

Low-intensity pulsed ultrasound enhances antibiotic release of gentamicin-loaded, self-setting calcium phosphate cement

Journal of International Medical Research

2018, Vol. 46(7) 2803–2809

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DOI: 10.1177/0300060518773023

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Abstract

Objective: This study aimed to investigate the effect of low-intensity pulsed ultrasound on antibiotic release from gentamicin-loaded, self-setting calcium phosphate cement.

Methods: A gentamicin-loaded calcium phosphate cement cylinder was eluted in stimulated body fluid. Low-intensity pulsed ultrasound (46.5 kHz, 200 mW/cm²) was used to produce a sinusoidal wave in the experimental group. Non-gentamicin calcium phosphate cement was used in the control group.

Results: The transient concentration and cumulatively released percentage of gentamicin in the ultrasound group were higher than those in control group at every time point. The duration of gentamicin concentrations over the level of the minimum inhibitory concentration was significantly prolonged in the ultrasound group compared with the control group. Antibacterial efficacy of gentamicin in the ultrasound group was significantly better than that in the control group with the same concentration of gentamicin.

Conclusion: Low-intensity pulsed ultrasound enhances antibiotic release, providing sustained antibiotic release at high concentrations. This increases the antibacterial effect of gentamicin.

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Keywords

Low-intensity pulsed ultrasound, gentamicin, calcium phosphate cement, antibiotics, bacteria, bone

Date received: 30 December 2017; accepted: 4 April 2018

Introduction

Bone cements based on calcium phosphate cement (CPC) have been successfully used to fill bone defects, such as osteomyelitis and infected non-union.^{1,2} Local antibiotic release, which aims to provide local high antibiotic levels to damage bacterial bio-film, is an important adjuvant therapy for osteomyelitis and infected non-union.³ A recent study reported that antibiotic-loaded CPC released a large amount of antibiotics *in vitro*.⁴ However, another study reported that the duration of antibiotic release of CPC was shorter than 1 month *in vitro*.⁵ How to prolong the term of release with high local levels of antibiotics has been recently studied. Ultrasound has been found to increase antibiotic release from bone cement and to enhance antibacterial efficacy of antibiotics.⁶ We previously reported that low-intensity pulsed ultrasound (LIPUS) increased gentamicin release from polymethyl methacrylate *in vitro*.⁷

We hypothesised the following in the present study: (1) LIPUS enhances gentamicin release and prolongs its duration with high concentrations to 3 months in gentamicin-loaded CPC; and (2) LIPUS enhances antibacterial efficacy of gentamicin released from CPC.

Materials and methods

Materials and microorganisms

Staphylococcus aureus ATCC 13565 (Biological Authentication Research

Institute, Shanghai, China) was incubated and then diluted to produce a suspension of 10^8 colony forming units (CFU)/mL of exponential-phase bacteria. CPC powder (Shanghai Rebone Biomaterials, Shanghai, China) was mixed with gentamicin powder (Amresco, Houston, TX, USA) in a ratio of 19:1. The mixture was then suspended with distilled water at a powder/liquid ratio of 3:1 to form a paste in stainless steel. This comprised a gentamicin-loaded CPC cylinder that was 5 mm in diameter and 5 mm in height (Figure 1). This cylinder was then vacuum-dried and sterilized in irradiation of 25 kGy ^{60}Co . Non-gentamicin CPC cylinders were obtained using the same procedure as gentamicin-loaded CPC cylinders without gentamicin. Stimulated body fluid⁸ (SBF) was prepared by dissolving 7.9946 g NaCl, 0.3529 g Na_2HPO_4 , 0.2237 g KCl, 0.3050 g $\text{MgCl}_2 \cdot \text{H}_2\text{O}$, 0.7102 g Na_2SO_4 , and 0.1742 g K_2HPO_4 in deionized water to be diluted to 1000 mL and then sterilized. An ultrasonic generator (Nexus; Hexin Biomedical Devices, China) was used with 30-mm-diameter unfocused transducers, and it produced a sinusoidal wave with 200 mW/cm² intensity, 46.5 kHz frequency, and the duty cycle of the pulse was 1:3.

Ethical approval for the study was not required because no animals for humans were studied.

Gentamicin release

Gentamicin-loaded CPC cylinders and SBF were placed in six-well cell culture (one cylinder and 3 mL SBF for each well). Twenty-four cylinders were divided into two groups

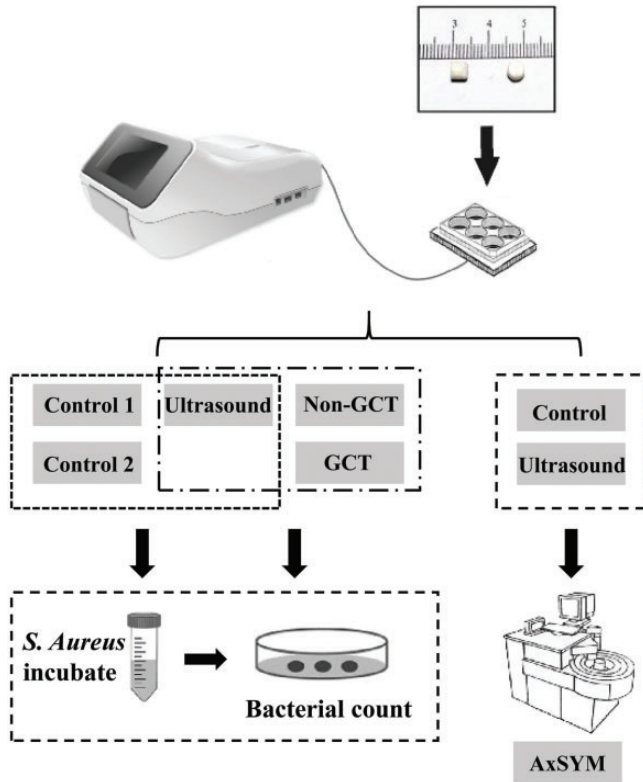


Figure 1. Flow chart of the present study

as follows: control group ($n = 12$) and ultrasound group ($n = 12$). The CPC cylinder in the ultrasound group was insolated by the ultrasound exposure system for 4 hours and SBF was replaced daily. SBF was also replaced daily in the control group. The eluted SBF from the ultrasound and control groups was tested on days 1, 3, 7, and 14, and at weeks 4, 8, and 12. Gentamicin concentrations in the eluted samples were measured using a fluorescence polarization immunoassay⁷ (AxSYM; Abbott Laboratories, Abbott Park, IL, USA) (Figure 1).

Antibacterial efficacy

Twenty-four gentamicin-loaded CPC and 12 non-gentamicin CPC cylinders, which

were cultured in six-well cell plates with 3 mL SBF, were divided into three groups as follows: non-gentamicin control group (non-GCT, $n = 12$), gentamicin-loaded control group (GCT, $n = 12$), and ultrasound group ($n = 12$). Cylinders were positioned in a normal incubator in the non-GCT and GCT groups. In the ultrasound group, cylinders were positioned on an ultrasound transducer and insolated for 2 hours daily. SBF was replaced daily in all of the groups. The eluted SBF samples were changed to a suspension of 3 mL Mueller-Hinton (MH) broth with 10^8 CFU/mL of exponential-phase *S. aureus* at months 1, 2, and 3. The suspension in each well was incubated for 24 hours and then obtained. The MH broth was changed back to SBF on the next day. The numbers

of bacteria in each well were determined by the plate count method (Figure 1).

In the ultrasound group, 3 mL MH broth was used as eluting solution at the 12 week, with 10^8 CFU/mL of *S. aureus*, and insonated by ultrasound for 2 hours. Samples were then incubated at 37°C for 24 hours. Another 24 tubes with 10^8 CFU/mL of exponential-phase *S. aureus* in 3 mL MH broth were divided into two control groups as follows: control group 1 (n=12) and control group 2 (n=12). In control group 1, tubes were incubated at 37°C for 24 hours. In control group 2, tubes had the same concentration of gentamicin added as that of the ultrasound group (concentrations of gentamicin = c1, c2... c12), and were incubated at 37°C for 24 hours. The numbers of bacteria in each tube in the ultrasound group, and in control groups 1 and 2 were calculated by the plate count method (Figure 1).

Statistical analysis

Data are expressed as mean \pm standard deviation. One-way analysis of variance was used to compare differences in gentamicin concentrations and clonal formation

units. Analysis was performed using SPSS v17 (Chicago, IL, USA).

Results

LIPUS-enhanced gentamicin release

Local drug release in the control and ultrasound groups at 12 weeks is shown in Figure 2. Gentamicin concentrations in the ultrasound group were significantly higher than those in the control group at every time point (all $p < 0.05$). The minimum inhibitory concentration of gentamicin was $1\ \mu\text{g/mL}$ and gentamicin concentrations in the control group were less than this level after 4 weeks. Gentamicin concentrations in the ultrasound group exceeded this level at the end of 12 weeks. The cumulatively released percentage of gentamicin in the ultrasound group was significantly higher than that in the control group at every time point (all $p < 0.05$).

LIPUS enhanced antibacterial efficacy

There was no significant difference in the antibacterial effect between the GCT and

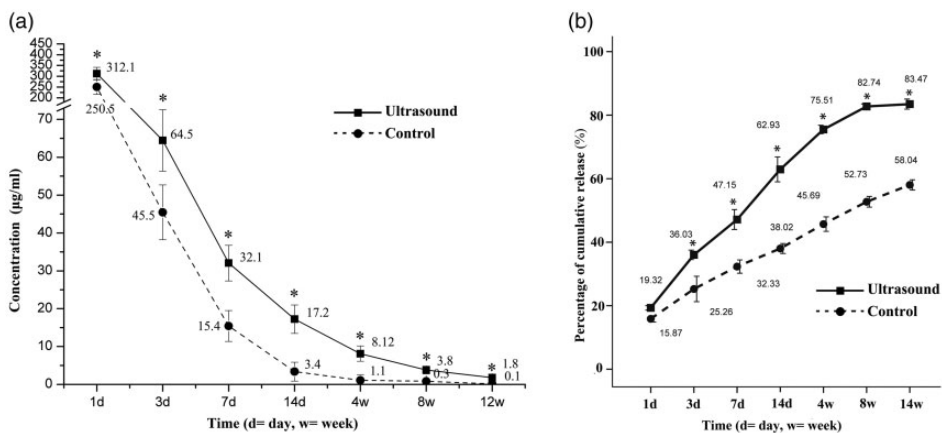


Figure 2. LIPUS-enhanced gentamicin release. (a) Gentamicin concentrations after LIPUS treatment were higher than those in the control group. (b) The percentage of cumulative gentamicin in the ultrasound group was significantly greater than that in the control group (* $P < 0.05$)

ultrasound groups in the first month, and both of these groups showed significantly lower bacterial density than did the non-GCT group ($p < 0.01$, Figure 3). There were no significant differences in bacterial density between the GCT and non-GCT groups after 2 and 3 months. The antibacterial effect in the ultrasound group was significantly better than that in the GCT and non-GCT groups after 2 and 3 months (all $p < 0.05$). We also found that antibacterial efficacy of gentamicin in the ultrasound group was significantly better than that in control groups 1 and 2 with the same concentration of gentamicin (both $p < 0.05$).

Discussion

Self-setting CPC has been developed as an advanced bone regeneration implant and delivery system in osteomyelitis. CPC has a crystal and chemical structure, which is similar to bone. Antibiotic-loaded CPC can refill defects and deliver antibiotics locally. Many studies have been conducted

to achieve stable and long-term release of the antibacterial effect of CPC.^{6,7,9–11}

Ultrasound enhances antibiotic release from bone cement.^{9,10} We previously found that ultrasound increased gentamicin elution from polymethyl methacrylate *in vitro*.¹¹ To the best of our knowledge, this is the first study on the effect of LIPUS on drug release of self-setting CPC. We hypothesized that LIPUS enhances the effect of gentamicin-loaded CPC in the following two different ways: (1) enhancing gentamicin release from CPC and prolonging the duration of high concentrations, and (2) enhancing the antibacterial activity of gentamicin.

The mechanism of ultrasound improving release of gentamicin from CPC remains complex. Gentamicin release from CPC is a diffusion-controlled process. Ultrasound might induce microstreaming because of stable cavitation caused by high- and low-pressure areas in a fluid.¹² Another possible mechanism is that ultrasound increases the local temperature around CPC, which

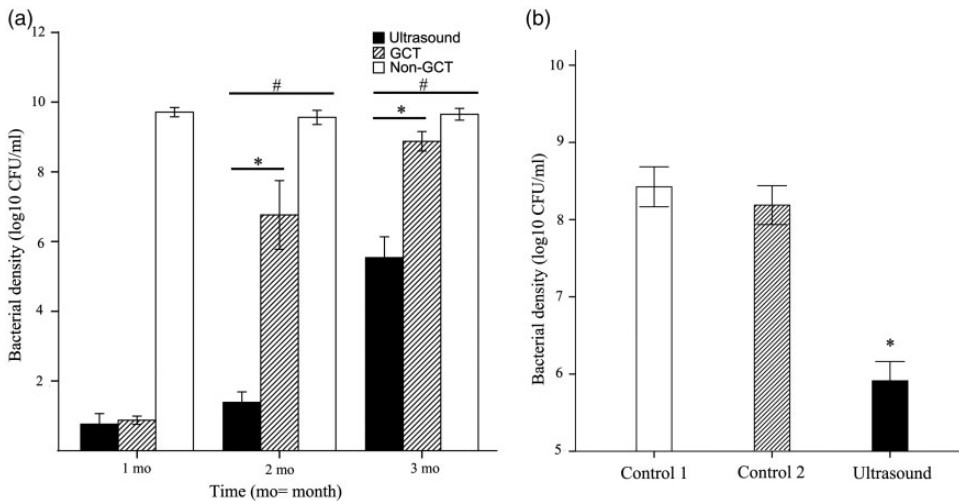


Figure 3. LIPUS-enhanced antibacterial efficacy. (a) The antibacterial effect in the ultrasound group was significantly better than that in the gentamicin-loaded control and non-gentamicin control groups after 2 or 3 months. (b) After 3 months, under the same gentamicin concentration, the antibacterial efficacy of gentamicin in the ultrasound group was significantly better than that in the control groups (**# $P < 0.05$)

results in an increase in gentamicin release.¹³

Ultrasound enhances antibacterial activity of gentamicin.¹⁴ The phenomenon of “sonoporation” occurs when ultrasound increases the permeability of bacterial cell membranes, which causes increased bactericidal action of antibiotics.¹⁵ Other studies have shown that ultrasound damages the bacterial biofilm and increases transportation of antibiotics throughout the biofilm.¹⁶

The present study has some limitations. The results of this *in vitro* study should be verified *in vivo*. Additionally, the involved cellular and molecular mechanisms are unknown and require further investigation.

In conclusion, LIPUS can increase gentamicin release, prolong the duration of gentamicin at high concentrations, and enhance the bactericidal effect in gentamicin-loaded CPC.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This present study was supported by Zhejiang Province Natural Science Foundation of China (Grant No. LQ16H060002) and the Medical and Health Science and Technology Project of Zhejiang Province (Grant No. 2016KYB120).

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