group 1 (P1) was a tooth graft measuring 20 mesh, equivalent to 841 µm; Treatment Group 2 (P2) was a tooth graft measuring 40 mesh, equivalent to 400 µm; and Treatment Group 3 (P3) was a tooth graft measuring 60 mesh, equivalent to 250 µm. Based on the results, Treatment Group 2 (P2) had the highest number of new blood vessels, followed by Treatment Group 1 (P1) and then Treatment Group 3 (P3). This difference can occur due to the role of calcium in regulating the angiogenic process, where the calcium content in the Hap tooth graft may help with the formation of new blood vessels.[21] HAp can induce angiogenensis by increasing VEGF expression. VEGF stimulates and initiates the function of endothelial cells to migrate, proliferate, and differentiate to increase the formation of new blood vessels.[22]

Blood vessels were observed in the femur preparations of Wistar rats after bone injury across all treatment groups. After the end of the inflammatory phase, around Weeks 2–3, there was a proliferative or granulation phase. The proliferative phase has several sub-phases: fibroplasia, matrix deposition, epitalization, wound contraction, and angiogenesis. Angiogenesis is an important phase in wound healing with the formation of a network of blood vessels around the injury.[23]

In this study, the treatment group with 40-mesh tooth graft implantation showed a higher number of new blood vessels than the other groups. This is because the particle size of the graft has an important role in the bone healing process through bone grafting. This is in line with research conducted by Pafumi et al.,[24] who state that particle size can have an influence on both the aspects of angiogenesis and the strength of the tooth graft itself as a scaffold.

Several researchers have reported that the particle size of the graft material plays an important role in activating osteoconduction, which affects the quantity of new bone.[25,26] Kim et al.[25] report that demineralized dentin matrix is a good material for bone formation because it contains osteoconductive material.

Recent studies suggest that there is a relationship between the particle size of the graft material and the formation of new bone.[21,27] Graft particle sizes of 250–1000 microns are considered to be ideal for activating bone growth.[21] Meanwhile, in research by Wadhwa et al.[27] using grafts in powder and block form, it was found that a smaller graft particle size (100–300 microns) enhanced osteogenesis as compared to block type particles.

Cellular components of bone include osteoblasts, osteocytes, osteoclasts, and osteogenic precursor cells. The function of these cells is the synthesis, regulation, deposition, and mineralization of the extracellular matrix. These cells also have a role in calcium–blood homeostasis and serve as mechanosensors for bone.[28]

In this study, the largest number of osteoblasts was in P3 (equivalent to 250 µm), followed by P2 (equivalent to 400 µm) and then P1(equivalent to 800 µm). This is in line with the study by Malinin et al., who show that particle sizes in the range of 90–300 microns result in rapid healing by direct ossification. Particles smaller than 90 microns have significantly reduced osteoinductive potential. Particles larger than 300 microns are much slower in healing and bone fusion compared to 90–300-micron particles. Particles smaller than 75 microns cause small osteogenesis similar to small HAp particles that can inhibit osteoclastic activity.[29]

The highest average number of osteoclasts (20.38) was found in the 20-mesh group (P1). In the 40-mesh group (P2), the average number of osteoclast cells (15.33) decreased. Meanwhile, the lowest average number of osteoclasts (11.82) was found in the 60-mesh group (P3). This indicates an effect of the particle size given in tooth grafting on the number of osteoclast cells. In a study that observed comparisons of osteoclast cells with HAp substitution and without HAp substitution, both showed that, on the 14th day, osteoclasts began their activity in resorbing bone. The number of osteoclasts without HAp substitution appeared to be much higher than the number of osteoclasts substituted by HAp.[30,31] The content of calcium and phosphate in HAp can reduce osteoclast resorption without inhibiting the process of osteoclastogenesis.[32]

Tooth graft has a composition that is almost identical to the composition found in bone. HAp is one of the inorganic substances contained in tooth graft. HAp has osteoconductivity that can stimulate osteogenesis, meaning that tooth grafts can become a medium for stem cells and actively stimulate osteoblasts from surrounding tissues to proliferate and differentiate in the regeneration of new bone. There is a calcium phosphate compound that is able to reshape bone when graft is implanted by interacting reciprocally.[11] The shape of the tooth graft used in this study was powder. Powder types were filtered based on particle size, inter-particle porosity, blood wettability, osteoconductivity, and slow substitution.[9]

In this study, there were several other factors that could have affected the results. One such factor was the size of the gap between the particles of the graft material, which might have affected the mechanism of bone healing. Nam et al.[21] state that the size of the gap between the particles is one of the factors that influence the ideal balance of angiogenesis and osteogenesis in the bone healing process. Proliferation and inflammation of the connective tissue formed in the space between the bone and bone graft proves the occurrence of fusion and osteogenesis. The

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research also found that graft particles measuring 250–1000 microns with a space between particles above 200 microns were effective in increasing the osteogenesis process. The distance between the particles plays an important role in the bone healing capability of a graft. The distance between large particles makes the blood clots retention is hindered, whereas the distance between particles that are too close together can prevent blood vessel ingrowth. This study has not provided information on the size of the gaps and pores in tooth graft–based bone graft, indicating an area for further research.[21]

Based on the results of the study, there were significant differences in the number of new blood vessels in each group. It can be concluded that tooth graft implantation of different particle sizes will result in different numbers of new blood vessels in the bone healing process for bone defects of Wistar rats, which validates the research hypothesis. The authors conclude that the 60-mesh group had the best osteogenesis capability with the highest number of osteoblasts and the least number of osteoclasts compared to the other treatment groups.

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