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Original article

The effect of nanoparticle tooth grafts on osteoblast stimulation in the first stages of the bone healing process in Wistar rats compared to the micro-tooth graft technique

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ABSTRACT

Background: *The use of a bone graft in bone regeneration is challenging. Tooth graft material has been used as a bone graft alternative due to its similar composition of organic and inorganic materials close to the bone. Recently, nanotechnology has been used to improve bone graft quality. The osteoconduction rate in the defect area represents the bone graft quality.* ***Purpose:*** *This study aimed to compare the number of osteoblasts using nano-tooth grafts and micro-tooth grafts in Wistar rats.* ***Methods:*** *Wistar rats were divided into six groups: the negative control groups (examined on days 7 and 14), the micro-tooth graft groups (examined on days 7 and 14), and the nano-tooth graft groups (examined on days 7 and 14). The control group received nothing, the micro-tooth group received a micro-size tooth graft, and the nano-tooth graft group received a nano-size tooth graft on the injured femur. Histological observations of osteoblasts were carried out using a light microscope with 1000x magnification. Data were analyzed using one-way analysis of variance and least significant difference tests.* ***Results:*** *On day 7, the nano-tooth graft group showed a higher osteoblast number (11.75) than the micro-tooth graft group (7.5) ($p = 0.039$). There was no significant difference in the micro-tooth graft group compared to the control ($p > 0.05$). On day 14, the nano-tooth graft group showed a decrease in osteoblast number close to normal (control) ($p > 0.05$), while the micro-tooth graft group still experienced significant elevation.* ***Conclusion:*** *Nano-tooth grafts accelerate the stimulation of osteoblasts in the first stages of the healing process compared to micro-tooth grafts.*

Keywords: *micro hydroxyapatite; nanohydroxyapatite; osteoblast; tooth graft*
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INTRODUCTION

The maxillofacial bone defect is common and can be caused by trauma, neoplasms, infections, and congenital abnormalities. Reconstruction of bone defects is still challenging, as the healing process is often impaired or even fails. Bone graft is often used in bone reconstruction, as it is expected to help accelerate the healing process.[1]

Bone grafting is a surgical procedure to replace missing or damaged bone using materials from the patient's body: artificial, synthetic, or natural.[2] A bone graft can be taken from a donor and substituted into the damaged bone tissue. Bone grafts should have three basic functions: osteogenesis, osteoinduction, and osteoconduction.[1] Osteogenesis is the graft's ability to produce new bone.[2] Osteoinduction is the differentiation induction of polyvalent stem cells from the surrounding tissue to an osteoblastic phenotype to form bone. Osteoconduction is the ability to carry out and promote bone growth by penetration of capillaries and bone-forming cells into the graft material.[3]

Bone grafts should be biocompatible, have good mechanical properties, and be easy to manipulate. There are four kinds of bone grafts: autograft, xenograft, allograft, and alloplastic or alloimplant synthetic material. Autografts, using bone graft from the patient's body, are still the primary option for bone defect reconstruction. Allografts use tissue from other donors within the same species, while xenografts use tissue from other species. Materials of tissue rehabilitation should have the same characteristics as natural bone.[1]

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Teeth are composed of both organic and inorganic mineral components which include calcium phosphate, collagen, and other organic elements. Several teeth components, such as hydroxyapatite (HA) and beta-tricalcium phosphate, are known to be osteoconductive and biocompatible to bone.[4] Kaur[5] found recycled dentin roots should be used in bone grafts since they induce new bone formation in the periodontium. Dental cementum and its progenitor cells also have several growth factors, including transforming growth factor (TGF)-beta and insulin-like growth factor (IGF)-I.[6] Among them, HA is one of the ceramics with good biocompatibility properties, as its mineral content is chemically and physically the same as human bones and teeth.[1] In recent years, nano-sized HA, with a particle size of 100 nm in at least one direction, is believed to have high osteoconduction and possibly can be used in bone grafting.[7]

Research has shown that micro-sized HA grafts have lower mechanical strength, so they cannot be used in areas that bear high loads. Nano-HA has biocompatibility, superior bioactivity, and osteointegration ability and causes a lower inflammatory reaction compared to micro-HA.[8,9]

Osteogenesis involves osteoblasts, osteoclasts, and osteocytes.[10] Osteoblasts contribute to bone matrix mineralization through the secretion of type I collagen and release calcium, magnesium, and phosphate ions. In both the embryonic and fetal stages, osteoprogenitor cells are active precursors to osteoblasts. However, when mature, these cells are resting and require stimulation to proliferate and differentiate into osteoblasts. The osteoconductive properties of HA promote the proliferation and differentiation of osteoblasts and accelerate vascularization.[11] HA particle size is considered to affect the substance absorption in the bone graft. Therefore, this study aims to determine the number of osteoblasts at the bone healing stage after implantation of nano- and micro-sized tooth graft materials in the femurs of Wistar rats.

MATERIALS AND METHODS

This research used 30 Wistar rats (*Rattus norvegicus*). The inclusion criteria were that they were male, three months old, and had a mass of 250–300 g. The rats were then divided into six groups. They were kept in the cage for seven days to adapt and fed with 50 g pellet each day. These study methods were approved by the Brawijaya University Research Ethics Commission (certificate number 008-KEP-UB-2021).

Tooth grafts were made by selecting post-extraction human teeth that had no filling. These were then washed and disinfected using 70% ethyl alcohol. Any soft tissue that still adhered was removed.[12] The teeth were then stored and sent to the National Nuclear Energy Agency of Indonesia (Badan Tenaga Nuklir Nasional) for powder production by ball-milling with a ratio of 1:5 (ball:powder) at 250 rpm for five hours. Next, the powder was filtered and dried (100°C, 24 hours), and then sieved until the microparticles formed. To convert the particles into nanoparticles, the sample was prepared, then micro-sized tooth graft powder was dried for 1.5 hours at 60°C. Wet milling was carried out with a ball-to-powder ratio parameter of 1:5 for 5 hours. After that, the suspension was filtered using filter paper and re-dried at 60°C for 1.5 hours. The powder was then ground with a mortar and sieved. X-ray diffraction was used to determine the chemical composition, phase identification, and crystallization size.

The rats were divided into six groups: the day 7 groups (control, nano-tooth graft, and micro-tooth graft) and the day 14 groups (control, micro-tooth graft, and nano-tooth graft). The bone defect in rats was made by applying 2.5 ml of ketamine and then antiseptic on the surgical area using 10% povidone-iodine. An incision was made horizontally on the femur of the Wistar rats. The full-thickness mucoperiosteal flap was opened with a rasparatorium. A circular defect was made in the femur with a diameter of 2 mm using a round bur on the right lateral femoral condyle and irrigated with 0.9% NaCl. On days 7 and 14, the defects in the control group were allowed to fill with blood; in the micro-tooth graft group, they were filled with tooth grafts measuring 250–710 μm, and in the nano-tooth graft groups, they were filled with tooth grafts with an average size of 484 nm. The tooth graft was applied while the rats were still under anesthesia. After being treated, the flap defect was closed and sutured using a simple interrupted technique. The rats were injected with 0.2 ml of amoxicillin and 0.2 ml of Novalgin for three days.

On days 7 and 14, after the administration of the tooth grafts, all rats were injected with ketamine and decapitated. The femur of the rat was removed and immersed in 10% formalin. The femur was then prepared for histological analysis of the osteoblast count using hematoxylin-eosin staining. Observations were carried out using a light microscope with 1000x magnification. Data were analyzed using one-way analysis of variance (ANOVA) and least significant difference (LSD) tests using the SPSS Version 19 software (IBM Statistic, USA). Statistical significance was determined when $p < 0.05$.

RESULTS

In this study, we counted the number of osteoblasts in the bone healing process in the femur of Wistar rats. The nano-tooth group showed the highest osteoblast mean number (11.75) on day 7 compared to the micro-tooth (7.5) and control (7.0) groups. Meanwhile, on day 14, the micro-tooth graft group showed a higher osteoblast number (11.75). On the other hand, the nano and control groups showed a lower osteoblast number (Figures 1 and 2).

The one-way ANOVA test showed there were significant differences in the osteoblast counts on both the day 7 and day 14 observations ($p < 0.05$). The LSD test showed that on day 7, there was a significant difference in

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the number of osteoblasts between the control group and the nano-tooth graft group ($p = 0.024$). On day 7, the micro-tooth graft group significantly differed from the nano-tooth graft group ($p = 0.039$). The control group had no significant difference from the micro-tooth graft group ($p > 0.05$). On day 14, the number of osteoblasts in the micro-tooth graft group was significantly higher than that in the control group ($p = 0.028$), while the control group and nano-tooth graft group had no significant difference (Table 1).

DISCUSSION

This study showed that the osteoblast count on the defective femur receiving the nano-tooth graft was higher than in the control and micro-tooth graft groups. This study supports the previous study that revealed that nano-HA could increase proliferation, differentiation, adhesion strength, osteoblast scattering, bone morphogenic protein (BMP) expression, and bone re-conduction properties.[13] Therefore,

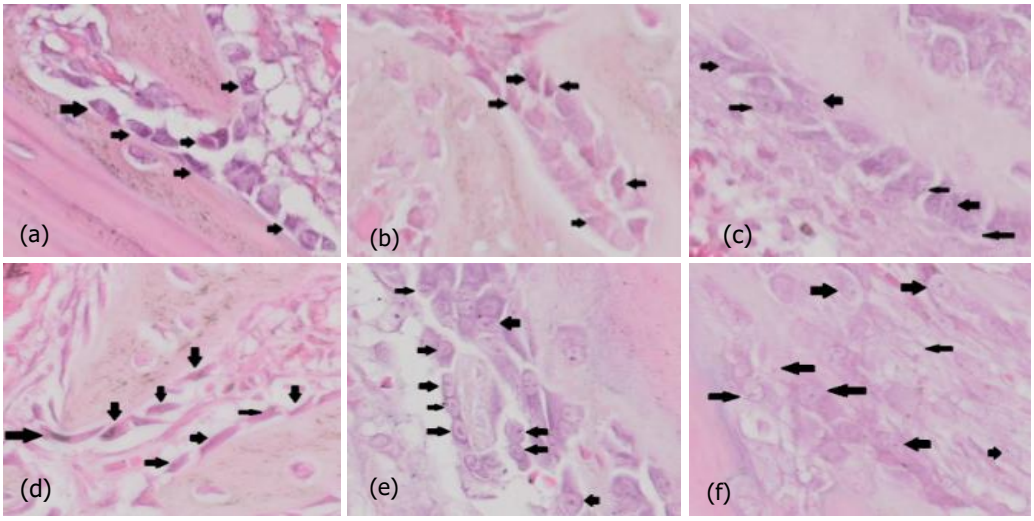

Figure 1. Osteoblasts (arrows) with 1000x magnification in the wound area of femurs of Wistar rats with hematoxylin-eosin staining: (a) Control group day 7, (b) micro-tooth graft group day 7, (c) nano-tooth graft group day 7, (d) control group day 14, (e) micro-tooth graft group day 14, (f) nano-tooth graft group day 14.

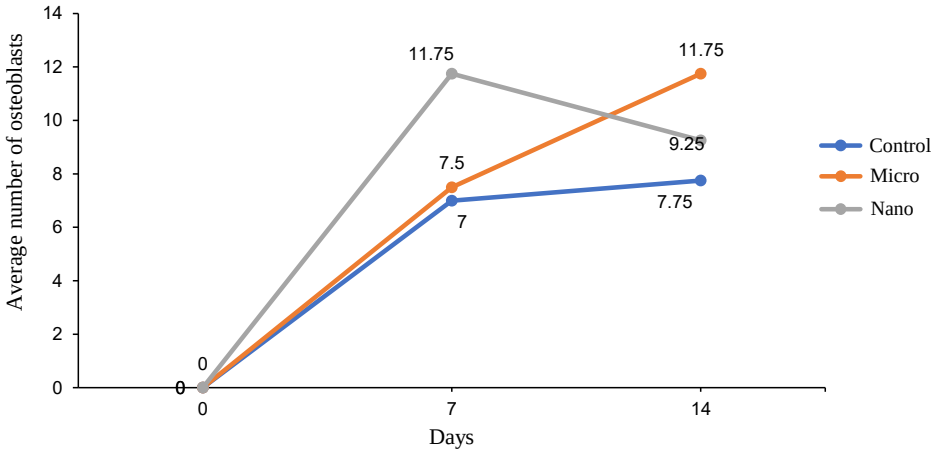

Figure 2. The average number of osteoblasts.

Table 1. The post hoc least significant difference test between groups

Observation time	Between groups		*p*-value
Day 7	Control	Micro-tooth graft group	>0.05
	Control	Nano-tooth graft group	>0.05
	Micro-tooth graft group	Nano-tooth graft group	0.039
Day 14	Control	Micro-tooth graft group	0.028
	Control	Nano-tooth graft group	>0.05
	Micro-tooth graft group	Nano-tooth graft group	>0.05

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using nanotechnology in tooth graft materials can improve the osteoconductive properties of bone grafts. The dimensions and shape of the pores are critical in the process of osteointegration. Nano-HA interconnected between pores with a rough surface will facilitate osteoblast cells' penetration and attachment. The surface of bone mineral crystals with nano-sized particles is large, and absorption by osteoclasts can occur evenly because these crystals present in the organic matrix have very loose connections to each other.[3] In vitro and in vivo studies of calcium ion release from HA powder using nanoparticles have shown similar results to bone apatite and are much faster than HA powder with microparticle size. The size reduction of enamel and dentin to the nanoparticle in nanotechnology applications can produce the crystalline degree and properties of the HA content close to natural sources of HA in bone.

Compared to nano-HA, microcrystalline HA has a low level of solubility, so it is not easily absorbed. Based on a study conducted by Pascawinata et al.[14], when implanted in bone defects, this HA can be well covered and even partially replaced by new bone. However, the resorption process takes a long time and is still seen in the experimental animal after nine months. Nanocrystalline HA (10–100 nm) resembles the natural form of HA found in bone more than microcrystalline HA. Moreover, it has tighter contact with the surrounding tissue, and many molecules on its surface are more quickly absorbed. The ideal implant material should have a resorption capacity equal to the time of new bone formation. Osteoblasts and osteoclasts that adhere more to the nanocrystalline HA accelerate the healing process of bone injuries.[14]

As well as the particle size, tooth graft powder also contains some beneficial components for cell proliferation. Ninety percent of the organic components of teeth are type I collagen and contain growth factors such as IGF-II, BMP2, and bone-like TGF-beta. BMP2 is present in the enamel and is potentially osteoinductive.[14] Cementum and dentin have other growth factors, including platelet-derived growth factor, IGF, and fibroblast growth factor. According to research, autogenous demineralized dentin matrix is chemotactic for osteoprogenitor cells and osteoblasts, encouraging the faster process of bone repair in bone defects.[15]

In this study, the number of osteoblasts in the nano-tooth graft group increased on day 7 compared to day 14, while the micro-tooth graft group found that the number of osteoblasts on day 7 was lower than in the nano-tooth graft. This shows that there is a faster maturation process of osteoblast precursors in nano- compared to micro-tooth grafts. Research conducted by Li et al.[16] showed that nanoparticles can accelerate surrounding stem cells to differentiate into osteoblasts. Research conducted by Mahmoud et al.[17] also proves that surface modification of titanium using nanotubes can increase and accelerate the differentiation of osteoblast cells. Furthermore, research conducted by Wu et al.[18] found a significant osteoblast proliferation acceleration by using titanium nanomaterials.

On day 14, there is a difference in osteoblast number between the nano-tooth graft and micro-tooth graft groups. Even though the nano-tooth graft group had the highest osteoblast number on day 7 compared to the other groups, it shows a depletion on day 14. However, the osteoblast number is still close to control. The micro-tooth graft still promotes osteoblast proliferation. The decrease of osteoblasts in the nano-tooth graft group may be explained by Brannigan et al.[19], who stated that in the bone healing process, the first two weeks are the most critical period, and the differentiation of mesenchymal cells into osteoclasts and osteoblasts occurs around the second week.[22] In the second week after injury, mature osteoblasts turn into osteocytes, while others remain on the surface of the periosteum and endosteum.[20]

According to a study conducted by Griffin et al.[21], nanoparticle grafts increase the adhesion and proliferation of osteoblasts compared to microparticle grafts. Nanostructure scaffold surfaces have increased surface area and improved protein attachment, which can stimulate cell attachment, compared to micro-size scaffolds.[21] Liang et al.[22] also demonstrated that the use of nano-HA increases the induction of osteoblasts.

This study also shows that nano-tooth grafts promote faster osteoblast proliferation in the first week after bone injury. A previous study showed cell proliferation in osteoblast cell cultures after seven days.[13] The activity of osteoblasts in bone remodeling then begins in the second week.[23] Micro-tooth grafts provide slower osteoblast proliferation in the first week after bone injury. Nano-tooth graft groups need one week to accelerate osteoblast proliferation, while the micro-tooth graft group needs two weeks. In conclusion, nano-tooth grafts accelerate osteoblast stimulation in the bone healing process more so than micro-tooth grafts. Nano-tooth grafts accelerate and shorten the healing process because osteoblasts are stimulated faster, i.e., on the 7th day, while the peak of osteoblast proliferation in micro-tooth grafts happens on the 14th day. Even though nano-tooth grafts have the potential to induce osteoblasts, further experiments are needed to evaluate toxicity and other negative effects.

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