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IN VITRO GENTAMICIN RELEASE FROM BIOACTIVE BHAG(LENA) IMPLANT AGAINST *Staphylococcus aureus*

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ABSTRACT

Osteomyelitis is a bone-related infectious desease which is difficult to treat, because the antibiotic reaches the target is lower than the MIC and bacteria can't be eradicated. This condition can cause the bacteria become resistant. To solve this problem, we can use local antibiotics as BHAG(ELENA) pellets implant, which can release gentamicin (GEN) continuously for more than a day with a concentration greater than MIC. BHAG(ELENA) pellet that have made contain BHA : GEL = 20 : 2 (dry state); GEN 10% and cross-linking with glutaraldehyde (GA) 0,5%, cylindrical weigh 100 mg; 4 mm in diameter and 3.2 mm thick. The release of GEN from BHAG(ELENA) pellet were tested in vitro, by soaking the pellet in phosphate buffer saline pH 7.4 at temperature 370C. The sample were sampled every day until 28 days. Then, the sample were tested by agar diffusion method that contain Staphylococcus aureus. Results showed that inhibition zone diameter greater than MIC GEN to S. aureus. Within 28 days, the release of GEN provide a total activity 99,24%, it showed that after 28 days, the pellets are still actively inhibit the bacterial growth. Furthermore, required to be tested in animal study (in vivo) with a defect in the femoral bone then filled with BHAG(ELENA) pellets as drug delivery system of GEN and bone fillers.

Keywords: agar diffusion method, Bovine Hydroxyapatite (BHA), gelatin (GEL), gentamicin (GEN), glutaraldehyde (GA) and drug delivery system (DDS).

INTRODUCTION

Osteomyelitis (OM) is a progressive infection of the bone marrow and cortex that resulting an inflammation and bone destruction (Nadeem *et al.*, 2010). If this condition is not handled properly, it can cause tissue damage, functional organ damage and also death of bone with a high degree of morbidity (McNally *et al.*, 2010). Osteomyelitis can occur due to trauma at the bone open fracture, closed fracture, joint replacement, postoperative bone, and infected diabetes mellitus (DM).The incidence of OM due to spinal surgery about 1-5% at the patients with closed fracture; open fracture at the I-III level about 3-50% and 5% of implant use (Suratun *et al.*, 2006). Patients who suffered an open fracture can be OM about 3-25%, whereas the diabetic patients with foot ulcers approximately 15% (Sia et al., 2006).

Bone is one of body part that has hard structure. Because of this condition, not all antibiotics can penetrate and bone trauma causes devascularisation so the antibiotic in the target has smaller concentration than MIC. It will become the antibiotics can not eradicate of bacteria, so this bacteria become resistant. Antibiotics can penetrate biofilms of bacteria colonies OM when the concentrations up to 10 times than the MIC (El-Ghannam *et al.*, 2005). While, the bacterial resistance to the antibiotics can occur due to improper use of antibiotic as too short and the doses are too low (Borhan, 2013).

On the use of parenteral and oral antibiotics, to obtain a sufficient dose to OM therapy needed in high dose and long term. Thus, the antibiotics can penetrate the biofilm of bacteria and bacterial colonies can be eradicated with 10 times MIC (El-Ghannam *et al.*, 2005). However, it will cause some problems include toxicity to the patient; less of compliance and will require a long residence time in the hospital that can harm the patient. One of alternative to solve this problem by using local antibiotic with BHAG (ELENA) pellet implant.

Gentamicin is one of antibiotics that has a broad-spectrum so that capable to control the growth of Gram positive and Gram negative bacteria, water soluble and resistant at body temperature for a long time. While *Staphylococcus aureus* is one of Gram positive bacteria that cause nosocomial infection in many hospitals, especially in the orthopaedi surgical (Jamel, 2011). The BHAG (ELENA) pellet made from bovine hydroxyapatite (BHA) is an of inorganic component of bone and gelatin (GEL) is an organic component of bone as drug delivery system (DDS) and Gentamicin (GEN) as a preventive or therapeutic antibiotics bone infection that has cross-linked with glutaraldehyde (GA) to delay the gentamicin release.

The cross-linking processed of BHA-GEL as drug delivery system with gentamicin (GEN) was done with the aim to forming a covalent bond between GEL and GEN both, of them using glutaraldehyde (GA) as a cross-link agent (Ginalska *et al.*, 2005) with a stationary phase of PET, whereas BHA that used in this study is biocompatible as silence and wrapped by GEL.

The formation of covalent bonds between GEL-GA-GEN lead BHAG(ELENA) pellet is not easily destroyed and will continuously release GEN for a long time with concentration greater than minimum inhibitory concentration (MIC) of *Staphylococcus aureus* (0.4 ppm).

The aim, of this study to make BHAG(ELENA) pellet formula that can eradicate the growth of *Staphylococcus aureus* in a period of 28 days, because the antibiotics for a bone infection is usually administered for 4 to 6 weeks. Test release of GEN from BHAG(ELENA) pellets performed by soaking the pellets in phosphate buffered saline pH 7.4 and temperature 370C and were sampled every day. The sampling results were tested by using agar diffusion method with the *Staphylococcus aureus* bacteria.

MATERIAL AND METHOD

Material

BHAG(ELENA) pellet made from 20 gr *bovine hydroxyapatite* powder (Dr Soetomo Hospital Tissue Bank Surabaya), 10 ml of gelatin 20 % (Rousselot Guangdong Chines), gentamicin 10 % (Arshine Technology CO, Limited Wanchai China) then make granules, then perfomed the cross-linking reaction by immersing the granules in 0,5% glutaraldehyde (E.Merck). A total of 100 mg of dried granul were made pellets and were pressed 3 ton, in order to obtain a cylindrical pellets with 4,0 mm in diameter and 3,2 mm thick.

The Release of GEN from BHAG(ELENA) (modification of Stallmann *et al*, 2006)

The release testing by immersing the pellets in 2 ml of phos-

phate buffer saline (PBS). The sample were sampled at 30, 90, 180 minutes and every 24 hour for 21 days and 28 days for about 0.5 ml. the sample were stored at -200C temperature until analysis. The analyzed of gentamicin concentration by using agar diffusion method with *Staphylococcus aureus* bacteria.

Agar diffusion method, modified of El-Ghannam *et al*, 2005

Antibacterial activities test with agar diffusion method. One oose *Staphylococcus aureus* (ATCC 25923) 5.106 colony-forming units/ml (cfu/ml) from primary culture were inoculated on slant agar medium. The tubes were incubated at 370C for 24 hour. Then added 15 ml of saline sterile solution and shaken until all the culture regardless from the medium. The bacterial suspensions were measured optical density (OD) at 580 nm, until 25% transmission.

For the antibiotic activity assay, as 6 test tubes of each10 ml antibiotic medium were cooled at 45 - 500C and poured into a 6 petri dish (second antibiotic medium) an allowed to solidify. A total of 5 µL cultures of *Staphylococcus aureus* (ATCC 25923) were suspended in 6 mL 1st antibiotic medium that have been melted and cooled at 45 - 500C for 6 tubes, then shaken until homogeneous and poured above 2nd antibiotic medium, and allowed to solidify. After the medium was solidified, then made some holes in the agar medium, 50 µL of sample or standard solution was poured into the hole. To obtain a calibration curve concentrations were used GEN 0; 1.25; 2.5; 5.0; 10.0; 20.0, 40.0 ppm in sterile saline solution and also to know the MIC GEN against *Staphylococcus aureus*. Samples were placed in different places in the petri dish and were incubated at room temperature for about 30 minutes. Then, the samples were incubated at 370C. After 24 hours incubation, the diameter of inhibition zone was measured (The experiment was done in triplo).

RESULT

Based on the observation result, some of GEN concentrations have a role as antibacterial. It show from the amount of inhibition zone diameter to the growth of *Staphylococcus aureus* (ATCC 25923), it show at Figure 1.

The result of gentamicin (GEN) release from BHAG(ELENA) pellet during 28 days immersion to the phosphate buffer saline (PBS) show at Figure 3. And the release result that were tested using agar diffusion method with *Staphylococcus aureus* (ATCC 25923) show in Figure 2.

BHAG(ELENA) contain 891.463±44.203 µg and based on Figure 3 show that the release of GEN from BHAG(ELENA) for about 89.24% during 28 days. At the 28 days, the inhibition zone diameter still greater than MIC. This suggests that the pellet was still actively releasing GEN to inhibit the growth of *Staphylococcus aureus*.

DISCUSSION

Based on Figure 1, regression equation obtained $y = 0.3194x + 12.7646$ and $R = 0.9528$ ($p < 0.05$), this indicated that if the GEN concentration increased it will increase the inhibition zone diameter. The equation can be used as a reference to calculate the concentration of GEN that release from the pellets with plate method. Based on the activity test with turbidimetry method, show that the minimal inhibitory concentration (MIC) of GEN against *Staphylococcus aureus* is 0.4 ppm, because the MIC is not clear with plate method (<1.25 ppm).

Thus the release of GEN from BHAG(ELENA) pellets can be controlled in the long term so that the bacteria can be eradicated or controlled perfectly in areas difficult to reach with the

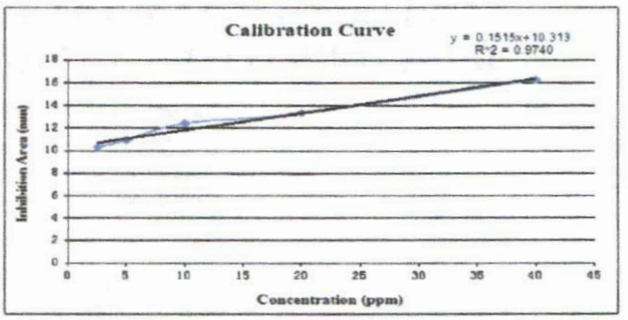

Figure.1 Calibration curve of concentration GEN (ppm) vs Inhibition Area (mm)

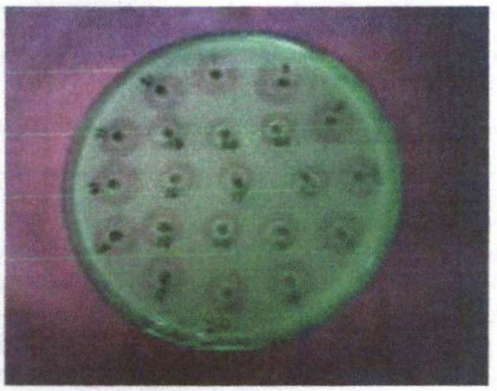

Figure 2. Inhibitor activity of GEN against *Staphylococcus aureus* in Nutrient Agar as sampling result of GEN release from BHAG(ELENA) pellet during

intravenously or orally GEN administered. To prove that, the agar diffusion method with Staphylococcus aureus were done and the result showed in Figure 1. While the results of the measurement of the inhibition zone diameter shown in Figure 2 for 28 days sampling. This suggests that GEN released during 28 days are stable and controlled with the concentrations greater than 10 times the MIC GEN (0.4 ppm) against *Staphylococcus aureus*.

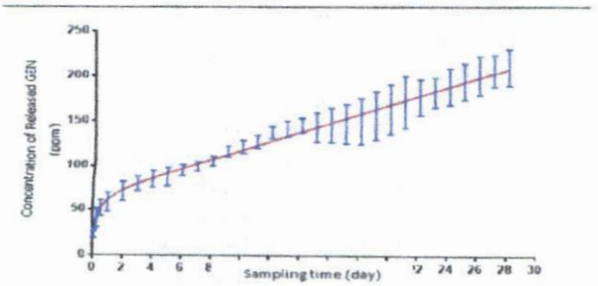

Figure 3. The BHAG(ELENA) pellet cumulative release GEN during 28 days (Sampling time vs Concentration of Released GEN)

Based on Figure 3, show that the stady state concentration of GEN (Css) = 41.47 ± 4. 19 ppm or 4.65% on day 2, which is referred to as the stady state time (tss) GEN release from the pellets. For the treatment of osteomyelitis (OM) takes a long time is 4 to 6 weeks consisted of parenteral administration for 2 weeks and then continued orally 4 weeks (Dipiro *et al*., 2011). Based on the result, need to do further testing in vivo of pellets BHAG (ELENA) by implantation in the femur of animals try as practiced by Messeguer Olmo *et al.*, (2002).

BHAG(ELENA) pellet can be controlled GEN release for more than 28 days with concentrations greater than the MIC *Staphylococcus aureus*. Thus the antibacterial activity can also be maintained for more than 28 days, in accordance with the purposes for local use

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