

BIOCOMPATIBLE COMPOSITE AS GENTAMICIN DELIVERY SYSTEM FOR OSTEOMYELITIS AND BONE REGENERATION

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ABSTRACT

Objective. The present study was designed to investigate the potency of BHA-GEL-GA-GEN pellet as gentamicin (GEN) delivery system matrix which has been cross-linked with glutaraldehyde (GA) 0.5%.

Methods. The composition of Bovine hydroxyapatite (BHA) compared to the composition of Gelatin (GEL) is 10 : 1. Pellets are cylindrical with 4.0 mm diameter and weighed 100.0 mg. GEN releasing is evaluated by in vitro and in vivo examinations based on the concentration of GEN in 28 days.

Results. Based on in vitro examination, it indicates 86% of GEN are released gradually in 28 days. Meanwhile, in vivo examination by implanting the pellet on rabbit femur indicates C_{max} of GEN release on proximal area reached 24.32 $\mu\text{g/L}$ in 14 days and 26.97 $\mu\text{g/L}$ in 28 days for distal area. X-ray examination indicates the pellets have circular form as its defects at the second day. At the 28th day examination, the pellets have transformed into compact form which filled the defects. This result indicates the pellets have been degraded and compatible with surrounding tissue. GEL is a type-1 collagen which is the main component of *softcallus* (osteoid). It undergoes calcification and transformed into *hardcallus* (osteocyte). High availability of calcium provided by BHA as delivery system accelerated this remodeling process.

Conclusion. It can be concluded that in 28 days BHA-GEL-GA-GEN as local delivery system releases higher concentration of GEN compared to MIC *Staphylococcus aureus*. BHA and GEL serve as bone remodeling template in osteomyelitis treatment.

Keyword: Bovine Hydroxyapatite (BHA); Gelatin (GEL); Gentamisin (GEN); Cross-link, Glutaraldehyde (GA); Drug Delivery System (DDS).

INTRODUCTION

Osteomyelitis is chronic bone infection disease which is hard to cure. It is caused by bone limited ability to deliver antibiotics. Commonly, the concentration of antibiotics reached target is below *minimum inhibitory concentration* (MIC) therefore it cannot eradicate infecting microorganisms. Traditional osteomyelitis treatment is done through debridement by removing infected bone, cleaning the area surrounding the wound aseptically, and providing antibiotics. However, local drug delivery system which enables higher concentration than MIC reaching target in long period has been developed in the last decades. About thirty percents of conventional osteomyelitis (OM) treatment is done by removing damaged bone (*necrosis*) and cleansing the defect area repeatedly (1,2). Annually, there are 22 child patients with OM in Caledonia Central Hospital (3). Osteomyelitis treatments are also done at Dr. Soetomo Teaching Hospital. There are 40 patients with OM during 2010 to 2012. Local treatments are given to 20% of them (4). A problem regarding OM treatment is that the defected bone may stop the continuity of bloodstream. This reduces the concentration of antibiotics reaching the target; therefore the infecting bacteria cannot be eradicated. To overcome this issue, high-dosage intravenous antibiotics intake needs to be given in a long period. However, this may cause adverse effects and toxicity to the patients.

To overcome this problem, a method in giving antibiotics locally by using bio-degradable and bio-compatible materials as antibiotics delivery system has been developed in past decades (5-7). The advantages of using biomaterial are: 1) antibiotics is released gradually in a long period; 2) the concentration of antibiotics reaching its target is higher than MIC; 3) additional operation to remove the material when the antibiotics runs out is not needed, because the material is degradable; and 4) the material is compatible with the surrounding tissues (8). This development is one step ahead compared to delivery system which is mostly used recently (PMMA). As a delivery system, PMMA contains non-degradable and non-compatible components which need to be removed after the treatment. Moreover, active compounds released by PMMA as delivery system is lower than MIC which causes in

delaying medication process and increasing the resistance of microorganisms (9). In Dr. Soetomo Teaching Hospital, PMMA as Gentamycin delivery system and bone filler is used in OM treatment. As reported by Fatehah (2011), there are 57 arthroplasty operations were done on 54 patients between 2008 and 2010 (10).

Biodegradable and biocompatible biomaterials have been developed based on compositions of bone organic elements (proteins, such as collagens and gelatin) and inorganic elements (hydroxyapatite) as done by Buranapanitkit *et al.*, (2004) and Hillig *et al.*, (2008) (11,12). In this research, hydroxyapatite is taken from bovine bones (*Bovine hydroxyapatite* = BHA). The *bovine hydroxyapatite* is porous. Its porosity is higher than synthetic hydroxyapatite. Therefore it has better osteoconductivity and may serve as bone cell scaffold during remodeling process which can regenerate damaged bone (13-15). Moreover, BHA has been produced by Tissue Bank of Dr. Soetomo Teaching Hospital. Because of its porous and fragile characteristics, BHA only adsorps drugs on its surface. In order to meet requirements as medium based on DDS, combination with gelatin (GEL) is needed. GEL addition improves the potential of BHA, changes the characteristics of BHA surface as place where the active compounds dispersed. However, BHA-GEL composite (combination) is easily degraded and hard to control the release of its active contents. Therefore, the composite and its active contents need to be cross-linked with glutaraldehyde which changes the characteristics of composite into expandable after absorbing surrounding fluids and degraded slowly (16).

In order to undergo cross-linking process, active compounds must have free groups of $-\text{NH}_2$ and $-\text{COO}^-$ (17). In this research, gentamycin is used as active compound for several reasons: its wide spectrum; its stability on body temperature for a long period; easily dissolved in water; having non-toxic effects on bone cells; and it does not interact with the composite (2,4). As a protein, GEL contains many free $-\text{COO}^-$ and $-\text{NH}_2$ groups while GEN also contains many $-\text{NH}_2$ groups. Therefore, both of them may undergo cross-linking process. The formula is made based on the composition of BHA : GEL = 10:1. GEN 10% is cross-linked with GA 0.5% molded into cylindrical pellets with 4.0 mm diameter and 100.0 mg weight.

MATERIAL AND METHOD

Materials

BHA was obtained from Tissue Bank of Dr. Soetomo Teaching Hospital. Gelatin 150 bloom was taken from cattle skin produced by Rousselot (Guangdong, China). Gentamicin was product of Arshine Technology CO, Limited, Wanchai China. Glutaraldehyde 25%, K_2HPO_4 , and KH_2PO_4 were products of Sigma-Aldrich. *Staphylococcus aureus* (ATCC 25293) was the collection of Microbiology Laboratory, The Faculty of Pharmacy, Airlangga University, Surabaya. The research has been approved by Animal Care and Use Committee (ACUC), Faculty of Veterinary, Airlangga University (ethical clearance number 237-KE).

Methods

BHA-GEL-GEN-GA Pellet Preparation

BHA (10.0 g) is mixed with 1.11 g GEN and added to 5 ml GEL 20%. The compound is stirred homogenously until granule mass formed. The compound is granulated with mesh diameter 1 mm. the resulted granules are dried in the oven at 40° C temperatures for 24 hours. Dried granules are cross-linked by soaking them into GA 0.5% at 37° C temperatures for 24 hours. Granules are washed in aquadest three times and rinsed with phosphate buffer saline (PBS). Granules are re-dried in the oven at 40°C temperatures for 24 hours. Dried granules are molded by using tablet machine (Greasebay Specac) with 4.0 mm diameter molds and 3 ton pressure.

Nutrient Agar Medium and *Staphylococcus aureus* Preparation

About 5.10^6 colony-forming unit/ml (cfu/ml) *Staphylococcus aureus* are swiped to nutrient agar (NA) askew antibiotics medium 1 and stored in incubator at 37° C temperatures for 24 hours. Then 5 ml sterile 0.9% NaCl solution is added and shaken until all cultures separated from NA. Optical density of the inoculum is measured at 580 nm until 25% of transmittant are obtained.

10 ml sterile NA from 6 reaction glasses which have been melted and cooled down until 45-50° C are poured into plate dish and solidified. 5 μ L *Staphylococcus aureus* are put into 6 reaction glasses containing 6 ml NA which have been melted and cooled down at 45-50° C. the glasses are shaken until the mixture becomes homogenous. The mixture is poured on plate dish containing solidified NA II. As a test for standard and sample, a hole is created on solidified NA which is filled by standard or sample. The plate dish is stored in incubator with 37° C temperatures for 24 hours and the diameter of inhibitory area is measured (in mm).

BHA-GEL-GEN-GA Implant Implantation

Twenty *New Zealand strain* rabbits (weighed about 2.5-3.0 kg) are used during in vivo examination whose femurs are implanted by implant. The rabbits are divided into five groups: 2-day, 7-day, 14-day, 21-day, and 28-day groups. For anesthetic purposes, xylazine (2mg/kg BW) is intramuscular injected, and ethamine sulphate (20 mg/ kg BW) is injected on left quadriceps continued by prevention dose 10 mg/ kg bw intramuscular injected to prevent any reactions made by the rabbits. Amphylline 24 mg/ kg bw is injected as prophylaxis. After the rabbits are anesthetized, its femur is lubricated by using antiseptic solution (savlon) to make shaving process easier. After the hair around the femur are removed, cancelous tissues are being incised layer by layer until femur bone is exposed. The femur bone is drilled by using 4.2 mm drill (see red hole on Fig 1). After defect is formed a 4.0 mm diameter pellet is implanted on the defect. After operation, the wound is stitched using 4.0 absorbable thread knots on muscular tissue and continuous nylon nylon 3.0 knots on skin tissue. Each group is terminated for certain period. One rabbit of each group is operated to examine how femur bone regenerated through radiological examination. Three of them are being measured the concentration of gentamicin released by taking bone around the

pellet, about 0.5 cm towards distal and proximal directions (Fig 1). The samples are cleared from its marrow and muscles and weighed out. The samples are homogenized in 2 ml PBS and stirred for 4 hours. The samples are centrifuged at 4000 rpm for 20 minutes until become extract. The extract is stored at -40° C temperatures before agar diffusion analysis by using *Staphylococcus aureus* (ATCC 25923). GEN standards are determined between 0.0 -40 μ g/ml to examine the linearity of GEN concentration and inhibitory zone diameter on *Staphylococcus aureus* in NA medium.

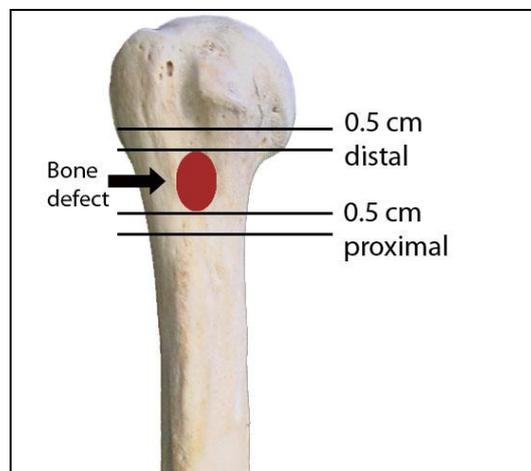


Fig. 1: Bone defect for implantation of BHA-GEL-GA-GEN

RESULT AND DISCUSSION

BHA-GEN-GA-GEL pellet implantation on rabbit cancellous femur is described by Fig 2: after being drilled (Fig 2A), and implanted on the defect (Fig 2B). In order to prevent infection on incised area, the covering gauze is replaced every two days. There are four replacements before the wound dried out.

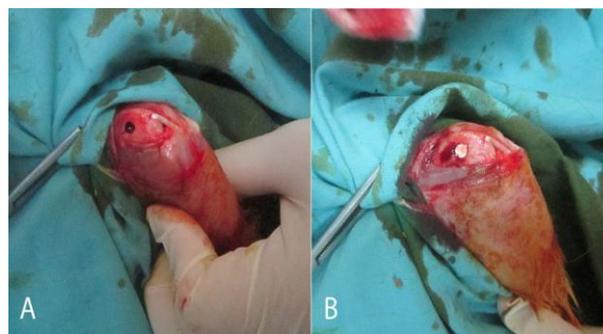


Fig. 2: A: Bone defect for implantation; B: Pellet BHA-GEN-GA-GEL implantation

Gentamicin Release and Biological Activity

GEN concentration inside bone is examined based on agar diffusion by using NA medium and *Staphylococcus aureus* micro-bacterium. The micro-bacterium is chosen because it is one of osteomyelitis main causes. About 30% of OM cases in hospital are caused by this bacterium (4,8). The correlation between gentamicin (GEN) concentration (ranged between 0.0 -40.0 μ g/ml) and the diameter inhibitory zone of *Staphylococcus aureus* is observed. Fig 3 showed linear relationship between the concentration of GEN and inhibitory zone diameter ($R^2= 0.9740$) and equation $Y= 0.1515X+ 10.313$.

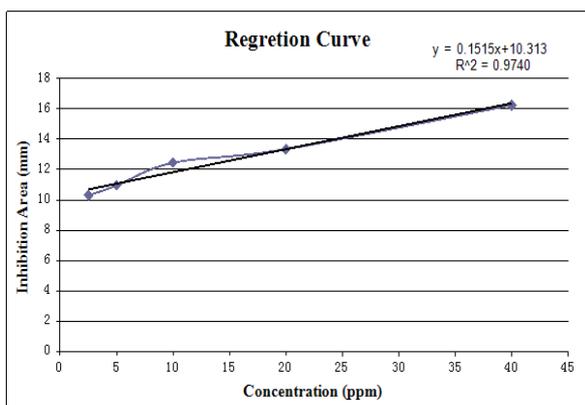


Fig. 3: Curve concentration of GEN vs Zone inhibition

GEN releasing process of implanted BHA-GEN-GEL-GA (CL) implant is initiated by absorbing surrounding bio-fluids. This happens because BHA-GEL as GEN delivery system has been cross-linked with GA. Therefore, it can absorb fluids ten times its weight which causes the implant to expand and slowly degrades (17). As the implant expands and degrades, its GEN content is released. Thus, GEN releasing process can be controlled and occurs in a long period. The released GEN penetrates the bone through its pores. It works based on Fick's Law which states drugs/ active compounds flow from higher concentration to the low concentration.

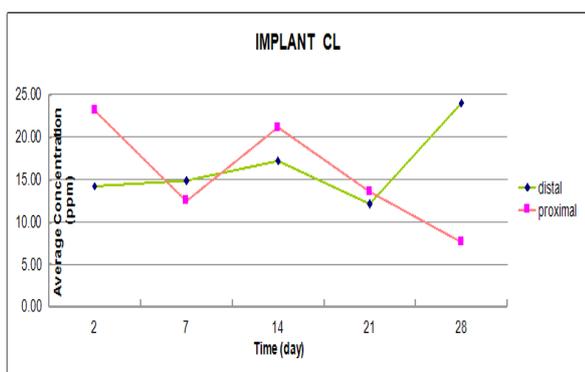


Fig. 4: Concentration of GEN in the distal and proximal of the rabbit femur that released at 2nd until 28th day from BHA-GEL-GA-GEN (cross-link = CL) implant.

GEN released profile of terminated rabbits at the 2nd, 7th, 14th, 21st, and 28th day showed at Fig 4. Three rabbit of each group are used as samples by cutting its femurs 0.5 cm towards distal and proximal directions from the implant. The samples are examined by using agar diffusion method. The concentration of GEN contained in the femurs is indicated by diameter inhibitory zone of *Staphylococcus aureus* on NA medium. Agar diffusion examination indicates that the concentration of GEN penetrated towards proximal direction at the second day is maximum because adsorbed GEN on the surface is directly released and dissolved by surrounding fluids. GEN diffuses and penetrated the proximal bone until reaching its high concentration at 14th day and go down until 28th day. Meanwhile, the concentrations of GEN penetrating distal area at the second, seventh and fourteenth days are similar. This indicates that blood flow towards distal area is stable. Optimum concentration is reached at 28th day. It indicates that blood flow in the bone has been improved due to the direction of bone blood flow is bottom-up. The lowest concentrations are reached at the 7th day on proximal direction and 21st day on distal direction. However, these values are ten times higher than MIC (< 1.25 µg/ml). These concentrations are able to eradicate *Staphylococcus aureus* as one of nosocomial bacteria which

cause OM (2,18). As the concentration of GEN penetrated into bone increased toward distal and decreased toward proximal. Which were showed that bone defect had been regenerated and neo-vascularization and blood circulation formed.

Radiological Examination of Bone Regeneration

From X-ray examination result at the second day, it appears that implant is circle-shaped which is indicated by higher intensity of X-ray compared to its surroundings (Fig 5A). the development of bone regeneration can be seen from intensity changes and implanted implant transformations: at the first week (Fig 5B); at the second week, absorbed x-ray intensity change is equivalent to homogenous gradual implant minimization (Fig 5C); at the third week, implant transformation started and minimization distributed unevenly (Fig 5D). A significant change appears at the fourth week. Circle-shaped implant is minimizing until unable to be seen (Fig 5E). It indicates implant has been degraded and partially fused with the surrounding tissues through bone defect remodeling process.

This remodeling process is accelerated by degrading pellet which contains gelatin (GEL) as collagen type 1 and BHA as calcium source. As a scaffold, BHA-GEL composite is osteo-conductive which enables surrounding tissues to migrate, penetrate, and proliferate with it. 90% of released GEL (collagen type 1) reacts with osteoblasts to form osteoids (*soft-callus*). Osteoids react with calcium (mineral) of BHA resulting osteocytes (*hard-callus*) (14,19). Based on X-ray intensity changes and radiological examination on BHA-GEN-GA-GEL implanted pellet, it can be concluded that the pellet is biodegradable. Its components are biocompatible which enables it to blend with surrounding cells. Hence, bone regeneration process may undergo faster. According to the previous literature, bone regeneration process takes between 8-12 weeks (13,18).

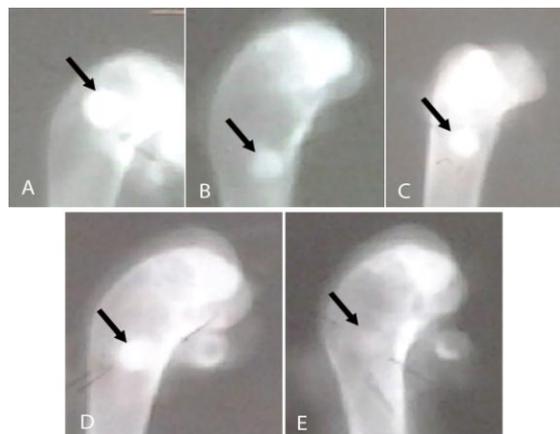


Fig. 5: X-ray images of the extracted femurs: (a) After 2 days, a circle image of homogeneous density is observed, which is similar to the implant. (black arrow), (b) After 1 week implantation, the image shows full circle, (c) After 2 weeks, gradually the image shows minimize; (d) After 3 weeks, the circle image is smaller than 2 weeks and (e) After 4 weeks, the circle image is smallest, and the implant component union with hostbone.

CONCLUSION

BHA-GEN-GEL-GA pellet can be used as GEN local delivery system matrix with gradual release on bone for 28 days. As GEN delivery medium, BHA-GEL improves and accelerates bone remodeling process.

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