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Chaiyakorn Thitiyanaporn

Pareeya Udomkusonsri

Naris Thengchaisri

Original Article

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Chaiyakorn Thitiyanaporn[1,2,3] Pareeya Udomkusonsri [4] Naris Thengchaisri [3*]

Abstract

Three types of gentamicin beads were compared in a rat osteomyelitis model. The gentamicin beads were prepared in cylinder shape (diameter 2 mm x height 4 mm). Gentamicin impregnated polymethylmethacrylate (GI-PMMA) beads were fabricated according to manufacturer's instructions. Gentamicin coated native calcium sulfate (G-NCS) bead and gentamicin coated high porous calcium sulfate (G-HPCS) bead were prepared in laboratory. Osteomyelitis was induced in the rat's tibias by using methicillin resistance *Staphylococcus aureus* (MRSA). After 3 weeks of infection, the infected tibias were implanted with GI-PMMA bead, G-NCS bead, G-HPCS bead or sham treatment (control). Radiographic change, white blood cell count and infection signs were weekly monitored for 6 weeks. At the end of the experiment, all tibias were collected for histopathologic examination and bone culture. Although white blood cell count and infection signs were not significantly different among different group of rats, the radiolucent area reduced significantly in GI-PMMA, G-NCS and G-HPCS compared to the control group. There was no significant difference in bacterial count among the groups, however, the histopathologic results revealed new bone development in G-NCS and G-HPCS groups, and a large bone defect in GI-PMMA group resulting from bead removal. This study suggests that the G-HPCS can be used as a local antibiotic carrier for management of osteomyelitis instead of calcium sulfate and polymethylmethacrylate beads.

Keywords: bead, gentamicin, osteomyelitis, rat

[1] *Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand,*
[2] *Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education. (AG-BIO/PERDOCHE),Thailand,*
[3] *Department of Companion Animal Clinical Sciences, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom, 73140, Thailand,*
[4] *Department of Pharmacology, Faculty of Veterinary Medicine, Kasetsart University, Bangkhen campus, Bangkok, 10900, Thailand*
***Corresponding author: E-mail:** fvetnrt@ku.ac.th, ajnaris@yahoo.com*

บทคัดย่อ

การเปรียบเทียบเม็ดลูกปัดโพลีเมดทิลเมดอะคริเลทที่เอิบชุ่มด้วยเยนต้ามิซินเม็ดลูกปัดแคลเซียมซัลเฟตแบบดั้งเดิมเคลือบด้วยเยนต้ามิซินและเม็ดลูกปัดแคลเซียมซัลเฟตแบบมีรูพรุนสูงเคลือบด้วยเยนต้ามิซินในการจัดการภาวะกระดูกอักเสบในหนูแรท

ชัยกร ฐิติญาณพร [1,2,3] *ปารียา อุดมกุศลศรี* [4] *นริศ เต็งชัยศรี* [3*]

เปรียบเทียบเม็ดลูกปัดเยนต้ามิซินสามชนิด ในการจัดการภาวะกระดูกอักเสบของหนูแรท เตรียมเม็ดลูกปัดเยนต้ามิซินในรูปทรงกระบอก (เส้นผ่านศูนย์กลาง 2 มม. x สูง 4 มม.) ขึ้นรูปเม็ดลูกปัดโพลีเมดทิลเมดอะคริเลทเอิบชุ่มด้วยเยนต้ามิซินด้วยวิธีที่บริษัทแนะนำ เตรียมเม็ดลูกปัดแคลเซียมซัลเฟตแบบดั้งเดิมและแบบมีรูพรุนสูงถูกเคลือบด้วยเยนต้ามิซินในห้องปฏิบัติการ ให้เชื้อสตาฟิลโลคอกคัสออเรียสที่ดื้อต่อยาเมททิซิลินเพื่อให้เกิดการอักเสบของกระดูกทิเบียแก่หนูแรท หลังจากนั้น 3 สัปดาห์ แบ่งหนูออกเป็น 4 กลุ่ม ได้แก่ กลุ่มที่รักษาด้วยเม็ดลูกปัดโพลีเมดทิลเมดอะคริเลทที่เอิบชุ่มด้วยเยนต้ามิซิน กลุ่มที่รักษาด้วยเม็ดลูกปัดแคลเซียมซัลเฟตแบบดั้งเดิมเคลือบด้วยเยนต้ามิซิน กลุ่มที่รักษาด้วยเม็ดลูกปัดแคลเซียมซัลเฟตแบบมีรูพรุนสูงเคลือบด้วยเยนต้ามิซิน และกลุ่มควบคุม (ไม่ทำการรักษา) ตามลำดับ ประเมินความเปลี่ยนแปลงของภาพถ่ายรังสี จำนวนเม็ดเลือดขาว และอาการการติดเชื้อ ในทุกสัปดาห์จนครบ 6 สัปดาห์ เก็บตัวอย่างกระดูกทิเบียเพื่อตรวจด้วยวิธีทางจุลพยาธิวิทยาและเพาะเชื้อจากกระดูกหลังการทดลองสิ้นสุด แม้ว่าจำนวนเม็ดเลือดขาวและอาการการติดเชื้อไม่มีความแตกต่างกันในแต่ละกลุ่ม แต่จากภาพถ่ายรังสีพบพื้นที่โปร่งในกระดูกลดลงอย่างมีนัยสำคัญในกลุ่ม GI-PMMA, G-NCS, G-HPCS เมื่อเปรียบเทียบกับกลุ่มควบคุม แม้ว่าผลของจำนวนแบคทีเรียไม่มีความแตกต่างกันในแต่ละกลุ่ม แต่ผลจุลพยาธิวิทยาพบการสร้างกระดูกขึ้นใหม่ในกลุ่ม G-NCS และ G-HPCS และพบว่ามีช่องว่างกระดูกขนาดใหญ่เกิดขึ้นเมื่อนำ GI-PMMA ออกจากกระดูก จากการทดลองนี้แสดงให้เห็นว่า G-HPCS สามารถใช้เป็นตัวนำพายาปฏิชีวนะในการจัดการภาวะกระดูกอักเสบทดแทนเม็ดลูกปัดแคลเซียมซัลเฟต และเม็ดลูกปัดโพลีเมดทิลเมดอะคริเลทได้

คำสำคัญ: เม็ดลูกปัด เยนต้ามิซิน กระดูกอักเสบ หนูแรท

[1] *ศูนย์เทคโนโลยีชีวภาพเกษตรมหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน นครปฐม 73140*
[2] *สำนักพัฒนาบัณฑิตศึกษาและวิจัยด้านวิทยาศาสตร์และเทคโนโลยี (สบว.) สำนักงานคณะกรรมการการอุดมศึกษา*
[3] *ภาควิชาเวชศาสตร์คลินิกสัตว์เลี้ยง คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน นครปฐม 73140*
[4] *ภาควิชาเภสัชวิทยา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตบางเขน กรุงเทพฯ 10900*
***ผู้รับผิดชอบบทความ E-mail:** fvetnrt@ku.ac.th, ajnaris@yahoo.com*

Introduction

Osteomyelitis is an inflammatory process accompanied by bone destruction and caused by microorganism infection (Lew et al., 2004). Treatments for osteomyelitis involve surgical debridement of necrotic tissue, irrigation, obliteration of dead space, bone repair, adequate soft tissue coverage and systemic antimicrobial administration for 4-6 weeks (Mader et al., 1999; Lazzarini et al., 2004). The necrosis of the infected bone tissue is a result of decrease in vascularity compromising the effectiveness of systemic antibiotic therapy (Mader et al., 2002).

Antibiotic impregnated polymethylmethacrylate (PMMA) beads are clinically used in various areas including joint replacement surgery and commonly used for standard treatment of local infected tissue, especially osteomyelitis (Kelsey et al., 1995; Roeder et al., 2000; Koo et al., 2001; Gondusky et al., 2009; Malizos et al., 2010). However, disadvantages of PMMA used in osteomyelitis management are requirement for surgical removal (Mader et al., 2002; Nelson et al., 2002), enhancement of bacterial colonization (Mader et al., 2002), higher cost of management and release of toxic substance during setting (Santschi et al., 2003). Unlike PMMA, calcium sulfate beads have been used in *in vitro* and *in vivo* studies as a vehicle to deliver antibiotics, growth factors and other pharmacologic agents (Santschi et al., 2003; Ham et al., 2008; Kanellakopoulou et al., 2009; Thomas and Puleo, 2009; Xie et al., 2009). Moreover, antibiotic impregnated calcium sulfate beads have been used in medical practices, especially for the treatment for osteomyelitis (Ham et al., 2008; Kanellakopoulou et al., 2009). A local antibiotic delivery system has been employed because it

provides higher local antibiotic concentration than parenteral antibiotic administration. Furthermore, an application of local antibiotics also reduces the risk of systemic side effects and aids bone defect management (Gitelis et al., 2002).

Calcium sulfate is a biomaterial that can provide a scaffold for osteogenic cell attachment and calcium ion needed for new bone regeneration. The implanted calcium sulfate bead can be totally reabsorbed with minimal inflammation (Thomas and Puleo, 2009). The osteoconductive mechanisms of calcium sulfate are involved in a direct source of calcium to bone defect (Al Ruhaimi et al., 2001) and in a rapid rate of reabsorption which allows earlier ingress of osteoprogenitor cells (MacNeill et al., 1999).

High porosity and interconnectivity pores are major requirements for bone substitute materials. Materials with proper porosities and pore sizes could facilitate cell attachment and cell ingrowth and promote a uniform cell distribution with adequate transportation of nutrients and cellular waste products (Hou et al., 2003). Micropores allow a migration of endothelial cells, promote a differentiation of osteoblasts and osteoprogenitor cells, promote vascularization, and contribute to an osteoblast proliferation and differentiation (Kusmanto, 2008). The porosity of the material can be fabricated with various techniques such as leaching of soluble particle (Hou et al., 2003; Joe¨l Reignier 2006; McLaren et al., 2007) and mechanical and chemical techniques (Shiramizu et al., 2008). Leaching of salt technique is an effective technique to control the amounts and sizes of pores, which could be determined by the amount and size of the particles (Hou et al., 2003). Besides the important role of porosity for osteogenesis, material porosity also influences dissolution permeability of drugs which in turn affects antibiotic elution from biomaterials (Schurman et al., 1978; McLaren et al., 2004).

The hypothesis of this study was gentamicin coated high porous calcium sulfate (G-HPCS) bead can be used as a local antibiotic carrier for management of osteomyelitis equally to gentamicin impregnate polymethylmethacrylate (GI-PMMA) bead. Furthermore, increased bead porosity of HPCS could improve bone formation at the infection site.

Materials and Methods

GI-PMMA, G-NCS and G-HPCS beads preparation:

GI-PMMA: Commercial polymethymetacrylate (PMMA) with gentamicin sulfate 3.8% (GENTAFIX®3, Teknimed S.A., France) was used. Sterile powder and sterile liquid were mixed according to the manufacturer's instructions. The mixture was poured into a silicone cylinder mold (2x4 mm, diameter x height) and left until the cement set. The PMMA beads were removed from the mold after setting.

G-NCS: Native calcium sulfate beads were prepared by mixing calcium sulfate hemihydrates powder (Sigma, USA) with sterile distilled water in ratio of 10 : 7 w/v. The mixture was poured into a silicone cylinder mold (2x4 mm, diameter x height). Calcium sulfate beads were immersed in gentamicin sulfate injection solution 40 mg/ml (T.P. drug Lab, Thailand) for 5 minutes and dried under blower overnight.

G-HPCS: For preparation of high porous calcium sulfate beads, calcium sulfate hemihydrated and sodium chloride (sigma, USA) were weighed and manually mixed in a ratio of 1 : 1 w/w. Sterile distilled water was added to the mixture in ratio 10:7 w/v of calcium sulfate hemihydrates and distilled water. After the mixture became homogenous slurry, it was poured into a cylinder mold. For salt leaching technique, the beads were placed with deionized water in an ultrasonic cleaner as described before (Thitiyanaporn et al., 2012). Calcium sulfate beads were immersed in gentamicin sulfate injection solution 40 mg/ml (T.P. drug Lab, Thailand) for 5 minutes and dried under blower overnight. GI-PMMA, G-NCS and G-HPCS beads were sterilized with ethylene oxide before use.

Animals: Male Wistar rats, approximately 3 months old, weighing 250-300 g, were used in this study. All rats were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The rats were randomly sorted into 4 groups, control (n = 10), GI-PMMA bead (n = 10), G-NCS (n = 10) and G-HPCS (n = 10). The rats were kept in individual cage with unlimited food and water. This study was approved by the Kasetsart University Animal Use Committee ID number ACKU 02753.

Osteomyelitis model: Osteomyelitis was induced in the left proximal tibia. The rats were anesthetized with pentobarbital (0.6 mg/kg) intra-peritoneal injection. The osteomyelitis induction method was modified from previous studies (Monzon et al., 2001; Orhan et al., 2009).Briefly, then left proximal tibias were prepared by hair shave, chlorhexidine scrub and alcohol as routine preoperative preparation. One centimeter skin incision was performed at the

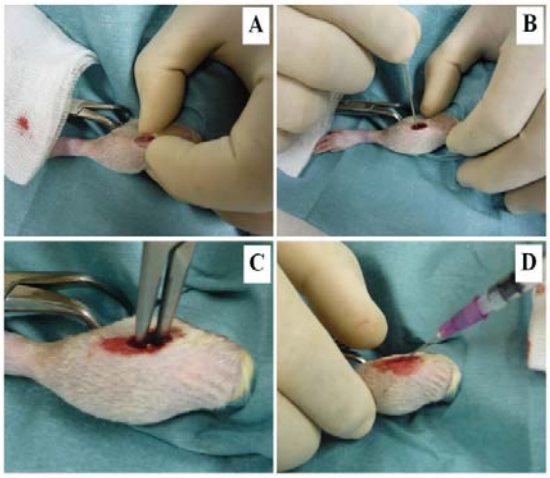

Figure 1 Osteomyelitis induction procedures on the left tibia. Proximal, craniomedian part of tibia was opened (A), median cortex was drilled by 18 gauge needle (B), Kirschner wire was inserted to the rat's tibia by needle holder, (C) and MRSA solution was injected into marrow cavity (D).

craniomedian area of proximal tibia. Soft tissue and muscle were dissected through the bone. One millimeter hole at median cortex was created with Kirschner wire (K-wire) connecting to bone marrow. A K-wire (5.0 x 1.0 mm) coated with methicillin resistance *Staphylococcus aureus* (MRSA) (National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand) biofilm was inserted into marrow cavity. Five hundred microliters of 4.0 x 10^7 CFU/ml MRSA was injected into the bone marrow cavity. The fascia and soft tissue were closed with polyglyconate (Maxon®, 4-0). Skin was sutured with nylon (Difilon®, 4-0) interrupted suture. After inoculation, the left tibia was monitored for osteomyelitis by gross appearance and radiographic examination weekly for 3 weeks (Fig 1).

Surgical procedure: After 3 weeks post-inoculation, the rats were anesthetized with pentobarbital (0.6 mg/kg) intra-peritoneal injection. The left proximal tibia was prepared by hair shave, chlorhexidine scrub and alcohol as routine preoperative preparation. Soft tissue and abscess capsule were removed and irrigated with 0.9% saline solution. The Kirschner wire was removed from the bone marrow cavity. The wound was sutured in the control group without any additional treatment. In GI-PMMA group, two GI-PMMA beads were implanted into the marrow cavity of the left proximal tibia. As GI-PMMA group, the G-NCS and G-HPCS beads were implanted into the marrow cavity of the left proximal tibia. Soft tissue and skin were closed routinely.

Infection signs and white blood cell count: Infection signs and white blood cell count were monitored weekly for 6 weeks. A 5-scale clinical sign scoring system (0 : no evidence of erythema, no pain and no swelling at infected site, 1 : erythema without abscess, no pain and mild swelling at infected site, 2 : erythema with swelling of the infected site, pain, 3 : purulent exudate, pain and swelling at infected site and 4 : purulent exudate, severe pain and tibia swelling) was used in the present study. The clinical sign scoring system was modified from a previous study (Rissing et al., 1985). Blood samples (0.5 ml) were collected from tail vein in EDTA container before MRSA inoculation at the day of treatment and every week post treatment for 6 weeks. Total white blood cell count was determined by automatic white blood cell counting machine (CELL-DYN® 3700, Abbott Lab, Illinois, USA). The cell was presented in total number of white blood cell x 10^3/µl.

Radiographic examination: Rats in each group were taken for radiographic examination (40 kV, 10 mAs, 100 cm) in anterioposterior and mediolateral views before and after the tibia was infected with MRSA weekly until the end of the study. The radiographic results were assessed as percentage of radiolucent area. Each parameter will be scored on a 5-scalescoring system (0 : no radiolucent area, 1 : 0-25% radiolucent area of tibia diameter, 2 : 25-50% radiolucent area of tibia diameter, 3 : 50-75% radiolucent area of tibia diameter, 4 : 75-100% radiolucent area of tibia diameter).

Bone culture: The whole right tibias of the rats were collected with sterile procedure after euthanasia. All soft tissues were removed from the tibia samples. The tibia samples were weighed and pulverized with mortar under sterile condition. One milliliter of sterile normal saline solution was added to the bone samples tube and quantitatively cultured by ten consecutive 1 : 10 dilution in sterile normal saline. A 0.1 ml aliquot of each dilution was plated onto Mueller Hinton agar (Difco, USA) and incubated at 37^0C for 18-24 hours. The colonies on the surface of agar plate were counted for calculating the colony forming unit (CFU) per weight (gram) of bone. Bacterial growth was expressed as log10 CFU/g tissue.

Histological examination: After euthanizing the rats with pentobarbital over dose, the tibias were stored in 10% neutral buffered formalin more than 24 hours. The samples were decalcified with 10% aqueous EDTA for a month and examined by a routine tissue processing for light microscopic procedure. The samples were stained with hematoxylin and eosin (H&E) for examining the cell types and Masson's trichrome for examine bone collagen. Histopathological osteomyelitis results were evaluated according the parameters including 1. Abscess formation, 2. Sequestrum formation, 3. Enlargement of corticalis, 4. Destruction of corticalis, and 5. General impression. The parameters 1 to 4 were scored with 0 (absent) or 1 (present). Parameter 5 was scored from 0 (absent), 1 (mild), to 2 (severe) for each ROI (Lucke et al., 2003). In addition, bone destruction and callus formation were scored by 0 : absent, 1 : involvement of 1-25% of bone cortex, 2 : involvement of 26-50% of bone cortex, and 3 : involvement of 51-100% of bone cortex.

Data analysis: The clinical sign score, white blood cell count, and radiographic scores were analyzed with Kruskal-Wallis One-Way ANOVA. Histopathological scores were analyzed with fisher's exact test. Data was expressed as the mean±standard deviation. Statistical difference was accepted with a $p < 0.05$ level.

Results

Infection signs: Infected tibias were slightly swollen and painful (score 1-2) in the last three days after MRSA inoculation and returned to normal (score 0) after two weeks. The infection sign scores of infected tibias were not significantly different between pre-MRSA inoculation (0±0), pre-treatment (0.04±0.21) and post-treatment (0±0) during the experiment in every group. Although the clinical signs were not present as osteomyelitis condition, the radiographic results revealed radiolucent area around the K-wire, indicating bone destruction.

White blood cell counts: The total white blood cell count was not significantly different between pre-inoculation and treatment period in every group. The results are shown in the Table 1.

Radiographic examination: The infected tibias were evaluated by radiographic picture after treatment. The

osteomyelitis of tibia was scored as percentage of radiolucent area in different levels between the groups. Sample radiographic pictures of control, GI-PMMA, G-NCS and G-HPCS groups are shown in Fig 2. The percentage of radiolucent area scoring result is shown in Fig 3.

Bone culture: After 6 weeks of treatment, bacteria colony forming unit (CFU) per gram of tissue was examined. Average of $\log_{10}$ CFU/g is shown in Table 2. The average of bacterial growth was not different among the control, GI-PMMA, G-NCS and G-HPCS groups.

Histopathological examination: All histopathological findings of the control, GI-PMMA, G-NCS and G-HPCS groups showed typical signs of bone infection such as abscess in bone marrow, bone sequestrum, destruction of the bone cortex and periosteal elevation. In the control group, the infected tibias presented multiple pyogranuloma, sequestrum and destruction of corticalis (Fig 4A and 5A). In the GI-PMMA group, the infected tibias showed multiple pyogranuloma with callus formation surrounding the GI-PMMA implantation site and cortical bone destruction (Fig 4B and 5B). In the G-NCS and G-HPCS groups, the infected tibias showed multiple pyogranuloma and new bone formation at the bone cortex without bone destruction (Fig 4C, 5C, 4D, and 5D). Histopathological scores indicated that osteomyelitis scores in the control group were significantly higher than the GI-PMMA, G-NCS and G-HPCS groups. Bone destruction scores in the G-NCS group was significantly lower than the control group and bone destruction scores in both G-NCS and G-HPCS groups were significantly lower than the GI-PMMA group. Interestingly, bone formation scores in both G-NCS and G-HPCS groups were significantly higher than the control and GI-PMMA groups.

Table 1 Total white blood cell count (x 10^3/µl) in pre-inoculation and treatment periods (6 weeks)

Time	White blood cell count (×10^3/µl)			
	Control	GI-PMMA	G-NCS	G-HPCS
Pre-inoculation	6.63±2.23	7.06±2.50	7.06±1.91	7.12±1.10
Week 0	5.83±2.56	6.48±1.92	7.54±2.39	6.57±2.72
Week 1	6.65±2.43	8.02±2.51	8.48±3.09	7.88±2.05
Week 2	6.98±1.08	6.25±1.26	7.21±2.38	7.93±2.36
Week 3	6.69±2.10	8.32±1.93	7.90±3.29	6.46±1.66
Week 4	7.58±1.43	7.09±1.38	7.10±1.46	6.77±3.35
Week 5	7.45±1.26	7.38±1.41	6.46±1.75	7.22±2.61
Week 6	5.25±2.18	4.93±1.48	5.10±1.43	6.82±3.81

Note: Pre-inoculation : Before inoculate MRSA, Week 0 : Treatment day, Week 1 : 7 days after treatment, Week 2 : 14 days after treatment, Week 3 : 21 days after treatment, Week 4 : 28 days after treatment, Week 5 : 35 days after treatment and Week 6 : 42 days after treatment.

Table 2 Bacterial growth (mean±SD of $\log_{10}$ CFU/g) from infected tibias after 6-week treatment period

Group	Control	GI-PMMA	G-NCS	G-HPCS
mean±SD of $\log_{10}$ CFU/g	3.64±0.38	3.59±0.63	3.24±0.37	3.43±0.66

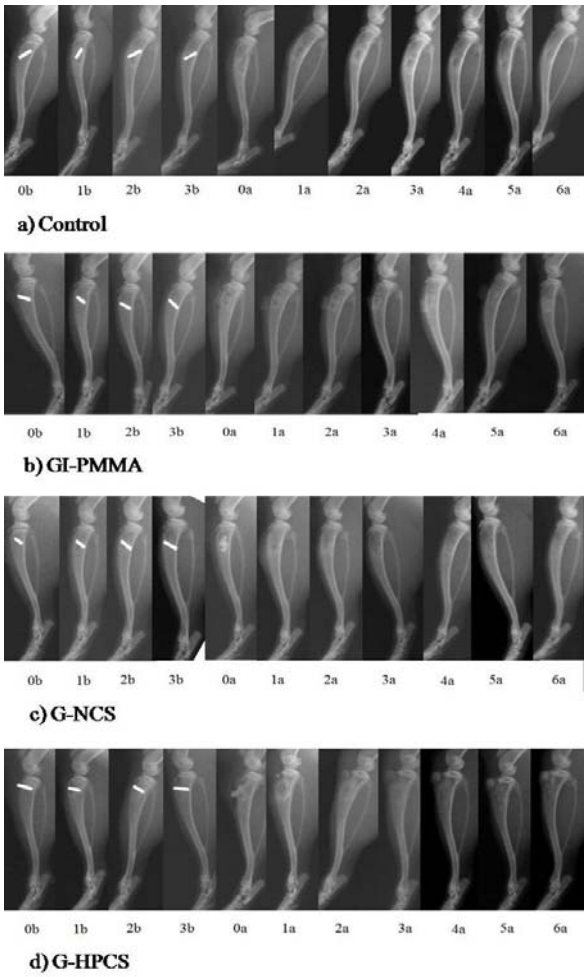

Figure 2 Radiographic pictures of the left tibia present in both osteomyelitis induction period and treatment period in control (a), GI-PMMA (b), G-NCS (c) and G-HPCS (d) groups. 0b : MRSA inoculation day, 1b : 1 week after MRSA inoculation, 2b : 2 weeks after MRSA inoculation, 3b : 3 weeks after MRSA inoculation, 0a : treatment day, 1a : 1 week after treatment, 2a : 2 weeks after treatment, 3a : 3 weeks after treatment, 4a : 4 weeks after treatment, 5a : 5 weeks after treatment, 6a : 6 weeks after treatment.

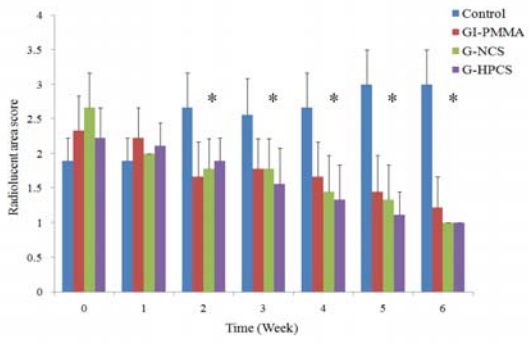

Figure 3 Radiolucent area scores of control, GI-PMMA, G-NCS and G-HPCS group. *p < 0.05 vs. control group.

Table 3 Histopathological scores

Group	n	Osteomyelitis	Bone destruction	Callus formation
Control	5	5±0[a]	1.6±0.55[a,b]	1.8±0.84[a]
GI-PMMA	5	3.8±0.45[b]	2.6±0.55[a]	0.4±0.55[a]
G-NCS	5	3.2±0.45[b]	0.2±0.45[c]	3±0[b]
G-HPCS	5	3.2±0.45[b]	0.4±0.55[b,c]	3±0[b]

[a,b,c] Different superscripts indicate a significant difference (p < 0.05) from control.

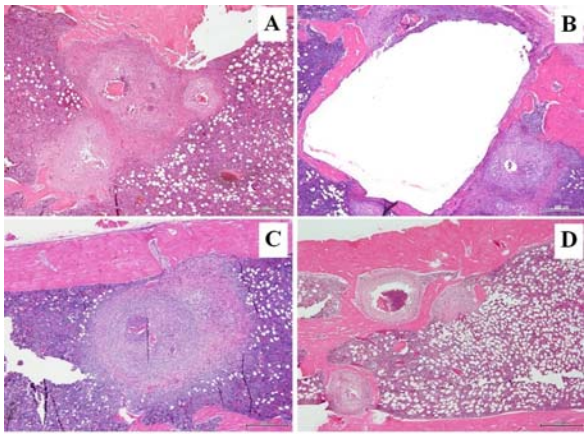

Figure 4 Bone marrow picture of control (A), GI-PMMA (B), G-NCS (C) and G-HPCS (D) H&E staining at x 400. Pyogranuloma and sequestrum were found in bone marrow in every group. Large space of GI-PMMA implantation site is presented in a Fig B.

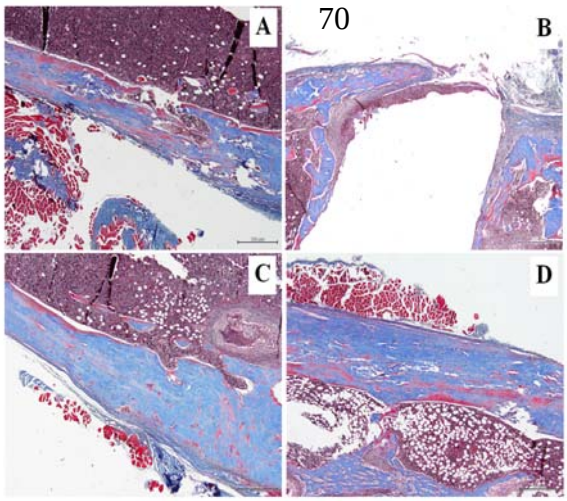

Figure 5 Bone cortex picture of control (A), GI-PMMA (B), G-NCS (C) and G-HPCS (D) Masson's trichrome staining at x 400. Both control and GI-PMMA groups showed incomplete bone cortex, while G-NCS and G-HPCS groups showed new bone formation at implantation sites.

Discussion

The management of ostemyelitis is quite difficult because of poor vascularization at the infected area due to the presence of necrosis of the surrounding soft tissue. Localized antibiotic administration is used for providing high concentration of antibiotic at the infected site without systemic side effect. Commercial gentamicin impregnated PMMA is widely used as commercial local antibiotic provider. Calcium sulfate bead is also used as a local antibiotic provider. The high porous calcium sulfate (HPCS) bead was developed in this study to reduce the side effect of calcium sulfate and to be used as a local antibiotic provider as same as calcium sulfate bead. A study showed that the difference of the porosity level of calcium sulfate bead had an effect on antibiotic release in an *in vitro* study and that high porous calcium sulfate bead could provide antibiotic in the highest concentration when compared in weight to calcium sulfate (Thitiyanaporn et al., 2012). However, an *in vitro* comparison study of gentamicin impregnated PMMA bead with gentamicin coated PMMA bead, gentamicin coated native calcium sulfate bead and gentamicin coated high porous calcium sulfate bead showed highly different patterns of gentamicin release and osteoblast attachment (Thitiyanaporn et al., 2013). However, the osteomyelitis management result of this study was similar in the GI-PMMA, G-NCS and G-HPCS groups. The GI-PMMA bead is a non-absorbable material; it had to be removed after a period of time, while G-NCS and G-HPCS are absorbable material. After GI-PMMA was removed, the wide space of the implanted bone cortex still remained. This space may be a result of the bone fracture from instability of the affected bone.

There are numerous reports of rat osteomyelitis model developed for studying in pathogenesis, diagnosis and osteomyelitis management. In our study, the rat osteomyelitis was modified from the experiment of Orhan et al. (2010) and Monzón et al. (2001). Orhan et al. (2010) developed the rat osteomyelitis model in rat tibia by injecting 200 µl of methicillin resistance *S. aureus* (MRSA) containing 1.0 x 10^7 CFU/ml and implanting Kirschner wire (5.0 x 1.0 mm) to the bone marrow cavity. The bone hole was sealed with dental gypsum and left for 3 months for development of the chronic osteomyelitis (Orhan et al., 2009). The experiment of Monzónv et al. (2001) developed the osteomyelitis model by implanting the Kirschner wire coated with biofilm of the *S.aureus* into the bone marrow and allowed 42 days of chronic osteomyelitis development (Monzon et al., 2001). Our study used 500 µl of MRSA 4.0 x 10^7 CFU/ml and implanted a Kirschner wire (5.0 x 1.0 mm) coated with biofilm of MRSA. The osteomyelitis was allowed to develop for 21 days. The osteomyelitis was developed in only bone marrow cavity and did not affect the other parts of bone. In a study of Fukushima et al. (2005). they injected *S. aureus* strain BB in Wistar rat tibia in many concentrations consisting of control : 0 CFU/5 µl, G1 : 6 x 10 CFU/5 µl, G2 : 6 x 10^2 CFU/5 µl, G3 : 6 x 10^3 CFU/5 µl, G4 : 6 x 10^4 CFU/5 µl and G5 : 6 x 10^5 CFU/5 µl and closed the tibial hole with bone wax. The osteomyelitis was allowed to develop within 1 week and the osteomyelitis was assessed in radiographic and histophatological examination. The recommended dose was G3 : 6 x 10^3 CFU/5 µl, sufficient to develop osteomyelitis condition in rat tibia (Fukushima et al., 2005). Last decade studies showed that the necrotic agent was not necessary for developing osteomyelitis condition in rat model (Nelson et al., 1990; Gisby et al., 1994; Monzon et al., 2001; Fukushima et al., 2005; Orhan et al., 2009) as the previous studies (Norden, 1970; Rissing et al., 1985; Mendel et al., 1999). The osteomyelitis model in rat was not created only in tibia but also in femur (Chen et al., 2005), mandible (Shvyrkov et al., 1981; Chistov, 1989) and hematogenous route (Kadyrov et al., 1966; Hienz et al., 1995).

The rat osteomyelitis model in the current study did not affect the weight, infection signs score

and white blood cell count. This result was due to the limited infection only in the marrow cavity. The deterioration of bone structure may happen if the osteomyelitis condition was allowed to continue for 42 days (Monzon et al., 2001; Brin et al., 2008) or 3 months (Orhan et al., 2009) as the previous experiments. However, after 3 weeks of osteomyelitis, the induction in this study showed osteomyelitis signs in the radiographic examination. The radiographic study showed the radiolucent area in the bone marrow cavity surrounding the inoculation site. After treatment, the radiolucent area in the control group was continuously widen, while radiolucent area in the GI-PMMA, G-NCS and G-HPCS groups were significantly decreased. This result suggested that the GI-PMMA, G-NCS and G-HPCS could be used for managing osteomyelitis. Histopathological study with H&E and Masson's trichrome staining revealed sequestrums, pyogranulomatous abscess and bone cortex deterioration in the control group, in contrast, new bone regeneration was found at the implantation site in the G-NCS and G-HPCS groups (Fig 4, 5 and Table 3). These results suggests that calcium sulfate beads could facilitate the new bone regeneration. Although the results of GI-PMMA on the treatment for osteomyelitis in this study were satisfying, the unabsorbable property of the PMMA could interfere with the bone healing process as found in this study.

Conclusion

High porous calcium sulfate bead was synthesized utilizing salt leaching technique to increase the porosity level and reduce the amount of calcium sulfate. G-HPCS is a new fabricated antibiotic bead that can be used as a local antibiotic provider for management of osteomyelitis comparable to the commercial antibiotic impregnated bone cement and the calcium sulfate coated antibiotic. In addition, G-HPCS has lower amount of carrier materials, thus helps reduce the inflammatory response to an implant material.

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