ABSTRACT

Purpose. To investigate the biocompatibility of hydroxyapatite composite (hydroxyapatite, plaster of Paris, and chitosan) impregnated with gentamicin, fosfomycin, imipenem, or amphotericin B.

Methods. The interactions of the extract from each drug against osteoblast were tested using the methylthiotetrazole test.

Results. Extracts from all drugs showed good biocompatibility at concentrations varying from 10 µg/ml to 1000 µg/ml. Imipenem and amphotericin B at a concentration of 1000 µg/ml had a significantly higher percentage of cell viability than the control group. No morphological change of osteoblast was observed in all drug tests at any concentrations.

Conclusion. The hydroxyapatite composite had a good biocompatibility for carrying gentamicin, fosfomycin, imipenem, or amphotericin B.

Key words: drug delivery systems; durapatite; hydroxyapatite cement

INTRODUCTION

Chronic osteomyelitis is a devastating orthopaedic problem,1 but can be treated medically and surgically. Prolonged administration of systemic antibiotics is usually used.2,3 Unfortunately, serious side-effects such as nephrotoxicity and hepatotoxicity are associated with such treatment.4 Local antibiotic treatment developed to minimise such systemic complications offers possible advantages including: cost-saving, decreased hospitalisation, and avoidance of parenteral administration.3–6 A number of materials have proved useful as a means of carrying antibiotics for this purpose, of which some are non-biodegradable such as polymethylmethacrylate (PMMA) and others are biodegradable such as hydroxyapatite, plaster of Paris, and chitosan. PMMA loaded with a variety of antibiotics (including antifungals) has been extensively studied.2,7–10 Its major drawback is the need for surgical removal and the risk of drug resistance,11 and only heat-stable drugs can be used due to the high-setting temperature of the process for PMMA polymerisation.1,12 Biodegradable materials including hydroxyapatite, plaster of Paris, and chitosan have been explored to replace PMMA, but drug elution
was not sufficiently prolonged. We developed a new biodegradable composite, which is composed of hydroxyapatite, plaster of Paris, and chitosan. Whether impregnated by antibacterials or antifungals, the efficacy of this composite has been demonstrated in our previous studies.\textsuperscript{10,13,14} The composite slowly releases the antimicrobial drug and inhibits organisms for more than one month. The lower-setting temperature of 30°C enables impregnation with heat-labile antibiotics such as imipenem.\textsuperscript{10} This study aimed to investigate the fundamental properties and biocompatibility of this composite impregnated with gentamicin, imipenem, fosfomycin, or amphotericin B in an \textit{in vitro} model, before conducting clinical studies.

\textbf{MATERIALS AND METHODS}

Hydroxyapatite (\(\text{CA}_{10}(\text{PO}_4)_6(\text{OH})_2\)) from MTEC, Bangkok, Thailand; plaster of Paris from Merck, Damstadt, Germany; and chitosan from Fluka, Buchs, Switzerland were used to make the hydroxyapatite composite and loaded with gentamicin from General Drug House, Bangkok, Thailand; fosfomycin from Meiji, Tokyo, Japan; imipenem from Merck, New Jersey, USA; or amphotericin B from Bristol-Myers Squibb, Madrid, Spain. They were prepared using a geometric dilution technique: 4.5 g of hydroxyapatite mixed with plaster of Paris (at a ratio of 15:85), then loaded with 10\% of antibiotic (gentamicin, imipenem or fosfomycin) or 45 mg of amphotericin B. An aqueous solution of 1\% chitosan was added to the mixture to make a smooth paste then poured into tablet moulds (6.5 mm in diameter and 5.2 mm in thickness). After hardening, the tablets were removed from the moulds and heated at 60°C for 2 hours.

Tablets of all materials were sterilised by soaking in 70\% alcohol for 30 minutes, followed by phosphate-buffer saline treatment (100 units/ml penicillin and 100 \(\mu\)g/ml streptomycin, pH 7.4) for 2 hours. The tablets were then washed with sterile Dulbecco’s modification of Eagle medium (DMEM). Extracts were prepared according to the recommendation of the British standard. The extractant used was DMEM supplemented with 10\% foetal calf serum (Gibco BRL, Life Technology, Paisley, Scotland). Finally, the extracts were passed through a 0.22-micrometre filter to remove any debris and stored at -70°C.

The cells used for assessment of the materials were immortalised human osteoblasts (MC3P3-E1, ATCC, USA), grown as monolayer cultures in DMEM containing 10\% (v/v) foetal calf serum in an atmosphere of 5\% \(\text{CO}_2\) in air. They were passaged routinely every 7 days.

The effect of extracts of these materials on osteoblast morphology were observed following staining of the osteoblasts for alkaline phosphatase using Sigma Diagnostics Procedure. Cells were seeded on glass slides for 48 hours before staining. The osteoblasts were examined under light microscope to detect morphological changes after exposure to the extracts of each drug at various concentrations.

Cell viability was quantified by the methylthiotetrazole (MTT) test (Sigma Chemical, St Louis [MO], USA). Staining cells were grown in a 96-well plate and inoculated to a point in the exponential growth phase. The plates were incubated for 24 hours at 37°C in a humidified atmosphere of 5\% \(\text{CO}_2\) in air. The medium was then replaced by the previously prepared diluted extract. After a 72-hour incubation, the cell culture was treated with 0.1 ml of MTT and incubated for further 2 hours at 37°C. MTT was removed and 600 \(\mu\)l of sodium dodecyl sulfate reagent added. The absorbance was measured using a microplate reader (EL312 Biokinefes, BioTek, USA) at 570 nm.

Data were reported as means at a significance level of 0.05. The Chi squared test was used to compare the percentage of viable cells present at each drug concentration versus the percentage of viable cells present in a control setting with zero drug concentration (blank).
RESULTS

Extracts from composite impregnated with gentamicin, imipenem, fosfomycin, or amphotericin B had no effect on osteoblast morphology when incubated for a period of 48 hours (Fig. 1). The osteoblast behaviour showed that the percentage of viable cells was similar to the controls evaluated by the MTT test. The percentage of viable cells was greater than 80%, compared to the control group, in elutes from every drug test and in each concentration ranged from 10 µg/ml to 1000 µg/ml. Elutes from imipenem-loaded composite had a significantly higher percentage of viable cells than the control group at every concentration, while those from amphotericin B showed a significantly higher percentage of viable cells than the control group at every concentration, while those from amphotericin B showed a significantly higher percentage of viable cells than both blank and control groups at a concentration of 1000 µg/ml, and elutes from fosfomycin had a significantly higher percentage of viable cells than the control group at a concentration of 100 µg/ml (Fig. 2a). Among the 3 concentrations (10, 100, and 1000 µg/ml) of extracts for each drug, the percentage of viable cells decreased at higher drug concentrations except for imipenem and amphotericin B. Imipenem at a concentration of 1000 µg/ml had a significantly higher percentage of viable cells than at lower concentrations (Fig. 2b).

DISCUSSION

Hydroxyapatite composite (hydroxyapatite, plaster of Paris, and chitosan) impregnated with gentamicin, fosfomycin, imipenem, or amphotericin B had a good cytotoxicity in our in vitro test. This composite did not affect osteoblast proliferation or cell morphology any differently to controls. The composites showed sustained elution characteristics; the antibiotics eluted for more than 6 weeks and inhibited common pathogens giving rise to musculoskeletal infections. Therefore, this carrier can function as scaffolds for bone regeneration and eradicate infection at the same time. It is especially beneficial in chronic osteomyelitis with bony defects, and may alleviate the need for additional bone grafts.

Hydroxyapatite is a good biocompatible material, but when used as a carrier in pure form, impregnated antibiotics can elute only for a short period.
Plaster of Paris contains calcium sulphate and has transient cytotoxic effects. Its dissolution leads to an acidic microenvironment responsible for a transient inflammatory response. When combined with hydroxyapatite and chitosan, its cytotoxic effect is neutralised. Chitosan—the aminopolysaccharide used as a binder in this composite—is also biocompatible and biodegradable. It also has antimicrobial effects and osteoconductive properties. Our composite has a low-setting temperature of 30°C and is therefore suitable for heat-labile antibiotics such as imipenem.

Despite a high local concentration of drug, we demonstrated that there was no impact on the percentage of viable osteoblasts. This is in contrast to previous studies showing gentamicin, fosfomycin, imipenem, and amphotericin B having cytotoxicity to osteoblasts in an in vivo test.

The hydroxyapatite composite (hydroxyapatite, plaster of Paris, and chitosan) impregnated with gentamicin, fosfomycin, imipenem, or amphotericin B showed good biocompatibility in our in vitro test and appeared to be a suitable drug delivery system for the selected antimicrobials. However, an in vitro test may not totally simulate the in vivo condition, so in vivo testing is needed to confirm these results.

REFERENCES