The Efficacy and Biocompatibility of Hydroxyapatite Composites Impregnated with Vancomycin as a Local Drug Delivery System

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Objective: *This study investigated the efficacy and biocompatibility of local biodegradable composites composed of hydroxyapatite, plaster of Paris, and binders (chitosan or alginate) impregnated with vancomycin.*
Material and Method: *Local biodegradable tablet composed of hydroxyapatite, plaster of Paris, and binders (chitosan or alginate) were prepared and mixed with vancomycin. The tablet was tested for releasing profile using dissolution apparatus for 1 week. Biocompatibility of each composite was tested using MTT test.*
Results: *Vancomycin could be released from the composite from both using binder either chitosan or alginate for at least one week but the release profile of the composite using alginate showed a better pattern of elution than those using chitosan. Biocompatibility test against osteoblast demonstrated good biocompatibility of the both composites at different concentration of drug use comparing to the control group.*
Conclusion: *The local hydroxyapatite composite composed of hydroxyapatite, plaster of Paris and binder either chitosan or alginate seems to be a promising local biodegradable delivery system for vancomycin in treating of methicillin-resistant Staphylococcus aureus osteomyelitis.*

Keywords: *Hydroxyapatite, local drug delivery*

The Thai Journal of Orthopaedic Surgery: 36 No.3-4: P1-8
Full text. e journal: *http://www.rcost.or.th, http://thailand.digitaljournals.org/index.php/JRCOST*

Methicillin-resistant Staphylococcal aureus (MRSA) is a high virulent organism that can cause a various site of infection including musculoskeletal system. The incidence of this causative organism in musculoskeletal system has been increasingly reported all around the world[1-5]. MRSA osteomyelitis had a higher morbidity and need a higher cost compared to nonMRSA osteomyelitis[6,7]. Treatment of this infection required both medical and surgical modalities.The standard drug for eradicating this infection is vancomycin. However, prolonged parenteral used of this antibiotic has been reported to be associated with a number of minor and major complications such as redman syndrome and nephrotoxicity[8-11]. Local antibiotic treatments have been approved for their efficacy and benefits in terms of shorter hospitalization and lower systemic side effects compared to conventional parenteral antibiotics for treatment of chronic osteomyelitis[1,9]. Nevertheless, commercially available local antibiotic cement, Septopal® (Merck, Darmstadt, Germany) is ineffective in treating chronic osteomyelitis caused by methicillin-resistant Staphylococcal aureus (MRSA), whereas homemade polymethylmethacrylate (PMMA) impregnated with antibiotics has been effective in treatment of this condition[6]. However, PMMA is a nonbiodegradable carrier which requires surgical removal; therefore numerous biodegradable materials such as hydroxyapatite (HA), plaster of Paris, chitosan, collagen sponge, polymers, and fibrin glue have been studied for use as local drug delivery systems for antibiotics[12-18]. Among these, HA and plaster of Paris are highly biocompatible and biodegradable materials. In addition, a composite of HA and plaster of Paris has a porous structure, which might be suitable for osteoblast proliferation and has been used clinically as a bone substitute. Chitosan and alginate are biodegradable materials that have been extensively used as a drug delivery system, which may be used as a binder to HA and plaster of Paris to improve the elution characteristics[17,19,20]. The objectives of this study were to test the release profile of vancomycin from the HA, plaster of Paris, and binders (chitosan and alginate) composite as the carrier and to investigate

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biocompatibility of these carriers when incorporated with vancomycin.

Material and Method

Hydroxyapatite (Ca_{10} $(PO_4)_6$ $(OH)_2$) was obtained from MTEC (Bangkok, Thailand); plaster of Paris from Merck (Darmstadt, Germany); chitosan (medium viscosity, 80% degree of acetylation) and alginate from Fluka (Buchs, Switzerland); vancomycin from Eli Lilly(Indianapolis, IN, USA).

Preparation of cement tablets

The cement powder for the hydroxyapatite composite consisted of HA: plaster of Paris at a ratio of 85:15. Chitosan and alginate gel were prepared by dispersing chitosan in distilled water and adjusting the pH to 4.5 with acetic acid. In each preparation, the amounts of the solid and liquid phases were fixed at 4.0 g and 4.5 g, respectively. Each antibiotic was loaded into the powder before mixing with chitosan gel, then poured into a tablet mold (6.5-mm diameter and 5.2-mm thickness).

Each carrier without antibiotic served as a control. After the cement tablets had hardened, they were removed from the mold and dried at 60^{o}C for 2 hours. All tablets were sterilized by ethylene oxide before testing.

Drug release test

The tablet was tested for releasing profile using dissolution apparatus II with phosphate buffer saline pH 7.4 under the temperature of 37 ^{o}C at the speed of 50 cycles per second. The elute was sampling at various time points (6, 12, 24 hours and then everyday until completed at day 7). Then, the elute was examined for the drug concentration with spectrophotometer at the wave length of 280.5 nm.

Biocompatibility test

MC3T3.E1 preosteoblast cells and cell culture conditions

MC3T3.E1 preosteoblast cells, kindly provided by Dr. P. Arpornmaeklong (Prince of Songkla University, Thailand), were used in our experiments between passage 4 and 10. MC3T3.E1 cell line is composed of osteoprogenitor cells derived from newborn mouse calvaria that are known to differentiate along the osteoblast pathway in the presence of ascorbic acid. The cells were grown in alpha Modified Eagle Medium (MEM, Gibco®), supplemented with ascorbic acid (50µg/ml) (Sigma®), 10 mM β-glycerophosphate (Sigma®), 50 IU/ml penicillin-streptomycin (Sigma®), and 10 % fetal calf serum (Gibco®). The cells were maintained at 37° in a fully humidified atmosphere at 5% CO_2. The media was changed every 48 hours after 3 days in culture. The cells were passaged with trypsin-EDTA (Gibco-BRL®), counted on Malassez and plated at a density of $2x10^4$ cell/cm^2 in 60 mm diameter Petri dishes. The granules were added (20 mg/culture dish) to the wells 24 hours later. Cultures were observed under phase contrast microscope up to day 21 and micrographs were taken on days 6 and 12.

The MTT assay

Cells were seeded into 96-well plate at a density of 1×10^4 cells per well in 100 µl medium. After 48 hours, when the cells were in the log phase of growth, the medium was replaced with 100 µl of the extracts or control medium. The cells were exposed to the extracts for 24 hr., and then the liquid was aspirated and 50 µl of 10 ml MTT in PBS was added to each well. After an incubation period of 4 hours, the liquid was aspirated and the fromazan reaction product dissolved in 200 µl dimethyl sulphoxide. The optical densities were measured using a Titertek multiscan (MCC 340) microspectrophotometer at 540 nm.

Cell morphology

Osteoblast morphology was observed and pictured under contrast light microscope for detecting morphological change after exposed to the extract of each drug at various concentrations.

Statistical Analysis

Mean inhibition zone widths were calculated for each condition every day and the inhibition profile with time was examined graphically. To evaluate the statistical significance of differences in inhibition zones between the two carriers for each antibiotic, the generalized estimating equations method of linear regression was used, in which the week of testing of each carrier and the interaction between the two carriers were included as explanatory variables. This method was used to account for the lack of independence among measures of the outcome with time for the same tablet.

Results

From the drug release test, the cumulative amount of vancomycin eluted from the chitosan-hydroxapatite composite showed a sharp increasing pattern at the first few days and then gradually decrease until 1 week period of testing.The amount of drug release depend on the amount of drug loaded. Chitosan-hydroxapatite composite with 10 percent of drug loaded showed a significantly higher than those loaded with 5 and 2.5 percent (Fig.1) The release pattern of vancomycin from alginate-hydroxapatite composite demonstrated a different pattern compared with chitosan-hydroxapatite composite. The rate of release was constant all over the period of testing. The drug release was significantly higher in the composite loading with the higher amount of vancomycin compared to those with lower amount of drug loaded (Fig.2). When compared between the drug release from chitosan and alginate-hydroxypaptite composite at the same timepoint, chitosan-hydroxapatite composite revealed a higher drug concentration than vancomycin eluted from alginate-hydroxypaptite composite (Fig.3).

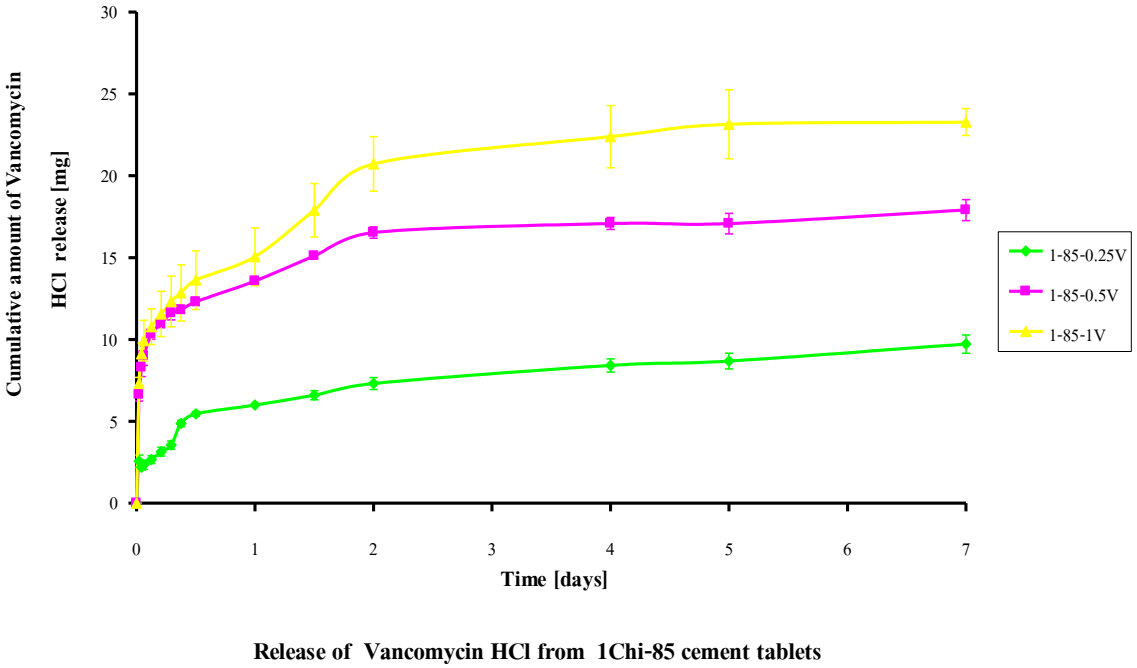


Fig. 1 Vancomycin releasing profile from chitosan-hydroxyapatite composite with different drug loading at various timepoints

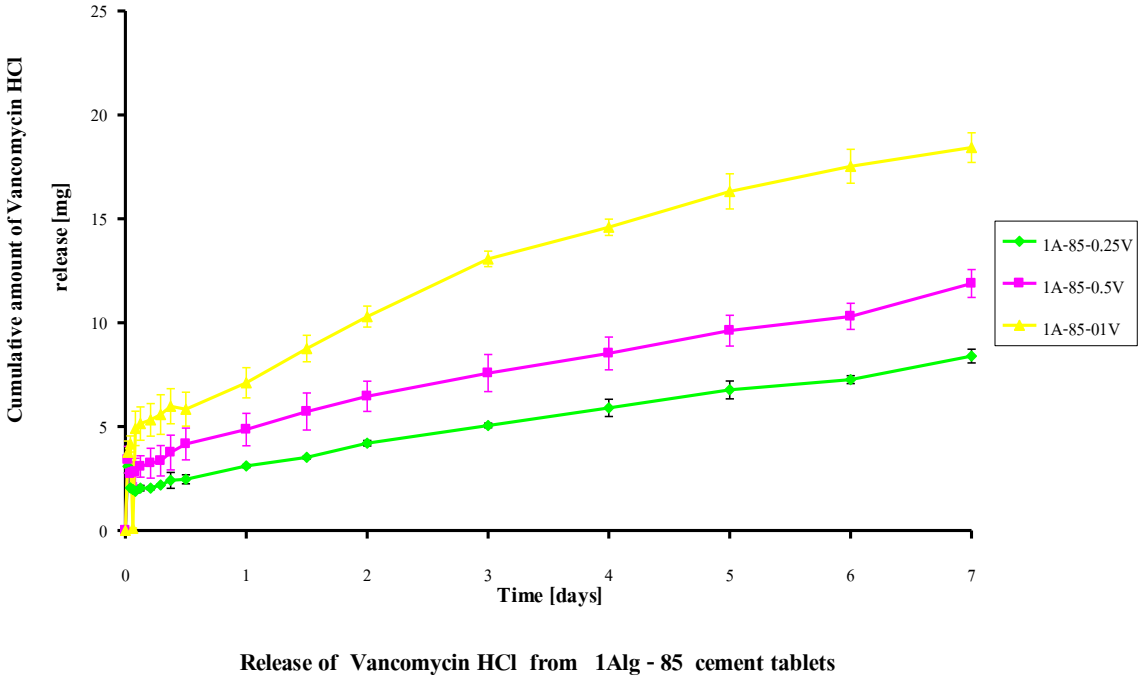
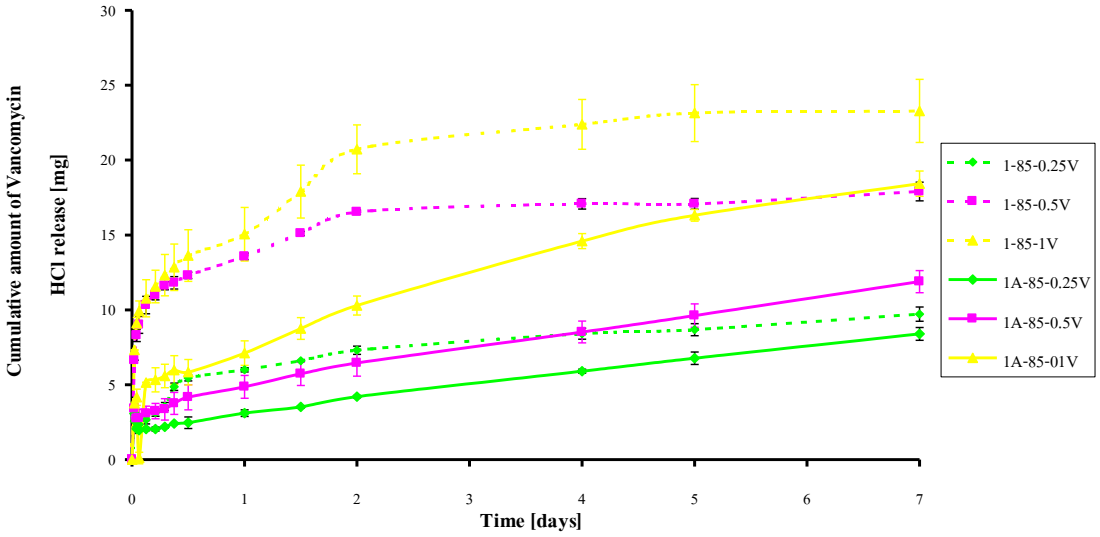


Fig. 2 Vancomycin releasing profile from alginate-hydroxyapatite composite with different drug loading at various timepoints



Comperative of Vancomycin HCl release from cement tablets with chitosan and sodium alginate as liquid phase of cements

Fig. 3 Comparison of vancomycin release profile in different concentrations from alginate and chitosan hydroxyapatite composites

From the biocompatibility test using osteoblast, the percentage of cell viability was above 80 percent in both control and hydroxyapatite composite loaded with vancomycin groups. When comparing chitosan and alginate-hydroxyapatite composite with the control group, there was no significant difference in percentage of cell viability in all groups. Furthermore, when increasing the amount of vancomycin from 10 to 1,000 mcg/ml, there was no deterioration in the percentage of cell viability in both groups (Fig.4). Histological examination of osteoblast morphology showed no gross morphological change after exposed to all of the carriers loaded with vancomycin (Fig.5).

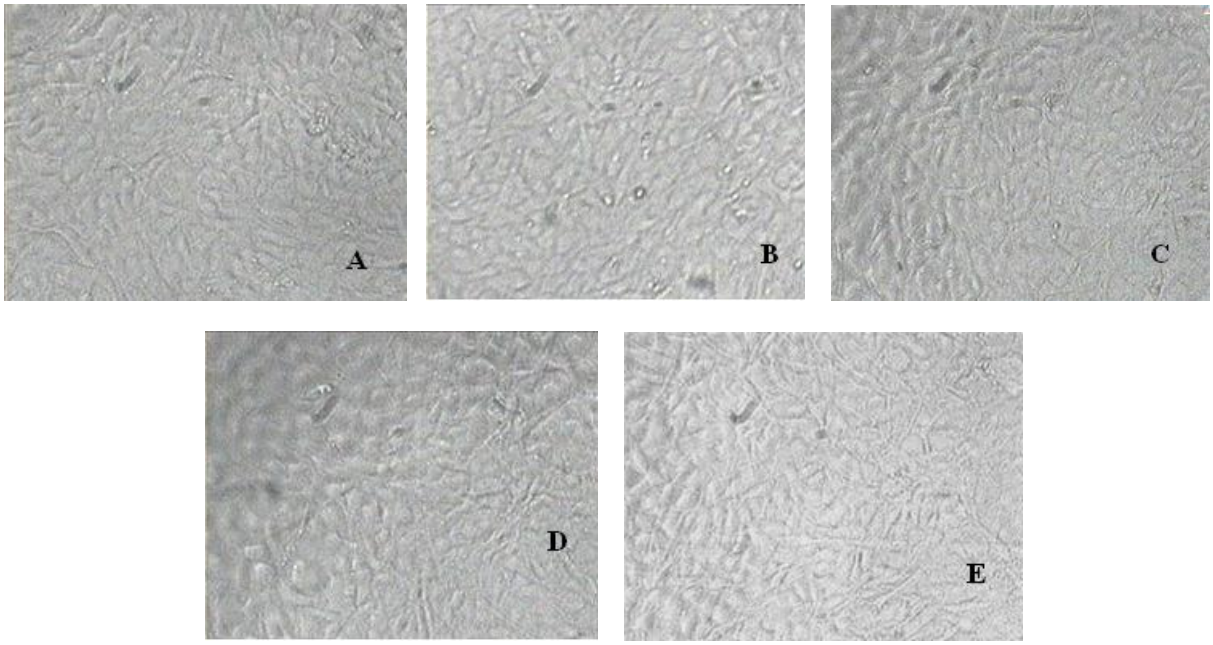


Fig. 4 Cell histology of osteoblast in different conditions, normal osteoblast (A), osteoblast exposed to chitosan-hydroxyapatite composite(B), osteoblast exposed to alginate-hydroxyapatite composite(C), osteoblast exposed to chitosan-hydroxyapatite composite loaded with vancomycin(D), osteoblast exposed to alginate-hydroxyapatite composite loaded with vancomycin(E)

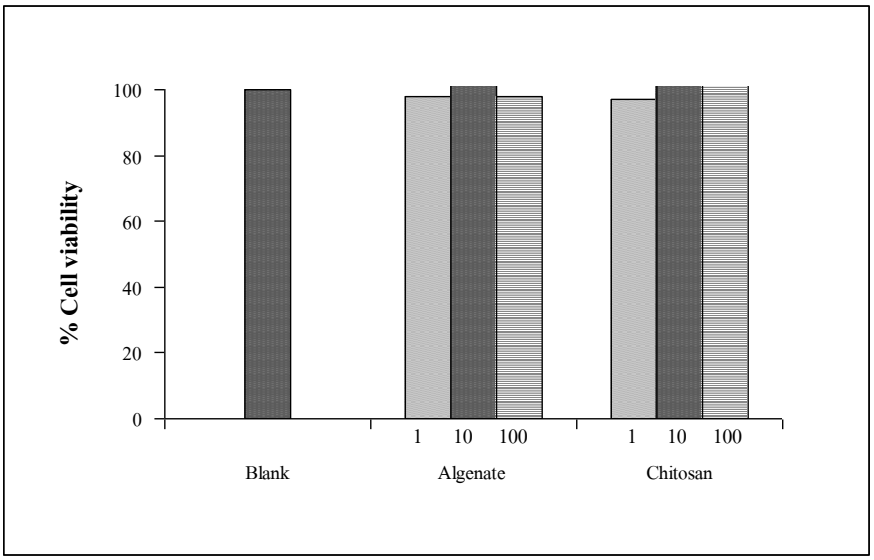


Fig. 5 Biocompatibility of alginate and chitosan hydroxyapatite composites impregnated with vancomycin to osteoblast at different concentrations of drug load

Discussion

This study demonstrated that local biodegradable composite composed of hydroxyapatite, plaster of Paris, and binders either chitosan or alginate might be used as a skeletal drug delivery system for antibiotics to treat MRSA osteomyelitis. Both carriers demonstrated sustained drug elution of vancomycin and showed no deteriorating effect on osteoblast cell.

Local antibiotic delivery systems have been approved for management of musculoskeletal infections[1]. Thorough debridement is required before augmenting with high sustained local antibiotics. Local delivery has advantages over the parenteral route because it can decrease systemic side effects, decrease hospitalization and save costs[1]. Although PMMA, a local nonbiodegradable carrier, has been studied and incorporated with various antibiotics with good results,[21,22] its main disadvantages are the need for surgical removal, the risk of recolonization of bacteria,[23] and thermal effects during polymerization[10,23]. Biodegradable carriers have the advantages of releasing antibiotics without requiring additional surgery for removal. There are some studies using biodegradable carriers as a skeletal delivery system. Mousset et al[2] showed that vancomycin loaded in plaster of Paris could be released for 1 week. Aimin et al[12] reported that chitosan incorporated with gentamicin released antibiotic for 2 weeks. Our study showed that HA, plaster of Paris, and binder either chitosan or alginate composite impregnated with vancomycin could release vancomycin. This benefit of the composite may be because of the porous structure of hydroxyapatite in which plaster of Paris can infiltrate and because the chitosan acts as a binder for two materials and promotes the sustainability of the antimicrobial effect. The aminopolysaccharide, chitosan, which has been used for a slow sustained drug carrier system, is inexpensive, biodegradable, and biocompatible[12,19,25]. It can be hydrolized by human lysozyme, chitinase, and chitosanase[26]. Previous studies have shown that chitosan also has antimicrobial, immunologic,[27] and osteoconductive properties,[19] and therefore is useful as a drug carrier and for bone substitution, which can decrease the surgical risks of additional bone grafting procedures[16].

The amount of antibiotic released from a local carrier system depends on several factors, such as type of antibiotic loaded, surface area of the carrier, and implant material. In this study, the shape and size of the carrier and the amount of each antibiotic were controlled; therefore the result of elution is related directly to the types of drugs and carriers. Furthermore, the release profiles of drugs from the composite are determined by the cross linked density of chitosan and initial drug content, which also was controlled. The modified disc diffusion technique used for determination of the inhibitive effect of each antibiotic yield only semiquantitative results. However, it correlates well with the antibiotic concentration as measured by high performance liquid chromatography. All antibiotic tablets were sterilized by ethylene oxide instead of gamma radiation because our previous study showed no deteriorating effects on their elution characteristics[6].

Our study has demonstrated that hydroxyapatite composite using either chitosan or alginate as a binder showed was not toxic to osteoblast at any concentration compared to control. This will ensure that these local biodegradable drugs will not disturb normal process of bone healing. Our result was quite similar to the recently reported by Rauschmann MA, et al. which showed

good biocompatibility of nanocrystalline hydroxyapatite and calcium sulphate carrying vancomycin[17].

The limitations of the study are that an in vitro study cannot totally simulate in vivo conditions because of such factors as lack of host immunity and local pH changes. The duration of the study was quite short, therefore, longer period of release study and additional in vivo testing are needed to confirm the efficacy, side effects, and biodegradable properties of this material.

We demonstrated that local biodegradable composite composed of HA, plaster of Paris, and binder either chitosan or alginate is a promising carrier for vancomycin.

Acknowledgement

The authors would like to thank Faculty of Medicine, Prince of Songkla University for financial support to this study.

References

1. Blaha JD, Nelson CL, Frevert LF, Henry SL, Seligson D, Esterhai JL Jr, et al. The use of septopal (polymethylmethacrylate beads with gentamicin) in the treatment of chronic osteomyelitis. Instr Course Lect 1990; 39: 509-14.
2. Mousset B, Benoit MA, Delloye C, Bouillet R, Gillard J. Biodegradable implants for potential use in bone infection: An in vitro study of antibiotic-loaded calcium sulphate. Int Orthop 1995; 19(3): 157-61.
3. Musher DM. The gram-positive cocci III: Resistance to antibiotics. Hosp Pract (Off Ed) 1988; 23: 105-7.
4. Sheftel TG, Mader JT, Pennick JJ, Cierny III G. Methicillin-resistant Staphylococcus aureus osteomyelitis. Clin Orthop Relat Res 1985; (198): 231-9.
5. Watanakunakorn C. Treatment of infection due to methicillin-resistant Staphylococcus aureus osteomylitis in a neonate. Ann Intern Med 1982; 97: 376-8.
6. Buranapanitkit B, Wongsiri S. Home–made local antibiotic cement for chronic osteomyelitis caused by resistant organisms. J ASEAN Orthop Assoc 2001; 14(1): 27-31.
7. Buranapanitkit B, Wongsiri S. MRSA (methicillin-resistant Staphylococcus aureus) osteomylitis: Factors and results compared to non-MRSA osteomyelitis. J ASEAN Orthop Assoc 2000; 13(1): 34-6.
8. Adams K, Couch L, Cierny G, Calhoun J, Mader JT. In vitro and in vivo evaluation of antibiotic diffusion from antibiotic–impregnated polymethylmethacrylate beads. Clin Orthop Relat Res 1992; (278): 244-52.
9. Cole WG. The management of chronic osteomyelitis. Clin Orthop Relat Res 1991; (264): 84-9.
10. Jain AK, Panchagnula R. Skeletal drug delivery systems. Int J Pharm 2000; 206(1-2): 1-12.
11. Mader JT, Wang J, Calhoun JH. Antibiotic therapy for musculoskeletal infection. J Bone Joint Surg 2001; 83A: 1878-901.
12. Aimin C, Chunlin H, Juliang B, Tinyin Z, Zinchao D. Antibiotic loaded chitosan bar: An in vitro, in vivo study of a possible treatment for osteomyelitis. Clin Orthop Relat Res 1999; (366): 239-47.
13. Buranapanitkit B, Wongsiri S, Ingviya N, et al: In vitro elution characteristic of antibiotic cement on MRSA organism. J ASEAN Orthop Assoc 2000; 13: 53-6.
14. Buranapanitkit B, Srinilta V, Ingviga N, Oungbho K, Geater A, Ovatlarnporn C. The efficacy of a hydroxyapatite composite as a biodegradable antibiotic delivery system. Clin Orthop Relat Res 2004; (424): 244-52.
15. Calhoun JH, Mader JT. Treatment of osteomyelitis with a biodegradable antibiotic implant. Clin Orthop Relat Res 1997; (341): 206-14.
16. Ebraheim NA, Elgafy H, Xu R. Bone-graft harvesting from iliac and fibular donor sites: Techniques and complications. J Am Acad Orthop Surg 2001; 9(3): 210-8.
17. Rauschmann MA, Wichelhaus TA, Stirnal V, Dingeldein E, Zichner L, Schnettler R, et al. Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections. Biomaterials 2005; 26(15): 2677-84.
18. Yamashita Y, Uchida A, Yamakawa T, Shinto Y, Araki N, Kato K. Treatment of chronic osteomyelitis using calcium hydroxyapatite ceramic implants impregnated with antibiotic. Int Orthop 1998; 22(4): 247-51.
19. Oungbho K, Muller BW. Chitosan sponges as sustained release drug carriers. Int J Pharm 1997; 156(2): 229-37.
20. Tomihata K, Ikada Y. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. Biomaterials 1997; 18(7): 567-75.
21. Greene N, Holtom PD, Warren CA, Ressler RL, Shepherd L, McPherson EJ, et al. In vitro elution of tobramycin and vancomycin polymethylmethacrylate beads and spacers from Simplex and Palacos. Am J Orthop (Belle Mead NJ) 1998; 27(3): 201-5.
22. Kuechle DK, Landon GC, Musher DM, Noble PC. Elution of vancomycin, daptomycin, and amikacin from acrylic bone cement. Clin Orthop Relat Res 1991; (264): 302-8.
23. Kanellakopoulou K, Giamarellos-Bourboulis EJ. Carrier systems for the local delivery of antibiotics in bone infections. Drugs 2000; 59(6): 1223-32.
24. Kendall RW, Duncan CP, Smith JA, Ngui-Yen JH. Persistence of bacteria on antibiotic loaded

acrylic depots. Clin Orthop Relat Res 1996; (329): 273-80.
25. Sidqui M, Collin P, Vitte C, Forest N. Osteoblast adherence and resorption activity of isolated osteoclasts on calcium sulphate hemihydrate. Biomaterials 1995; 16(17): 1327-32.
26. Muzzarelli R, Tarsi R, Filippini O, Giovanetti E, Biagini G, Varaldo PE. Antimicrobial properties of N-carboxybutyl chitosan. Antimicrob Agents Chemother 1990; 34(10): 2019–23.
27. Peluso G, Petillo O, Ranieri M, Santin M, Ambrosio L, Calabró D, et al. Chitosan-mediated stimulation of macrophage function. Biomaterials 1994; 15(15): 1215-20.

ประสิทธิผลและความเข้ากันได้ของสารพาหะไฮดรอกซี่อาปาไทท์ที่ผสมด้วยยาแวนโคมัยซิน สำหรับเป็นแบบนำส่งยาเฉพาะที่

ประภากร กลับกลาย, พบ, ขวัญจิต อึ๊งโพธิ์, ภบ, บุญสิน ตั้งตระกูลวนิช, พบ

วัตถุประสงค์ *: เพื่อศึกษาประสิทธิผลและความเข้ากันได้ของสารพาหะชนิดใหม่ที่มีส่วนประกอบของไฮดรอกซี่อาปาไทท์ ปูนพลาสเตอร์ และสารผสม (binder) ทั้ง ไคโตซานและอัลจีเนต*

วัสดุและวิธีการ*: เม็ดยาประกอบด้วยไฮดรอกซี่อาปาไทท์ ปูนพลาสเตอร์ และสารผสม ทั้ง ไคโตซานและอัลจีเนต ถูกผลิตและผสมด้วยยาแวนโคมัยซิน ก่อนนำไปศึกษาประสิทธิผลการปลดปล่อยยา โดยใช้เครื่อง dissolution apparatus เป็นเวลา 1 สัปดาห์ นอกจากนี้ศึกษาความเข้ากันได้ (biocompatibility) ของเม็ดยาโดยวิธี MTT test*

ผลการศึกษา*: พบว่าเม็ดยาสามารถปลดปล่อยยาแวนโคไมซินได้ในระยะเวลาอย่างน้อย 1 สัปดาห์ โดยเม็ดยาที่มีสารอัลจีเนตปลดปล่อยยาได้ดีกว่าไคโตซาน อย่างไรก็ดีพบว่าเม็ดยาชนิดใหม่มีความเข้ากันได้ กับเซลล์ ออสติโอบลาสในทุกความเข้มข้นยา*

สรุป*: คณะผู้วิจัยได้สร้างสารพาหะชนิดละลายได้เฉพาะที่ชนิดใหม่ ซึ่งมีองค์ประกอบของไฮดรอกซี่อาปาไทท์ ปูนพลาสเตอร์ และสารผสมสามารถปล่อยสารยาแวนโคมัยซินได้ น่าจะนำไปใช้ในการรักษาโรคติดเชื้อกระดูกและข้อที่เกิดจากเชื้อ MRSA ได้แต่ต้องมีการศึกษาต่อทางคลินิก*