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Research Paper

Prolonged anti-bacterial activity of ion-complexed doxycycline for the treatment of osteomyelitis

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ABSTRACT

The main purposes of the present study are the fabrication of an ion-complexed antibiotic which allows for the continuous release of the drug for sufficient periods of time without any additional matrix leading to unfavorable tissue responses, and the feasibility study of the ion-complexed antibiotic as a therapeutic system for osteomyelitis using an animal model. An ion-complexed doxycycline (*ic*DX) as an antibiotic was prepared by simple mixing of positively charged doxycycline hyclate (DX) and negatively charged multivalent Na_2HPO_4 ($2Na^+$ HPO_4^{2-}) aqueous solutions. The *ic*DX showed a controlled release of the DX up to 6 weeks. From the *in vivo* feasibility study using an osteomyelitis rat model, the *ic*DX group showed a more effective therapeutic effect for the osteomyelitis, at 3 and 6 weeks, compared to the non-treated control and free DX groups. This was due to the sustained release of DX from the *ic*DX in the osteomyelitis bone (medullary cavity) without migration. These findings suggest that the *ic*DX may be a promising local delivery system in the clinical field for the treatment of the osteomyelitis.

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1. Introduction

Osteomyelitis is defined as a bone infection by bacteria or fungi. Although the understanding of infectious diseases and the development of surgical/pharmaceutical therapeutic techniques are rapidly growing, osteomyelitis continues to be a challenge for orthopedic surgeons [1–3]. Osteomyelitis leads to severe osteonecrosis associated with devascularization in bone [4,5]. Trauma, replacement of artificial hip and knee joints, bone surgery, and soft tissue infection (particularly in diabetic patients) are considered major causes of the disease [4]. For the treatment of osteomyelitis, a combined surgical (debridement of infected bone) and pharmaceutical (parenteral long-term delivery of antibiotic) method has been accepted as a gold standard in clinical practice. Waldvogel et al. [6–8] reported that the parenteral delivery of antibiotics into the target region for 4 –6 weeks is necessary for the appropriate therapeutic effect. However, the inadequate delivery of antibiotics to the disease site with limited blood vessels (much lower drug concentration than the circulation system) and systemic toxicity (i.e., ototoxicity and nephrotoxicity) by large and long-term drug administration to compensate for the insufficient drug concentration at the lesion are still regarded as inherent limitations [9–11]. To overcome these, the direct local delivery of the antibiotic in high doses, which allows sufficient concentration of the drug on the target region despite limited vascularity as well as minimizes the systemic toxicity, garnered increasing interest as an alternative [12]. After the first use of the antibiotic-eluting bone cement in 1970 for the treatment of osteomyelitis by Buchholz and Engelbrecht [13], the antibiotic-containing matrices including polymethyl methacrylate (PMMA) [14,15], calcium sulfate [16,17], hydroxyapatite (HA) [18], tricalcium phosphate (TCP) [19], poly (lactic-co-glycolic acid) (PLGA) [11,20,21], polycaprolactone (PCL) [22], collagen [23], fibrin glue [24–26], and cross-linked hyaluronic acid [27] have been widely investigated as a local delivery system in laboratories and clinics for the treatment of osteomyelitis. Among them, the antibiotic/PMMA system has been predominantly applied in clinical fields over the others for the last 40 years [28]. Several products including Simplex P (Stryker), Palacos G (Zimmer), and Prostalac prosthesis/SmartSet GHV/SmartSet MHV (Depuy Orthopaedics) are approved for human use from the U.S. Food and Drug Administration (FDA) [28]. However, the short-term (i.e., within few days [29,30]) or limited (i.e., few percentages of total antibiotic [31,32]) release of the incorporated antibiotic cannot guarantee anti-bacterial effects and could stimulate antibiotic

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resistance, the necessity of a secondary surgery to remove the non-degradable matrices which may lead to another infection [33]; the undesirable inflammatory responses by used matrices themselves or by-products during degradation [34] remain as inevitable huddles for clinical applications. On the basis of the literature, it was hypothesized that if a sustained releasing antibiotic could be developed for sufficient periods of time (i.e., more than one month) without any additional matrix (free from adverse responses by matrix and a cumbersome removal surgery for the non-degradable matrix), it may be a promising therapeutic for the treatment of osteomyelitis.

In our previous study [35], it was demonstrated that a water-soluble (ionized) drug could form an ion-complex with multivalent ions (counter ions of a drug) and could have a sustained release from the ion-complex allowing a prolonged therapeutic effect. It was recognized that the ion-complexed drug could be a simple drug delivery system even without any additional materials. Therefore, the main purposes of the present study are as follows: (i) the fabrication of an ion-complexed antibiotic, which can allow the continuous release of the drug for sufficient periods of time without any additional matrix leading to unfavorable tissue responses and secondary surgery to remove them and (ii) the feasibility study of the ion-complexed antibiotic as a therapeutic tool for osteomyelitis using an animal model. Doxycycline hyclate (DX), which is a drug water-solubilized by ionization of hydrophobic doxycycline, was chosen as a model antibiotic in the present study to treat the osteomyelitis caused by bacterial infection in bone, because of its chemotherapeutic effects in bone [36–39] and ionic property (positive charge) in the aqueous solution which leads to the formation of an ion-complex by multivalent counter-ions. An ion-complexed doxycycline (*ic*DX) was fabricated by the mixing of aqueous solutions containing positively ionized DX and multivalent counter-ions (HPO_4^{2-} dissolved from Na_2HPO_4). The *in vitro* DX release, cytotoxicity, and anti-bacterial activity of the *ic*DX were investigated. Their *in vivo* therapeutic efficacy for osteomyelitis was also estimated using an osteomyelitis rat model.

2. Materials and methods

2.1. Materials

Doxycycline hyclate (DX; Sigma, USA) as a positively charged antibiotic and sodium phosphate dibasic (Na_2HPO_4; Sigma–Aldrich) as a negatively charged divalent ion source, were used to prepare an ion-complexed doxycycline (*ic*DX). Hyaluronic acid (HA; MW ~ 4800 kDa; Genewel, Korea) was used as a carrier for the homogeneous delivery of the *ic*DX into an osteomyelitis animal model through a needle.

2.2. Fabrication of ion-complexed doxycycline (icDX)

Five wt% DX aqueous solution and Na_2HPO_4 aqueous solutions with different concentrations (3, 5, 7 and 10 wt%) were prepared, respectively, and two solutions were mixed with the same volume at room temperature. The yellowish powder (*ic*DX) was precipitated by the formation of an ion complex between positively charged DX and divalent HPO_4^{2-} (dissociated from Na_2HPO_4). The *ic*DX was obtained after washing with excess water to remove free DX and Na_2HPO_4, and freeze-drying (Fig. 1). The total amount of obtained *ic*DX powder was weighed. To determine the yield of *ic*DX, the *ic*DX powder (1 mg) was dissociated in dimethyl sulfoxide (DMSO, 5 mL) and the total amount of DX in *ic*DX was estimated using a UV/VIS spectrometer (absorbance at 273 nm; UV-3600, Shimazu, Japan).

2.3. Drug release study

Each *ic*DX (10 mg) and each free DX (10 mg) powder were incubated in phosphate buffered saline (PBS; pH ~ 7.4, 1 mL in 2 mL polypropylene conical tube) at 37 °C for up to 6 weeks under mild shaking (100 rpm) to investigate the release patterns, respectively. At designated time intervals (1, 2 h; 1, 3, 5, 7, 12, 14, 21, 28, 30, 32, 35, 37, 39, 42 days), a tube with *ic*DX or free DX was centrifuged (12,000 rpm), the whole supernatant was collected and fresh PBS was added with same volume. The amount of released DX in the collected medium was determined by a UV/VIS spectrometer.

2.4. Cytotoxicity of icDX

The cytotoxicity of *ic*DX and free DX was compared using an extract of the drug in cell culture medium. For this, the drug with different concentrations in DMEM culture medium (Gibco Laboratories, USA) (*ic*DX or free DX; 0.1, 0.5, 1.0, 3.0, 5.0, 10.0 and 20.0 mg/mL) was incubated at 37 °C for 1 day under mild shaking (100 rpm), and the medium containing DX released (or dissolved) from the *ic*DX or free DX was collected after centrifugation (12,000 rpm). To estimate the cytotoxicity of the DX in the collected medium, the murine calvaria pre-osteoblast (MC3T3-E1; CRL-2593, ATCC, USA; immortalized osteoblastic-like cells) was used as a model cell. The MC3T3-E1 cells in DMEM culture medium supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories), 0.1% gentamicin sulfate (Sigma), and 1% penicillin G (Sigma) were seeded in 96-well cell culture plates (Corning, USA) at a density of 6.6×10^3 cells/well. At 1 day after the cell seeding, the medium was removed from the wells and replaced with the collected DX-containing mediums (200 μL, 10% FBS added), respectively. After 1 day incubation, the cell viability was determined by an MTS assay. To accomplish this, 40 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution (MTS, Promega, USA) was added in each well, and incubated for 2 h at 37 °C. The absorbance of the contents in each well was measured using a microplate reader at 490 nm (Molecular Devices, USA). The normal cell culture medium group without DX was used as a control (cell viability, 100%).

2.5. Anti-bacterial activity of icDX

The *in vitro* anti-bacterial activity of *ic*DX or free DX with time was estimated using a paper disk diffusion inhibition test. The designated concentration of *ic*DX or free DX (0.1, 0.5, 1.0, 3.0, 5.0, 10.0 and 20.0 mg/mL) was incubated in 5 mL PBS (in 15 mL conical tube) at 37 °C for up to 6 weeks under mild shaking (100 rpm), because the antibiotic delivery of 4–6 weeks is usually necessary for the effective treatment of osteomyelitis [6–8]. At predetermined time intervals (1, 7, 21, and 42 days for *ic*DX; 1 and 2 days for DX), the tube containing antibiotic was centrifuged, and the whole supernatant was collected and replaced with fresh PBS. To evaluate the anti-bacterial activity, *Staphylococcus aureus* (*S. aureus*) KCTC1621, which is a predominant bacteria in osteomyelitis [40], was selected as a model microorganism. A single colony of *S. aureus* grown on nutrient agar (3 g beef extract, 5 g peptone, 15 g agar, and 1 L water) was inoculated into 3 mL of nutrient broth and cultured with rotary shaking (170 rpm at 37 °C) for 12 h. Overnight cultured bacteria were inoculated on nutrient agar plates using a sterilized cotton swab, and the sterile paper disks (thickness 0.7 mm, Ø 8 mm) soaked in the collected medium for 2 h were put on the Petri-dishes. After incubation for 1 day at 37 °C, the anti-bacterial activity of the DX released (or dissolved) from *ic*DX or free DX was determined by zone of inhibition (ZOI). The diameter of the inhibition zone was measured using an image analysis program (*i*-Solution, IMT, Republic of Korea).

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