ORIGINAL ARTICLE

In vivo study of bovine hydroxyapatite-gelatin-hydroxypropyl methylcellulose with alendronate as injectable bone substitute composite in osteoporotic animal model

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ABSTRACT

The injectable bone substitute (IBS) is a self-setting local drug delivery system that adjusts the shape of the bone gap in the fracture. This study aimed to examine the effectiveness of IBS composites of bovine hydroxyapatite (BHA) and alendronate (Ale) in accelerating bone growth in osteoporotic rats. IBS was made by mixing BHA with gelatin 5%, hydroxypropyl methylcellulose (HPMC) 2%, and Ale 10%. The physical properties of IBS were viscosity, injectability, and density tests. Twenty-four female Wistar rats were divided into four groups. After 8 weeks, 2 mm gap was made in the right femur of all rats and filled with IBS. The healing process was observed after 6 weeks with X-ray imaging and H and E staining. The obtained results showed viscosity, injectability, and density value of IBS from 30.4 to 39.4 dPa.s, 98.22%–98.64%, and 0.6325–0.8409 g/cm^3, respectively. X-ray imaging and histology results proved the condition of osteoporosis in rats with ovariectomy. The addition of BHA-Gel-HPMC-Ale significantly affected the number of osteoblasts, osteocytes, and osteoclasts ($P < 0.05$). After 45 days of observation, the addition of BHA-Gel-HPMC-Ale showed the highest mean number of osteoblasts, osteocytes, and osteoclasts, which were 25.00 ± 3.00, 64.33 ± 11.15, and 5.67 ± 0.58 compared to BHA-Gel-HPMC and positive control groups. The BHA-Gel-HPMC-Ale IBS has the potential to reverse osteoporosis. Nevertheless, the underlying potential of these biomaterials to reverse osteoporosis needs further research.

Key words: Alendronate, bovine hydroxyapatite, fracture, injectable bone substitute, osteoporosis

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INTRODUCTION

Osteoporosis is a systemic disease characterized by a gradual decrease in bone density (T-score ≤−2.5) and the damage to the bone tissue microarchitecture, resulting in an increase in bone fragility and the risk of fractures or large fractures gap formation. Osteoporosis occurs due to an imbalance

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between osteoclast and osteoblast activity, making the resorption process higher than the bone formation process. Data from the International Osteoporosis Foundation in 2015 stated that 1 in 3 women and 1 in 5 men experienced an osteoporosis-related fracture at 50 years worldwide.[1-3]

The injectable bone substitute (IBS) is a local drug delivery system that adapts the shape of the bone gap to fracture, a minimally invasive treatment method, and an alternative route for delivery of high drug concentration.[4,5] The injectable system was aimed at the bone gap due to osteoporotic cases, which contained inorganic and organic components that resemble the structure of the bone matrix.[6,7]

Alendronate (Ale) is a bisphosphonate drug used to prevent and treat osteoporosis. There are two mechanisms of Ale acting as an inhibitor of the farnesyl pyrophosphate synthase enzyme. This enzyme increases osteoclast activity and binds to calcium from bone hydroxyapatite. As a result, osteoclast activity for bone resorption decreased.[8] In oral use, Ale has low bioavailability (1%–5%). In addition, it also causes side effects, such as gastrointestinal disorders and osteonecrosis of the facial jaw, if consumed for a long time.[3,7,9]

Delivery of Ale in the bone tissue can be supported by bovine hydroxyapatite (BHA). BHA is hydroxyapatite from bovine bone, which has chemical and physical properties similar to human bone. Besides, gelatin (Gel) is a polymer that resembles an organic component of bone tissue. BHA and Gel help accelerate bone growth in damaged bone tissue.[7] In addition, hydroxypropyl methylcellulose (HPMC) is an inert, viscoelastic polymer used for controlled delivery.[7,10] BHA-Gel-HPMC composite could be an excellent delivery system for Ale in the bone tissue.

This study aimed to examine the BHA-Gel-HPMC IBS composite in osteoporotic bone tissue engineering as a filler to strengthen bone mechanical properties and Ale delivery system.

MATERIALS AND METHODS

Synthesis dan characterization of injectable bone substitute

The IBS was synthesized by dissolving Gel powder 5% into distilled water at 40°C for 1 h. BHA was added to the Gel solution with a ratio of 45:55 and stirred for 1 h. Then, Ale was mixed with the BHA-Gel solution with the composition of 10% from the mass of BHA in each sample and stirred until homogeneous. HPMC 2% (w/v) was dissolved with distilled water at 90°C until the temperature decreased to 40°C. The HPMC solution was poured into the BHA-Gel-Ale mixture at 40°C and stirred for 6 h. The physical characterization was carried out by using a viscometer.

The injectability test was carried out by inserting the suspension into a 10 ml syringe with an inside diameter of 2 mm and a 1.2 mm inner diameter syringe needle within 2 min with variations of temperature: 15°C, 25°C, 35°C, and 45°C. The setting time test was carried out by injecting IBS into the HA substrate, then observing the setting process that occurred. The injected suspension in the HA substrate was conducted at 25°C close to room temperature and 35°C close to body temperature.

In vivo test

200–250 g 3 months old Wistar rat (*Rattus norvegicus*) were used in the experiments. This research has been approved by the Ethical Committee in the Faculty of Veterinary Medicine in Airlangga University, Indonesia, with reference number 2.KE.075.05.2018. After an adaptation period of 1 week, 24 rats were randomly divided into four groups: control negative group (healthy group, P1), control positive group (P2), BHA-Gel-HPMC Group (P3), and BHA-Gel-HPMC-Ale (P4). The positive control group underwent ovariectomy without bone filler. After 8 weeks, the pre-surgery treatment was performed by anesthetizing the rat with ketamine (25 mg/kg) and xylazine (8 mg/kg) I. M in the left leg.[11] The incision in the abdomen was performed through linea alba. The ovarium was removed, and the open abdomen was sutured. The osteoporosis took place after 8 weeks.

After 8 weeks of ovariectomy, the treatment of bone filler was performed by anesthetizing the rat.[11] The fracture was made on the rat's femur in the lengthwise direction and incision was performed in the femur until the bone. The drilling on the bone was performed to make a hole with a diameter of 2 mm. The IBS was then injected into the hole, and the opened skin was sutured.

Histopathology

After 6 weeks of bone filler injection, the rats were terminated by using ether anesthesia. The femur bone was stored in the 10% BNF. Bone decalcification was performed in 10% Ethylenediaminetetraacetic acid solution. The bone cell observation was observed with H and E (HE) staining, and the bone gap was observed radiologically. The bone cell observation included osteoblast, osteoclast, and osteocytes. They were observed under a light microscope with a magnification of 400 times.

Statistical analysis

Results obtained are showed as mean ± standard deviation. Independent sample *t*-test was used to evaluate the difference between control positive and negative group. One way analysis of variance was to analyze osteoblast, osteoclast, and osteocytes scoring. $P < 0.05$ was stated statistically significant differences.

RESULTS AND DISCUSSION

Injectable bone substitute characteristics

The viscosity test was carried out to determine the thickness of the suspension synthesized as a function of temperature and ensure the viscosity of the HPMC suspension viscosity standard value of 2% (w/v) as a bone substitute application, which is 40 dPa.s.[7] The IBS viscosity test results obtained viscosity between 30.4 and 39.4 dPa.s. The viscosity of IBS and the temperature of IBS greatly affected by injectability.[5] The percentage of injectability is between 98.22% and 98.64% at 25° C–35°C was able to set and increased the substrate density from 0.6325 g/cm^3 to 0.8409 g/cm^3 according to normal bone density. The physical properties of IBS allowed IBS BHA-Gel-HPMC to be applied to treat osteoporosis.

Anatomical pathology test results

The results of X-ray imaging on the rat's femur after 6 weeks in the P1, P2, P3, and P4 groups are shown in Figure 1. The gap size was calculated using ImageJ v1.44p software, as shown in Table 1. The results showed that osteoporosis had occurred in positive control. In addition, the results of the analysis of P2 showed a significant difference ($P < 0.05$) in the bone gap size compared to P1 [Table 1]. Ovariectomy has been shown to cause osteoporosis. The ovariectomy triggers excessive osteoclast activity, which causes an imbalance of bone remodeling, promoting bone resorption and decreasing bone regeneration.[12] Osteoporosis-related estrogen deficiency suppressed the osteocyte life sustainability and disrupted the physiological response of osteoblasts for mechanical stimulation, damage detection, and bone repair.[13]

Moreover, as shown in Table 2, the positive control groups P3 and P4 were not significantly different ($P > 0.05$) in compare to P2. Ale concentration 10% in BHA-Gel-HPMC used was less than optimal for the balance of bone formation, and the lack of observation time affects the number of cell growth. Ale would suppress osteoclast activity excessively which made bone remodeling suppressed and decrease the bone formation and caused a delay in bone grafting.[14,15] The observation's times played a vital role in assessing the effectiveness of Ale. Based on the remodeling process, bone takes 3–4 months or years to complete the fully regenerated bone structure.[16-18]

Observation of bone cell growth was also carried out for the femoral gap through a histopathological test with HE staining. The cells observed included osteoblast, osteocyte, and osteoclast with a magnification of × 400. The results are shown in Figure 2, and the mean numbers of osteoblasts, osteocytes, and osteoclasts between treatment groups are shown in Table 3. HE staining results showed the number of osteoblasts and osteoclasts in P2 significantly different ($P < 0.05$) compared to P1. The average number of osteoblasts in P2 is 14.33 ± 2.52, while the average number of osteoblasts in P1 is 9.00 ± 1.73 and the average number of osteoclasts in P2 is 10.00 ± 2.00. The number of osteocytes in the P2 was not significantly different ($P > 0.05$) compared to P1. Based on the result of the independent *t*-test in P2, the addition of BHA-Gel-HPMC-Ale (P4) significantly affected the number of osteoblasts, osteocytes, and osteoclasts ($P < 0.05$). After 45 days of observation, the

Table 1: Injectable bone substitute physical properties

Parameter	Value
Viscosity	30.4-39.4 dPa.s
Injectability	98.22-98.64%
Density	0.6325 g/cm^3 to 0.8409 g/cm^3

Table 2: Bone gap size between positive control, bovine hydroxyapatite-Gel-hydroxypropyl ethylcellulose, and bovine hydroxyapatite-Gel-hydroxypropyl ethylcellulose-Ale (mean±standard deviation)

Group	Bone gap size (mm)
Negative control	1.046±0.013
Positive control	1.784±0.027[a]
BHA-Gel-HPMC	1.750±0.017
BHA-Gel-HPMC-Ale	1.752±0.022[b]

[a]$P<0.05$ versus negative control, [b]$P>0.05$ versus negative control and positive control. HPMC: Hydroxypropyl ethylcellulose, BHA: Bovine hydroxyapatite

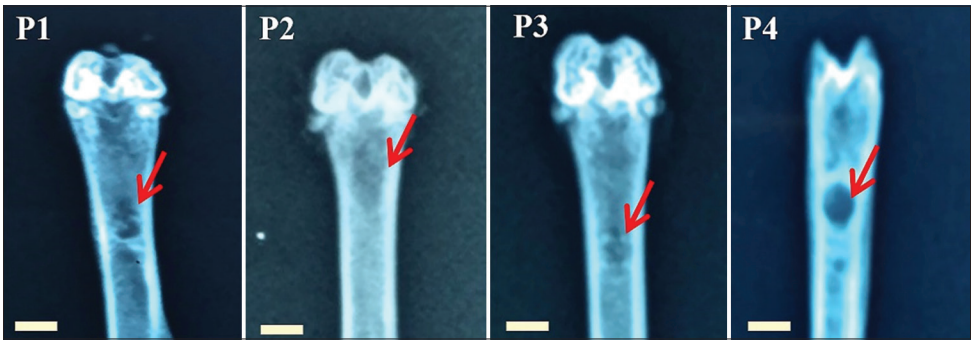

Figure 1: Radiological X-ray image representation of bone gap size: Negative control (P1), positive control (P2), BHA-Gel-HPMC (P3), BHA-Gel-HPMC-Alendronate (P4). HPMC: Hydroxypropyl ethylcellulose, BHA: Bovine hydroxyapatite

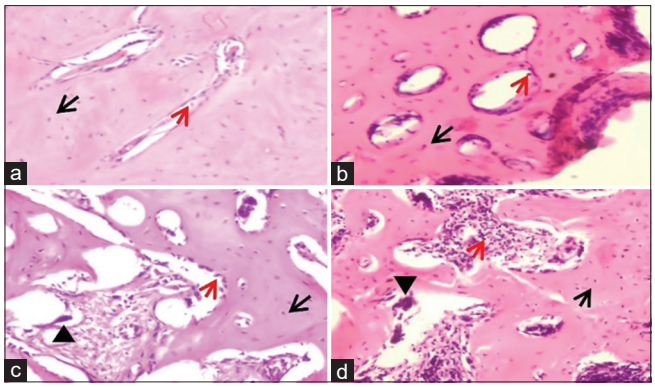

Figure 2: H and E staining results; osteoblasts (red arrow), osteocytes (black arrow), osteoclasts (triangle). (a) Negative control; (b) Positive control: (c) BHA-Gel-HPMC (d) BHA-Gel-HPMC-Alendronate. HPMC: Hydroxypropyl ethylcellulose, BHA: Bovine hydroxyapatite

addition of BHA-Gel-HPMC-Ale (P4) showed the highest average number of osteoblasts, osteocytes, and osteoclasts 25.00 ± 3.00, 64.33 ± 11.15, and 5.67 ± 0.58, respectively, in compared to P3 and P1.

The result showed that the number of osteoblasts, osteocytes, and osteoclasts in group BHA-Gel-HPMC with Ale (P4) showed a significant difference ($P < 0.05$) in compared to P2 and P3. This study showed the same results as the research conducted by Toker *et al*. After 8 weeks, they evaluated the local effect of Ale and calcium phosphate combination on calvarial bone defects in rats.[19] The results showed no significant difference between the two groups (P3 and P4) on the number of osteoblasts or osteoclasts. This result showed that the bone formation process was still happening. When injected into the bone gap, Ale binds to BHA form a very strong bond, while Ale distributed in the Gel-HPMC released and binds to the calcium hydroxyapatite of the bone around the gap. It caused the bone around the gap became solid and hard. Gel-HPMC interacted with osteoblasts to form osteoid. Osteoid turn into osteocytes with bone calcium and calcium from BHA.[7,20,21]

CONCLUSIONS

The results of physical characteristics test showed that BHA-Gel-HPMC-Ale has potential as treatment for osteoporosis. Moreover, X-ray imaging and histopathological tests proved that ovariectomy in Wistar rat resulted in an osteoporosis model. The IBS combination did not accelerate narrowing the bone gap based on the results of X-ray imaging. The results of HE staining showed that the injectable BHA-Gel-HPMC increased the number of osteoblasts, osteocytes, and osteoclasts in ovariectomy rat models. The addition of Ale did not increase the number of osteoblasts, osteocytes, and osteoclasts within 6 weeks of observation in compared to BHA-Gel-HPMC. It is recommend to observe more than 6 weeks to prove the addition of Ale in accelerating of bone growth.

Table 3: The number of osteoblasts, osteocytes, and osteoclasts between positive control, bovine hydroxyapatite-Gel-hydroxypropyl ethylcellulose, and bovine hydroxyapatite-Gel-hydroxypropyl ethylcellulose-Ale (mean±standard deviation)

Group	Osteoblast	Osteocyte	Osteoclast
Negative control	9.00±1.73	15.33±4.51	0.00±0.00
Positive control	14.33±2.52	16.67±5.03	10.00±2.00
BHA-Gel-HPMC	20.33±4.51	55.67±6.03^{b}	2.67±0.58^{c}
BHA-Gel-HPMC-Ale	25.00±3.00^{a}	64.33±11.15^{bb}	5.67±0.58^{cc}

$^{a}P<0.05$ versus positive control and negative control, $^{b}P<0.05$ versus positive control and negative control, $^{bb}P<0.05$ versus positive control and negative control, $^{c}P<0.05$ versus positive control and negative control, $^{cc}P<0.05$ versus positive control and negative control. HPMC: Hydroxypropyl ethylcellulose, BHA: Bovine hydroxyapatite

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Conflicts of interest

There are no conflicts of interest.

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