In Vitro Antimicrobial Activity of Phramongkutklao Hydroxyapatite Antibiotic Pellet

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Objective: *Assess the antimicrobial activity of Phramongkutklao antibiotic hydroxyapatite pellets in vitro.*
Material and Method: *The selected bacteria used in the present study were the standard and drug-resistant strains of Staphylococcus aureus and Pseudomonas aeruginosa, which are the common organisms causing infection in Orthopedics. Phramongkutklao antibiotic hydroxyapatite pellets developed by the Orthopedics Department, Phramongkutklao Hospital contained either Vancomycin, Fosfomycin, or Gentamycin. Each preparation was placed on an agar plate inoculated with each bacterium. The inhibition zones were monitored in 24 hours. Then the pellets were moved onto the new inoculated plate every day for 28 days.*
Results: *Compared with the control group, Phramongkutklao antibiotic hydroxyapatite pellets had good inhibitory effect against S.aureus. Vancomycin hydroxyapatite pellets were able to maintain their activity for 28 days whereas Gentamycin hydroxyapatite pellets was effective for only three days. Fosfomycin hydroxyapatite pellets could inhibit MSSA for 13 days and MRSA for 25 days. On the contrary, all three Phramongkutklao antibiotic hydroxyapatite pellets had very low efficacy against P.aeruginosa.*
Conclusion: *Phramongkutklao antibiotic hydroxyapatite pellets were able to inhibit the growth of S. aureus both MSSA and MRSA while the activity against P.aeruginosa needs to be developed.*

Keywords: *Hydroxyapatile, Antibiotic pellet, Infection*

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Nowadays, many bone substitute materials are available in Orthopedics. Hydroxyapatite is a one of the bone substitutes that can induce osteoconduction and has neither toxicity nor immune response stimulating effect[1-4]. However, in Thailand the cost of hydroxyapatite is high, as this has to be imported. For this reason, this is not commonly used in the authors' practice.

Since 1993, the Orthopedic Department, Phramongkutklao Hospital has developed hydroxyapatite produced from bovine bones. This material has high purity and excellent effect of osteoconduction[5,6]. It gave the satisfactory results in the clinical trials such as total hip revision and comminuted fracture of distal end radius[6,7].

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In 1999, the antibiotic sustained release hydroxyapatite pellet was developed. This material serves as two major functions. One is to carry the antibiotic to the target and the other is to act as an osteoconductor[8]. Formerly, polymethylmethacrylate has been used as a carrier. However, this material is needed to be removed because it cannot be degraded and eventually became the foreign body.

Many international studies have shown the effectiveness of hydroxyapatite containing antibiotics *in vitro* and *in vivo*. A variety of hydroxyapatites and antibiotics had been reported for the treatments of osteomyelitis and bone defect, and the prevention of infections in opened fracture[9-11].

In the present study, Phramongkutklao hydroxyapatite mixed with calcium sulfate and either vancomycin, fosfomycin, or gentamycin were compared for antibacterial activity against microorganisms often

causing infections in orthopedics[12,13]. These included *S. aureus* and *P. aeruginosa* both standard and drug-resistant strains. Activity of each antibiotic hydroxyapatite preparations were observed every day for 28 days. The results will be useful for further development of Phramongkutklao antibiotic hydroxyapatite preparations.

Material and Method

Preparation of Phramongkutklao hydroxyapatite

Phramongkutklao hydroxyapatite was prepared from bovine bones. The bones were collected, boiled for 10 hr, air-dried at room temperature, and soaked in concentrated HCl for 48 hr to remove unwanted organic substances. Then, double sinter technique was performed by burning prepared bones at 550°C for 6 hr, followed by burning at 1300°C for 6 hr yielding Phramongkutklao hydroxyapatite. Crude Phramongkutklao hydroxyapatite was ground into fine powder before use.

Preparation of hydroxyapatite pellets containing antibiotics

To prepare antibiotic hydroxyapatite pellets, 20 g of Phramongkutklao hydroxyapatite and 20 g of calcium sulfate were mixed in a rotor mixer for 10 min. The binder, sodium silicate, was slowly added until getting damp mass to prepare granules. Next, wet granules were heated until dried at 50°C for 4 hr. After sifting, same size granules were collected and mixed with either 1 gram of vancomycin, 1 gram of fosfomycin, or 1 gram of gentamycin. The mixture was compressed by a high-pressure tabletting machine yielding 6 mm-diameter antibiotic hydroxyapatite pellets. The pellets then were made sterile by gamma ray at 25 kGy for 25 hr.

Preparation of microbacterias

Two microbacterias were included in the present study, both standard strain and resistant strain as shown in Table 1.

Each of Phramongkutklao Hydroxyapatile Antibiotic pellets (Vancomycin, Fosfomycin, and Gentamycin) and one Hydroxyapatile pellet without antibiotic for control group were prepared. Each was placed on an agar-plate that inoculated each bacterial strain. After that, there were incubated at 37°C and transferred to new same prepared agar-plate every 24 hours for 28 days (Fig. 1). Antibiotic assay was performed by inhibition zone (millimeters).

Table 1. Bacterial strains in this study

1. Methicillin sensitive *Staphylococcus aureus* (ATCC) standard strains	: MSSA
2. Methicillin resistant *Staphylococcus aureus*	: MRSA
3. *Pseudomonas aeruginosa*	: ATCC
4. *Pseudomonas aeruginosa*	: MDR

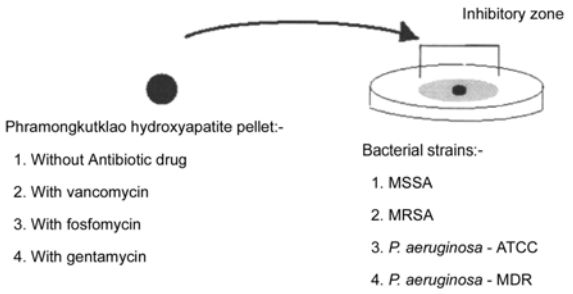

Fig. 1 Diagrams of hydroxyapatite pellets containing antibiotics and Bacterial strains

Statistical analysis

Repeated Measures Analysis of Variance was used to analyze covariance of inhibition zones of antibiotic pellets at various time points. Multiple comparisons were used to compare the day that have no difference in average inhibitory effect at the different time. Significant difference in statistics was accepted when $p < 0.01$.

Results

Inhibition zones of each antibiotic hydroxyapatite pellets had a similar pattern that showed the largest diameter size on the first day and then slowly decreased in the following day. The result showed that fosfomycin gave the largest inhibition zone size followed by vancomycin and gentamycin, respectively (Table 2-5).The difference between inhibition zones was compared at each time point. It showed that the difference of inhibition zone size decreased with increasing time.

Vancomycin pellets could effectively inhibit only the growth of *S.aureus* both MSSA and MRSA during 28 days. There were no statistical differences at day 7 for MSSA and at day 3 for MRSA. In contrast, Fosfomycin pellets could inhibit all tested bacteria but not all 28 days (13 days for MSSA, 25 days for MRSA, 7 days for *P. aeruginosa* ATCC, and 5 days for *P. aeruginosa* MDR). Gentamycin pellets could inhibit some strains but not *P. aeruginosa* MDR. Compared with Vancomycin and Fosfomycin, it showed the

Table 2. Comparison of inhibition zones diameter each antibotic pellet at various times for *S. aureus* (ATCC)

Day (s)	Inhibition zone diameter (mm) Mean (95% CI)		
	Vancomycin	Fosfomycin	Gentamycin
1	22.4 (21.2,23.5)	32.2 (31.0,33.3)	14.2 (13.0,15.3)
3	19.4 (15.6,23.1)	25.6 (21.8,29.3)	8.2 (4.4,11.9)
7	17.0 (15.4,18.5)	10.6 (9.0,12.1)	6.0 (4.4,7.5)
14	16.8 (16.5,17.0)	6.0 (5.7,6.2)	6.0 (5.7,6.2)
21	16.0 (15.6,16.3)	6.0 (5.6,6.3)	6.0 (5.6,6.3)
28	15.2 (14.7,15.6)	6.0 (5.5,6.4)	6.0 (5.5,6.4)

Table 3. Comparison of inhibition zones diameter each antibotic pellet at various times for *S. aureus* (MRSA)

Day (s)	Inhibition zone diameter (mm) Mean (95% CI)		
	Vancomycin	Fosfomycin	Gentamycin
1	22.4 (20.9,23.8)	34.4 (32.9,35.8)	16.0 (14.5,17.5)
3	17.0 (15.2,18.7)	27.0 (25.2,28.7)	6.0 (4.2,7.7)
7	15.8 (14.2,17.4)	15.4 (13.8,16.7)	6.0 (4.4,7.5)
14	14.4 (12.3,16.4)	9.2 (7.1,11.2)	6.0 (3.9,8.0)
21	14.8 (14.1,15.4)	7.8 (7.1,8.4)	6.0 (5.3,6.6)
28	14.6 (14.2,14.9)	6.2 (5.8,6.6)	6.0 (5.6,6.4)

Table 4. Comparison of inhibition zones diameter each antibotic pellet at various times for *P. aeruginosa*

Day (s)	Inhibition zone diameter (mm) Mean (95% CI)		
	Vancomycin	Fosfomycin	Gentamycin
1	6.0 (3.9,8.05)	36.0 (33.9,38.0)	33.4 (31.3,35.4)
3	6.0 (3.0,8.9)	24.2 (20.9,27.5)	24.8 (21.4,28.1)
7	6.0 (4.5,7.4)	6.0 (4.5,7.4)	14.8 (13.3,16.2)
14	6.0 (6.0,6.0)	6.0 (6.0,6.0)	6.0 (6.0,6.0)
21	6.0 (6.0,6.0)	6.0 (6.0,6.0)	6.0 (6.0,6.0)
28	6.0 (6.0,6.0)	6.0 (6.0,6.0)	6.0 (6.0,6.0)

Table 5. Comparison of inhibition zones diameter each antibotic pellet at various times for *P. aeruginosa* (MDR)

Day (s)	Inhibition zone diameter (mm) Mean (95% CI)		
	Vancomycin	Fosfomycin	Gentamycin
1	6.0 (4.9,7.0)	32.4 (31.4,32.4)	6.0 (4.9,7.0)
3	6.0 (4.5,7.5)	16.6 (15.1,18.1)	6.0 (4.5,7.5)
7	6.0 (6.0,6.0)	6.0 (6.0,6.0)	6.0 (6.0,6.0)
14	6.0 (6.0,6.0)	6.0 (6.0,6.0)	6.0 (6.0,6.0)
21	6.0 (6.0,6.0)	6.0 (6.0,6.0)	6.0 (6.0,6.0)
28	6.0 (6.0,6.0)	6.0 (6.0,6.0)	6.0 (6.0,6.0)

inferior inhibitory effect (5 days for MSSA, 3 days for MRSA, and 7 days for *P. aeruginosa* ATCC). Hydroxyapatite pellet without antibiotics, which was a control group, did not have an inhibitory effect.

There was no statistically difference inhibitory effect of Vancomycin pellet after 7 days for MSSA and 3 days for MRSA, Fosfomycin pellet after 14 days for MSSA and MRSA and after 7 days for ATCC and MDR, and Gentamycin pellet after 7 days for MSSA and ATCC, and after 3 days for MRSA (Table 6).

Repeated Measures Analysis of Variance revealed that the differences in types of antibiotics and incubation period significantly affected inhibition zones ($p < 0.001$).

Table 6. Periods of time which had no change in inhibition zone size of each antibiotic by multiple comparison (days)

	Bacterial strains			
	MSSA	MRSA	ATCC	MDR
Vancomycin	7	3	0	0
Fosfomycin	14	14	7	7
Gentamycin	7	3	7	0

Discussion

Hydroxyapatile has the excellent biocompatibility and compressive mechanical resistant which is very helpful for filling in the bony defects. With its interconnecting pore structure, the Hydroxyapatile should theoretically have a good antibiotic contained property.

The present study was focused on the assessment of antimicrobial activity of Phramongkutklao antibiotic hydroxyapatite pellets developed by Phramongkutklao Hospital. The selected bacteria

used in the present study were the common infectious organisms in Orthopedics. The experiment was performed by observing the antibiotic releasing ability of hydroxyapatite pellets and inhibitory efficiency of antibiotic hydroxyapatite pellets.

The results demonstrated that vancomycin pellet could continuously release antibiotics and had the inhibitory effect against MSSA and MRSA for the whole experimental period. In fact, it showed that Fosfomycin could inhibit MSSA and MRSA with higher extent than Vancomycin did at the early time points. This indicated that fosfomycin diffused from hydroxyapatite pellets into the agar medium much better than vancomycin. It might be possible due to the lower molecular weight and higher water solubility of Fosfomycin[14]. For Gentamycin, its inhibitory effect rapidly decreased. The factors that could affect the results were the physical factors between antibiotics and hydroxyapatite, the drug-resistant bacteria, the quantity of antibiotics, and the stability of antibiotics in different environmental conditions. The development of preparations, antibiotic contents, and stability tests are still required.

When compared with other studies using polymethylmethacrylate with four-times higher in concentration of the same kinds of antibiotics, they showed the lower efficacy in both releasing ability and inhibitory effect[15]. This suggested that hydroxyapatite had higher efficiency to be a carrier than that of the polymethylmethacrylate. The presented data also revealed that *P. aeruginosa* could not be inhibited by any tested antibiotics. This problem might be solved by using antipseudomonas antibiotics or mixing at least two drugs with different modes of action to make Phramongkutklao antibiotic hydroxyapatite pellets effective on a variety of microorganisms.

It is necessary that *in vivo* study mimic human body condition before using Phramongkutklao antibiotic hydroxyapatite pellets in clinical practice.

In conclusion, Phramongkutklao antibiotic hydroxyapatite pellets effectively release antibiotics and can inhibit the growth of bacteria causing infection in orthopedics such as *S. aureus* both MSSA and MRSA but it has no effect on *P. aeruginosa*. Therefore, further development is still required to broaden its efficacy.

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การศึกษาการยับยั้งเชื้อแบคทีเรียจากเม็ดแคลเซียมพระมงกุฎเกล้าที่มียาฆ่าเชื้อในหลอดทดลอง

ธไนนิธย์ โชตนภูติ, ปัญญา สุริย์จามร, สุริยา ลือนาม, พิพัฒน์ องค์น้ำทิพย์

วัตถุประสงค์*: คณะผู้นิพนธ์ได้ศึกษาความสามารถของ Phramongkutklao antibiotic hydroxyapatite pellet ซึ่งคิดค้นพัฒนาส่วนผสมโดยกองออร์โธปิดิกส์ โรงพยาบาลพระมงกุฎเกล้าฯ ในการยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียที่พบบ่อยในการติดเชื้อทางออร์โธปิดิกส์*

วัสดุและวิธีการ*: เป็นการทดลองในจานเพาะเชื้อ (in vitro study) โดยเชื้อแบคทีเรียได้แก่ Staphylococcus aureus และ Pseudomonas aeruginosa ทั้งสายพันธุ์มาตรฐาน (standard strain) และสายพันธุ์ดื้อยา (resistant strain) โดยเลือกใช้ยาปฏิชีวนะ 3 ชนิดคือ Vancomycin, Fosfomycin และ Gentamycin ผสมใน Phramongkutklao hydroxyapatite pellet ได้เป็น Phramongkutklao antibiotic hydroxyapatite pellet 3 ชนิด นำไปวางในจานเพาะเชื้อแต่ละสายพันธุ์ จากนั้นทำการวัดความสามารถในการยับยั้งการเจริญเติบโตของเชื้อแบคทีเรีย Inhibition zone (mm) จดบันทึกทุก 24 ชั่วโมง และเคลื่อนย้ายไปวางบนจานเลี้ยงอันใหม่จนครบ 28 วัน นำค่าที่ได้มาเปรียบเทียบกันระหว่างกลุ่ม และเปรียบเทียบกับกลุ่มควบคุมซึ่งใช้ Phramongkutklao hydroxyapatite pellet ที่ไม่ได้ผสมยาปฏิชีวนะ*

ผลการศึกษา*: พบว่า Phramongkutklao antibiotic hydroxyapatite pallet สามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียได้ดี โดย Staphylococcus aureus สามารถถูกยับยั้งได้ดีด้วย Vancomycin hydroxyapatite pallet โดยยับยั้งได้ตลอด 28 วัน, Fosfomycin hydroxyapatite pellet สามารถยับยั้ง MSSA 13 วัน, MRSA 25 วัน และ Gentamycin hydroxyapatite pellet สามารถยับยั้งได้ 7 วัน ขณะที่ Pseudomonas aeruginosa มีความสามารถยับยั้งการเจริญเติบโตได้ด้วย Phramongkutklao antibiotic hydroxyapatite ต่ำ*

สรุป*: Phramongkutklao antibiotic hydroxyapatite มีความสามารถในการปลดปล่อยยาปฏิชีวนะออกมายับยั้งการเจริญเติบโตของ Staphylococcus aureus, MSSA, MRSA ซึ่งเป็นเชื้อที่พบบ่อยในการติดเชื้อทางออร์โธปิดิกส์ได้จริง ส่วนการครอบคลุมเชื้อ Pseudomonas aeruginosa ยังจำเป็นต้องมี พัฒนาการต่อไป*