RESEARCH ARTICLE

The effect of bone graft substitute in healing fractures with bone defects through examination of alkaline phosphatase and radiology in the murine model (*Rattus norvegicus*) Wistar strain [version 1; peer review: awaiting peer review]

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Abstract

Background: A significant bone defect is a condition wherein the bone cannot repair spontaneously. Therefore, replacing bone defects with bone substitution remains a reconstructive concern for orthopaedic surgeons. Bone Graft Substitution (BGS) are classified broadly, such as bone grafts (autograft, allograft, and xenograft) synthetic ceramics (hydroxyapatite, calcium sulphate). This study aims to determine the effect of various Bone Graft Substitute on the healing process of bone defects assessed based on the area of callus formation and levels of alkaline phosphatase (ALP).

Methods: The study design was an in vivo laboratory experimental approach with a randomized post-test only control group design. The 20 experimental animals that matched the inclusion criteria were divided into five groups, in each one of control positive group, one of control negative group, and three of treatment group. The bone graft substitution used in this study is a synthetic ceramic, namely Synthetic HA-Ca₁₀(PO₄)₆(OH)₂ - BONGROS®, Bone Graft Substitution Nanocrystalline HA-CaSO₄-PEROSSAL®, and also hydroxyapatite Bovine. After selecting rats, we performed osteotomy on the femur to the made bone defect. After 30 days, murine models were harvested. Then, we measure callus formation using radiological examination and ALP level serum

Results: From Callus formation, Nanocrystalline HA-CaSO₄ is the highest (86.54 ± 4.24604) compared with other groups and significantly (p:0.021) increase in callus formation than the other experimental groups. Then, from the ALP level, Bovine is the highest (9.287 ± 0.58586) but did not significantly compare with K-neg, and the second one is Nanocrystalline HA-CaSO₄ higher than KP-1, and it has a significantly higher levels serum ALP rather than K-Neg.

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Conclusion: Bone Graft Substituted using Nanocrystalline HA-CaSO₄ is a good material that can repair and increase callus formation in fracture model rats with bone defects.

Keywords

Bone defect, Substitute bone graft, Tissue engineering, Nanocrystalline, Bovine bone, Hydroxiapatite

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Introduction

The human skeleton possesses an extraordinary capacity for self-regeneration injury.¹ However, spontaneous bone healing is not always appropriate in some conditions.² A significant bone defect is a condition wherein the bone cannot repair spontaneously. This disorder occurs as a consequence of severe trauma or malignancy that damages bone tissue.² If this condition is ignored, it will cause deformities that are significant causes of morbidity and a significant economic impact on health services.³ Therefore, Replacing bone defects using bone substitution remains a reconstructive concern for orthopedic surgeons, and decision-making on possible choices remains problematic.³

Bone substitutes are being significantly in the field of orthopaedic surgery such as, reconstruction, revision prosthetic surgery and spine surgery, etc.⁴ Bone Graft Substitution (BGS) are classified broadly as bone grafts (autograft, allograft, and xenograft), synthetic ceramics (hydroxyapatite, TCP, calcium sulphate), and growth factors (DBM, PRP, BMP'S).^{5,6} Bone substitutions ideally induce new bone formation neovascularization such as being osteoconductive and osteoinductive and structurally similar with originally bone^{7,8}

In the large bone defects condition, the bone healing process is insufficient to cover this damage. In these circumstances, they are the most preferred method for bone replacement. Autograft is the gold standard substance in bony defect repair.^{4,6} However, Autograft harvesting requires additional surgery at the donor site, which can result in some complications, most usually acute and potentially long term pain.⁹ On the other hand, allografts can also be used as bone substitutes, but it is reported that there is a risk of disease transmission and requires high costs^{6,10} Therefore, an alternative to bone graft is synthetic ceramics (SC) such as hydroxyapatite and calcium sulphate base. The SC also can be used as a consideration for bone substitution. SC typically promotes bone healing but have limited material properties, requiring stable hardware fixation.⁴ Due to their widespread availability, low cost, and low risk of morbidity associated with donor site and viral transmission can be alternative to both autologous and allogeneic graft choices.^{1,7} Not only SC but also xenograft can be alternative for bone substitution such as xenograft that consist of Bovine⁶

Within the broad of biomaterial used in orthopaedic surgery, most widely used. Because of the high temperature during production, the density may be elevated while the porosity is decreased.¹¹ Therefore, recently developed material with nanostructured material consisting of Nanocrystalline hydroxyapatite (HA-SiO) embedded in a silica gel matrix to solve this problem.¹² Not only (HA-SiO) but also bovine bone mineral (DBBM) reported well-documented as a xenogenic bone replacement. In addition, After organic components are removed, DBBM is essentially hydroxyapatite (HA) ceramic.^{7,13}

Of the various existing bone grafts, it is required to establish parameters to evaluate the efficiency of bone graft on bone healing. There are radiological and blood level parameters. One of these blood level parameters is Alkaline Phosphatase (ALP), in which changes in alkaline phosphatase (ALP) activity are identical to changes in ALP levels. Therefore, an increase of ALP is commensurate with an increase in callus formation.¹⁴

We hypothesizes that Bone Graft Substitute administration have several potentials to increase healing process for bone defect. Therefore, this study aims to determine the effect of various Bone Graft Substitute administration on the healing process of bone defects, which were assessed based on the area of callus formation and levels of alkaline phosphatase (ALP).

Methods

Study design and animal model

The study's design was an *in vivo* laboratory experimental approach with a randomized post-test only control group design. In this study, the parameters measured are the results of the treatment of researchers.

The study began by choosing rats that completed the inclusion and exclusion. The population was male rats, Rattus novergicus strain Wistar, aged about six months. The sample was used Wistar rats with male gender, aged $\pm$ three months or 12 weeks with a body weight of 200-280 grams, healthy, active, without limb deformities, and with no history of therapy or chemical administration that refers to inclusion criteria. Then, for the exclusion criteria were defects in the extremities, infection of the extremities before and after treatment, sick or died during the treatment period, and not having liver disorders.

The sampling technique in this study uses simple random sampling. We used Federer formula for determining a sample size, after the 20 experimental animals that matched the inclusion criteria, we divided into five groups using: four rats are the positive control group, four rats are the negative control group, and the rest twelve rats are the treatment group, with each group consisting of four rats. The Negative group is normal rats without fracture and bone defect. The positive group

is the murine model with fracture and large-sized bone defect but without bone graft substitution application. Lastly, the experimental group animal will be made to experience fractures with bone defects with substitution bone graft administration according to the group.

Then, we performed an examination, ALP levels and radiology to assess the extent of callus formation 30 days after surgery. The bone graft substitution used in this study is a synthetic ceramic, namely Synthetic HA- $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ - BONGROS®, Bone Graft Substitution Nanocrystalline HA- CaSO_4 -PEROSSAL®, and also hydroxyapatite Bovine. Therefore, the samples in this study were divided into five groups consisting of one control positive group, one negative control group, and three treatment groups. The treatment groups consist of K-P1 to K-P3 groups was a group of model rats that received synthetic HA- $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (K-P1), HA- CaSO_4 nanocrystalline (K-P2), and BGS bovine HA (K-P3), respectively.

Study procedures

The samples were acclimatized, maintained, and manipulated in the authors' institution's Physiology Laboratory. This study was conducted from March 2020 to December 2020. The Ethical Committee of Medical Research Faculty of Medicine Universitas Brawijaya has approved all protocols with number 160/EC/KEPK-PPDS/09/2020, and all subsequent experiments were carried out according to the ARRIVE guidelines and regulations. All animals were maintained in authorized vivariums under standard conditions with gentle handling, daily cage cleaning, and regular monitoring to minimize animal suffering.

Study procedures for fracture model with bone defect and application of bone graft substitution

After selecting rats that met the inclusion and exclusion criteria and were acclimatized for seven days, then, we carried out sterilization of all surgical instruments and instruments prior to surgery and made sure at the time of the operation, and keeping the weight of the rat's ranges from 200-250 grams because the heavier the rats are, the size of the fixator used will be different.

Fracture Surgical Procedure with bone defect and POP placement was a preparation the rats in the operating room, performing anaesthetic measures by administering an injection of ketamine 100 mg/kgBW and xylazine hydrochloride 10 mg/kg intraperitoneally, injection antibiotics (cefazolin, 20 mg/kgBW) into the right leg, disinfecting, placing the rat in the prone position and covered the operating area with a sterile cloth. Following made a 3-4 cm incision in the skin along the lateral cranio surface of the right femur from the greater trochanter to the supracondylar region of the knee using a scalpel. Exposure to the femur shaft with a gentle dissection separates the lata fascia and ensures that muscle tissue is not cut. After that, separate the vastus lateralis and biceps femoris muscles and lift the tensor fasciae latae to expose the entire length of the femur (make sure the sciatic nerve is preserved). In the area where osteotomy is performed, prepare the femur along the middle area of the diaphysis by separating the tissue around the femur. For a 3 mm bone defect, it is done by cutting the midshaft area of the femur with a 3 mm Kerrison, covering the muscle layer and fascia lata using ethibond vicryl suture 4-0 and skin with ethicon monocryl 3-0. Fixation is done using POP attached to the proximal femur to the ankle with the knee flexing 90°. In the first three days post-operative action, giving analgesics to rats every eight hours (ketorolac 5 mg/kgBW IM) and antibiotics 24 hours post-action (cefazolin 20 mg/kgBW IM) and monitoring rats regularly. Monitoring was done periodically for 30 days.

Laboratory analysis with ELISA method

After 30 days, murine models were harvested. The area of bone defect with callus formation was collected by taking the femur of the rats and then measured with the radiological examination. Then, the levels of serum ALP were assessed using the laboratory examination method.

Statistical analysis

The steps for testing the comparative hypothesis are as follows: data normality test, variant homogeneity test, comparative test using One-way ANOVA test. The data obtained normal distribution but not homogeneous. Therefore, a non-parametric analysis is carried out using the Kruskal Wallis method. All technical data processing results were analyzed computerized using Statistical Product and Service Solution (SPSS) 23.0 with confidence interval 95% (RRID: SCR_002865).

Results

In this study, the first step is to conduct a Kruskal-Wallis test to determine the normality of data,³⁰ and the result is $p > 0.05$, which means the data are distributed normally. Then, a homogeneity test is conducted, and the result is $p < 0.05$, which means the data are not homogenous. Afterwards, the Kruskal-Wallis test is done, and the result is $p < 0.05$. This means there is a significant difference between groups.

Table 1. A comparative test of Bone Graft Substitution on the area of callus formation with Kruskal Wallis Test.

Treatment	Mean $\pm$ SD	p-value
K-Neg	89.725 $\pm$ 17.89	0.006
K-Pos	62.9975 $\pm$ 3.74835	
K-P1 (Bone Graft Substitution with Synthetic HA-Ca ₁₀ (PO ₄) ₆ (OH) ₂ - BONGROS ®)	83.62 $\pm$ 4.09827	
K-P2 (Bone Graft Substitution Nanocrystalline HA-CaSO ₄ - PEROSSAL ®)	86.54 $\pm$ 4.24604	
K-P3 (Bone Graft Substitution with Bovine HA)	79.5725 $\pm$ 6.39162	

Table 2. A comparative test of treatment between Bone graft substitution on ALP level.

Treatment	Mean $\pm$ SD	p-value
K-Neg	11.4983 $\pm$ 0.97128	0.004
K-Pos	2.9 $\pm$ 0.16668	
K-P1 (Bone Graft Substitution with Synthetic HA-Ca ₁₀ (PO ₄) ₆ (OH) ₂ (BONGROS ®))	5.516 $\pm$ 2.37176	
K-P2 (Bone Graft Substitution Nanocrystalline HA-CaSO ₄ - PEROSSAL ®)	5.7928 $\pm$ 0.11703	
K-P3 (Bone Graft Substitution with Bovine HA)	9.287 $\pm$ 0.58586	

Callus formation

In this study, based on the analysis results using Kruskal-Wallis, the p-value is 0.006. it means smaller than $\alpha = 0.05$ ($p < 0.05$). So, from this result, it can be concluded that there is a significant effect of giving Bone graft Substitution on Callus Formation. Furthermore, we measure the area of callus formation with Bone Graft Substitution using Synthetic HA-Ca₁₀(PO₄)₆(OH)₂ is 83.62 $\pm$ 4.09827, using Nanocrystalline HA-CaSO₄ is 86.54 $\pm$ 4.24604, and using Bovine 79.5725 $\pm$ 6.39162, respectively.

Hereafter, we conducted a Post Hoc test for reveals a significant difference between the K-Positive group and the treatment group which received HA-Ca₁₀(PO₄)₆(OH)₂ ($p = 0.065$), nanocrystalline HA-CaSO₄ ($p = 0.021$), and Bovine-HA ($p = 0.0214$) respectively. From the area of callus formation, the highest group is the group that received Nanocrystalline HA-CaSO₄ (Table 1). Therefore, it can be concluded that Bone Graft Substitution using Nanocrystalline HA-CaSO₄ had a significantly greater increase in callus formation rather than the other experimental groups.

ALP Levels

Hereafter, we analyze the effect of the Bone Graft Substitute on alkaline phosphatase (ALP). After that, the Kruskal-Wallis test is conducted, and a p-value of 0.004 is obtained. It means a significant correlation between Bone Graft Substitution treatment with ALP level.

Hereafter, we measured serum ALP level when using Bone Graft and obtained the result with the highest ALP level on Bone Graft Substitution using Bovine 9.287 $\pm$ 0.58586. However, the Post Hoc test reveals no significant difference between the K- Positive group with Bovine (0.375). In contrast, based on post hoc result between K-Positive group with Bone Graft Substitution using Synthetic HA-Ca₁₀(PO₄)₆(OH)₂ ($p = 0.016$), and using Bone Graft Substitution Nanocrystalline HA-CaSO₄ ($p = 0.019$) has significant difference result with ALP level 5.516 $\pm$ 2.37176 and 5.7928 $\pm$ 0.11703, respectively.

From the test above, it can be concluded that Bone Graft Substitution with Nanocrystalline HA-CaSO₄ had significantly higher serum ALP levels than another experimental group because, despite the ALP levels of Using Bovine was higher, it was not statistically significant (Table 2).

Discussion

Ideally, biomaterials for bone graft substitution promote cell attachment, migration, and proliferation, interact actively with cells and tissues, and promote repair and regeneration.¹⁵ In the present study, we compared the effects of administering various types of bone graft substitution. The substitutions bone graft used here includes nanocrystalline HA-CaSO₄, synthetic bone graft HA-Ca₁₀(PO₄)₆(OH)₂ and the local Bovine HA from General Hospital Soetomo, Surabaya.

Previous research *in vivo* study was conducted by Dahabreh 2014 to find the ability of substitution bone graft to support osteoprogenitor cells Osteoconductive and Osteogenesis, Based on the many benefits obtained from this bone graft substitution.¹⁶ In this study, *in vivo* research was conducted to assess several research variables, namely the effect of bone graft substitution through radiological examination to assess the extent of callus formation and a marker of osteogenesis and ALP levels through laboratory examination.

Effect of Bone graft substitutions on Callus Formation

In this study, there was a significant difference between the area of callus formation on the provision of Bone Graft Substitution material ($p = 0.006$). This study's highest average callus formation area was in the K-P2 group that use Nanocrystalline HA-CaSO₄ materials. A study by Park *et al.* stated that nanocrystalline has the capability to provide a material suitable for healing bone defects, and also Nanocrystalline HA-CaSO₄ can provide as a reservoir and/or release agent for a growth factor that assists in bone regeneration or bone healing.¹⁷ In addition, Götz 2008, mentioned that nanocrystalline processes has osteoconductive capabilities and are rapidly absorbed into the host's natural bone turnover.¹¹ Therefore, it may be relevant to our finding in this study that nanocrystalline provided bone repair better than other bone graft substitutions. A study by Gerike *et al.* in line with our finding that bone defects treated with nanocrystalline were mostly totally repaired.¹²

Then, Synthetic HA-Ca₁₀(PO₄)₆(OH)₂ also be considered for the material as Bone graft Substitution. From this study, it was found that using bone substitution such as Synthetic HA-Ca₁₀(PO₄)₆(OH)₂ had a better area of callus formation (83.62 ± 4.09827) than without using Bone graft substitution (K-Pos) (62.9975 ± 3.74835). Although, the result below uses Nanocrystalline HA-CaSO₄. Some studies state that Synthetic HA-Ca₁₀(PO₄)₆(OH)₂ has several advantages, one of which is for osteomyelitis and increased healing rate in patients with spondylitis.¹⁸ Von Stechow in 2019 reported that in patients with osteomyelitis, it is reported that synthetics have been successful in treating 12 patients from 19 with osteomyelitis using Synthetic HA-Ca₁₀(PO₄)₆(OH)₂.¹⁹

Bovine-derived xenografts are reported that give structural integrity and simplicity of usage in reconstructive foot surgery. However, there are currently just a few trials evaluating the efficacy of bovine bone xenografts.²⁰ This study found that the lowest area of callus formation was the administration of Bovine HA only (K-P3, 79.5725 ± 6.39162). This result is relevant to the study by Mahadhipta and Kamal, 2013 shows that Bovine has lower biocompatibility compared to other synthetic ceramics HA.²¹ This is presumably because physically, the pores of the Bovine material are not as many as the synthetic HA. The pores on the bone graft allow vascular growth into the pores and aid in the bone healing process. This is in line with other studies showing that local Bovine has the lowest toxicity and viability with cell proliferation inhibition compared with other bone graft substitution (HA-CaSO₄, HA-nanoparticulate paste, HA-synthetic).²¹ However, the study conducted by Musson *et al.* reported that Bovine has a potent anabolic and catabolic activity that use for Osteoinductive activity, although in his study also did not find improving bone healing on bone defect condition.²² This statement in line with research was conducted by Musson, Kübler 2004 stated that bovine bone has a high porosity and big granule size, which promotes osteoblast adhesion and protein structure preservation. These morphologic features act as an effective spacer and as a bone formation-guiding matrix.²³

Effect of bone graft substitution on ALP levels

ALP has an important role in the osteoid formation and bone mineralization. Serum ALP can be used as a marker to monitor the bone healing process in bone fractures.²⁴ In this study, there were significant differences between ALP levels with the bone defect given bone graft substitution.

The activity of alkaline phosphatase (ALP) may be used to monitor the process and degree of bone healing after significant fractures.¹⁴ Therefore, it is important to examine the ALP level to determine the level of bone healing in fracture cases. ALP examination is recommended to be performed 21 days after injury. According to Bowles's study, in the first week, inflammation might decrease and is considered a consequence of the systemic inflammatory response.²⁵ Moreover, in this study, we examined the ALP level after 30 days to reduce the potential bias of the ALP levels.

The treatment group which received Bone Graft Substitution using Nanocrystalline HA-CaSO₄ has significantly higher levels of ALP than K-Negative, even though the highest were used with Bovine as a bone graft substitution (9.287 ± 0.58586). Chiang, 2016 stated that using nanocrystalline as bone graft substitution can increase the activity of ALP. He believes that Nanocrystalline released concentrations and multiple complex growth factors can be important elements regulating the microenvironment to promote bone mineralization.²⁶ This finding is in line with a study by Laurel, 2017. His study found that the level of alkaline phosphatase (ALP) activity in the nanocrystalline group was higher than that in the control group (cells only in culture well) and nanocrystalline and Collagen alone groups.²⁷

From callus formation and increasing ALP levels serum, nanocrystalline HA-CaSO₄ is a good bone graft substitution material for bone defects. This result may be explained by the fact that one of the nanocrystalline components contains calcium sulfate, where calcium sulfate has good characteristics, such as biodegradable, well-tolerated and osteoconductive bone graft substitution.²⁸ In another study, calcium pellets have been applied in patients with unicameral bone cysts to fill metaphyseal gaps in the humerus, femur, and calcaneus. These pellets are inserted into the cavity and, the cavity can be monitored due to radiopaque view for complete filling. After a 37-month follow-up, 80% of patients achieved a complete or partial response to one therapy, with a cumulative healing rate of 100% for patients who got up to three treatments.²⁹ Therefore, we can conclude that nanocrystalline HA-CaSO₄ is a good bone graft substitution material for bone defects, especially in bone femur defects.

This study also has some limitations, The basic material used in this study is a mixture of several materials, so there is a possibility of bias in this study. The preliminary research that discusses the biocompatibility, viability, and adhesion of the Nanocrystalline HA-CaSO₄ is needed to assess the ratio of each Bone Graft Substitution. Then, there is fluctuation in ALP levels, so further research should be carried out by examining serial ALP levels.

Conclusion

From callus formation and increasing ALP levels serum, nanocrystalline HA-CaSO₄ is a good bone graft substitution material for bone defects. Therefore, Bone Graft Substituted using Nanocrystalline HA-CaSO₄ can repair and increase callus formation in fracture model rats with a bone defect.

Data availability

Underlying data

Zenodo: The Effect of Bone Graft Substitute in Healing Fractures with Bone Defects Through Examination of Alkaline Phosphatase and Radiology in the Murine Model (*Rattus norvegicus*) Wistar strain, <https://doi.org/10.5281/zenodo.6204667>.³⁰

This project contains the following underlying data:

- Raw Data BGS.sav (ALP Level, Radiology data for callus formation)
- Raw Data BGS.xlsx (ALP Level, Radiology data for callus formation)

Reporting guidelines

Zenodo: ARRIVE checklist for The Effect of Bone Graft Substitute in Healing Fractures with Bone Defects Through Examination of Alkaline Phosphatase and Radiology in the Murine Model (*Rattus norvegicus*) Wistar strain, <https://doi.org/10.5281/zenodo.6204667>.³⁰

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