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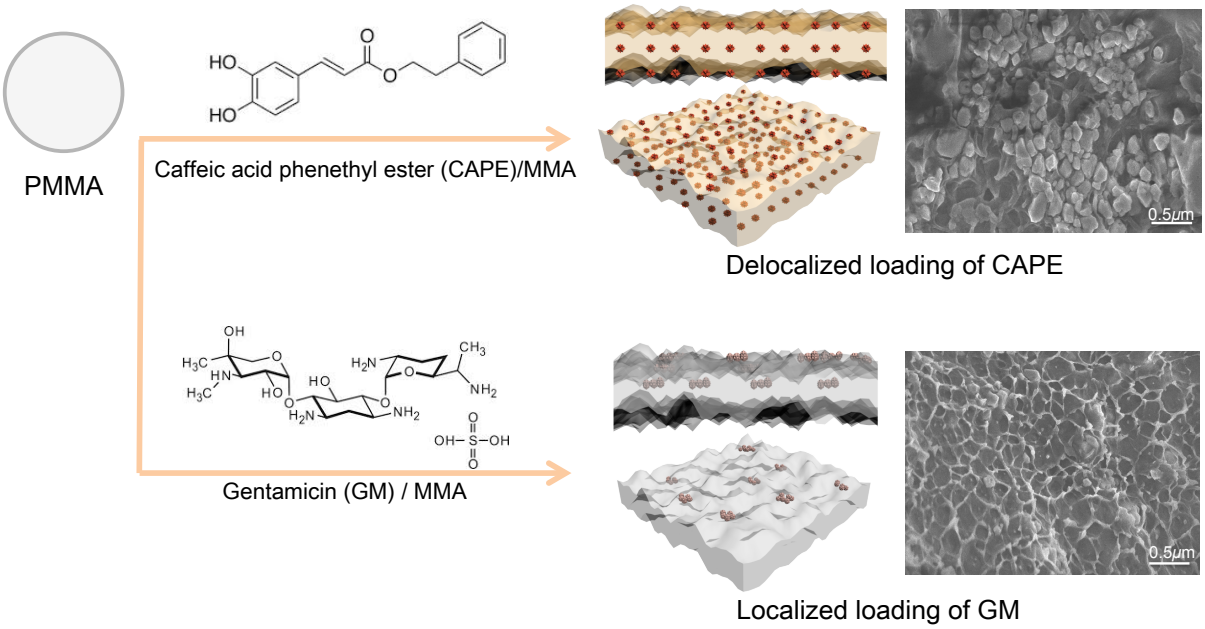
Graphical Abstract



Delocalized loading of CAPE

Localized loading of GM

(Full paper)

Antimicrobial spine-bone cement with caffeic acid phenethyl ester for controlled release formulation and *in vivo* biological assessments

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Abstract

The work report the preparation of antimicrobial spine-bone-cement with a highly controlled release formulation that was subjected to tests involving *in vitro* and *in vivo* biological assessments such as antimicrobial effect, cytotoxicity test, bacterial reverse mutation (Ames) assay, micronucleus assay, and implantation analysis. Two kinds of acrylic bone-cements were tested for the expression of antimicrobial and antibiotic activity. For these tests, new antimicrobial bone-cement was prepared using a caffeic acid phenethyl ester (CAPE) compound from natural propolis, and an antibiotic bone-cement was prepared by inclusion of gentamycin (GM). The antimicrobial ability, and controlled release property of both CAPE-loaded and GM-loaded bone-cements were demonstrated as a function of time, concentrations and optimization for maximizing their antimicrobial effect against *Staphylococcus aureus* bacteria. The compressive strengths of both bone-cements, at various CAPE and GM concentrations, were over 130 MPa and 80 MPa, for CAPE- and GM-loaded bone-cement, respectively. Moreover, the CAPE-loaded antimicrobial bone-cements showed a controlled release pattern better than that of the GM-loaded antibiotic bone-cement due to its homogeneous loading. Through various *in vitro* and *in vivo* biological assessments, the CAPE-loaded antimicrobial bone-cements showed positive results in all assessments, without any inflammatory response.

Introduction

The use of antibiotic-loaded bone-cement is becoming the standard practice for both primary and revision, knee and hip arthroplaties.[1-3] Like other biomaterials, PMMA-based bone-cement has an elevated risk of infection when implanted into the human body, when compared to autogenous vital tissue.[4-6] Therefore, there has been a focus on development of antibiotic-loaded PMMA bone-cement to reduce the infection rate. Since, the first significant reduction of the bone-cement infection rate by the use of gentamycin (GM) was reported,[7-9] various types of antimicrobial agents such as tetracycline hydrochloride, chlorohexidine, and cefotaxime sodium have been used to reduce bacterial infection.[10-12] However, worrisome factors in the application of GM-loaded bone-cement with chemical-based antimicrobial agents as prophylacs, is the chance of introducing resistant strains by releasing antibiotic/antimicrobial drug at sub-inhibitory concentrations and causing cancer in humans for many years.[13] Furthermore, antibiotic bone-cements still have some unresolved formulation issues regarding the enhancement of antibiotic/ antimicrobial efficiency and the compressive strength of the cement under given setting conditions. Therefore, the additional property of controlled release, and the use of natural antibiotic/antimicrobial agents should be included in the development of antibiotic/antimicrobial bone-cements.

To accomplish this, one approach is to screen natural extracts which represent a rich source of novel antimicrobial reagents. Such natural extracts have traditionally provided the pharmaceutical industry with some its most important sources of compounds with antimicrobial potential. Propolis, is a natural beehive product prepared by honey

bees (*Apis mellifera*) using resinous substances collected from various plants.[14] The propolis extract is an extremely complex mixture of natural substances including amino acids, phenolic acids, phenolic acid esters, flavonoids, cinnamic acid, terpenes, and caffeic acid.[15] In particular, this natural compound possesses antimicrobial, antioxidant, antivirus and anti-inflammatory effects.[16] For these reasons, propolis extract drew the interest of the pharmaceutical industries, and has since been introduced in products for human consumption, such as drinks, food and cosmetics.[17] Recently, caffeic acid phenethyl ester (CAPE), an active component of propolis extract, has been identified as one of the major biologically active principles in propolis, and has chemo-preventive and antitumor properties.[18,19] In addition, it has been suggested that the antimicrobial activity of propolis results from the combined action of flavonoids such as CAPE, pinocembrine and galangin, the action of which is based on the inhibition of bacterial RNA polymerase.[20]

Another approach is to create formulations which provide homogeneous loading and controlled release of antibiotics or antimicrobials.[21-24] The formulation of a drug carrier must provide the ability to incorporate a drug, to retain it at a specific target site, and to deliver it progressively over time to the surrounding tissues. When the drug is incorporated into bone-cement, it is necessary to verify that addition of the drug (either to liquid or solid phases of the cement) does not interfere in the setting and hardening reaction, and with its rheological behavior. Moreover, it is necessary to characterize the kinetics of the drug release. Subsequently, the effectiveness of the bone-cement to act as carriers for drug delivery must also be assessed.

Herein, we have reported creation of an antimicrobial bone-cement with homogeneous (delocalized) loading and highly controlled release of natural antimicrobials, and evaluated its antimicrobial activity and biocompatibility in comparison with localized GM-loaded, antibiotic PMMA bone-cement. The mechanical characteristics, specifically the controlled antibiotic/antimicrobial release profiles of the PMMA bone-cements, were measured as a function of time, and antibiotic concentrations. Based on these results, CAPE-loaded bone-cement formulation showed a better controlled release pattern than did the GM-loaded bone-cement. Consequently, we achieved the optimization and maximization of the antimicrobial effect against *Staphylococcus aureus* bacteria with the CAPE-loaded bone-cement. Furthermore, we completed several successful evaluations of biocompatibility, including an *in vitro* cytotoxicity test, bacterial reverse mutation (Ames) assay, micronucleus assay and *in vivo* implantation test.

Experimental section

CAPE and GM-loaded PMMA bone-cements and test microbes

Caffeic acid phenethyl ester (CAPE)- and GM-loaded bone-cements were prepared by dissolution of antibiotics or antimicrobials in methylmethacrylate (MMA). CAPE-loaded PMMA bone-cement was prepared by mixing PMMA beads (20 g), and various concentrations of CAPE (20 to 80 mM) dissolved in methylmethacrylate (8.5 g). The GM-loaded PMMA bone-cement was prepared by mixing PMMA beads (20 g), gentamycin powder (10mM) and methylmethacrylate (8.5 g). The liquid cement was spread over a cement plate mold (6 mm diameter and 3 mm thickness). The filled mold was manually

pressed between two glass plates, covered with overhead projector film to facilitate removal after hardening. After 15 min, the cement discs were pulled out of the mold and stored under dark, sterile conditions at room temperature. The sustained release of antibiotics and antimicrobials from the CAPE- and GM-loaded PMMA bone-cements was achieved in phosphate buffered saline (pH 7.4, 10 mL), and was performed in a temperature-controlled incubator at 37 °C. The total quantity of antibiotics and antimicrobials released from the bone-cements was determined using a UV-Vis. spectrophotometer over different time intervals at 340 nm and 250 nm, for CAPE and GM, respectively. After each measurement, the PBS buffer was replaced. *Staphylococcus aureus* was the bacterial species in this study, of which the strain was obtained from a stock culture on Luria-Bertani (LB) medium (Becton, Dickinson Co., USA) at 37 °C and 180 rpm on a rotary shaker to an optical density of 0.8, at a wavelength of 600 nm.

Bacterial growth inhibition assay

The assay for cell growth inhibition by antibiotics and antimicrobials was performed using flask tests. Initially, the *S. aureus* strain was streaked from glycerol stocks onto agar plates. One colony, grown overnight, was inoculated into a culture tube containing 2 mL of LB medium and cultured at 30 °C with 200 rpm shaking until reaching the late exponential phase. From this culture, 500 μL was inoculated into a new culture flask containing 50 mL of LB medium and grown to an early exponential phase. After reaching 100 μL, the culture was transferred to new culture tubes (10 mL) containing different concentrations of GM- and CAPE-loaded bone-cements (1 g). The new cultures were all grown until the control culture reached to an optical density of 0.8 at a wavelength of 600 nm. At this cell density, the

optical densities of all the cultures were measured using a spectrophotometer. As a control experiment, the same conditions were used without GM and CAPE. All data were obtained from three independent samples measured simultaneously for error analysis, and the standard deviations of the results are shown as error bars within the graphs.

In vitro cytotoxicity assay and bacterial reverse mutation (Ames) assay

The L929 mouse fibroblasts were cultured to about 80 % confluence in a T75 flask containing DMEM culture medium supplemented with 10 % FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-Glutamine, at 37 ^{o}C in a humidified atmosphere of 5 % CO_2 and 95 % air.[25] The cells were washed with fresh DMEM, trypsinized, centrifuged, re-suspended and counted under an inverted microscope using a haemocytometer. After this, cell viability was determined using the tryphan blue exclusion method. Twelve T-25 flasks were seeded with 1×10^5 cells/mL and incubated. The same amounts of zinc dibutyldithio carbamate polyurethane and high-density polyurethane film were used as negative and positive controls, respectively.[26,27]

The Ames assay was conducted by the pre-incubation method using *Salmonella typhimurium* strain TA 98 and TA 1537 for the detection of frame-shift mutations and TA 100, TA 1535, and *E. coli* WP 2uvrA for the detection of base-pair substitutions.[28] In this study, upon a layer of histidine-free agar (minimal glucose agar) for the *S. typhimurium* strain, and upon tryptophan-free agar for the *E. coli* strain, a second layer of top agar containing 0.6 % Bacto-Agar, 0.5 % NaCl, 0.5 mM L-histidine or L-tryptophan, 0.5 mM biotin, 0.5 mL of phosphate buffer, 0.1 mL of cultured bacteria (O.D. = 0.8) and 50 μL of test

samples or control (positive or negative) was incubated at 37 °C for more than 48 h. In the assay with metabolic activation, 0.5 mL of phosphate buffer was replaced by 0.5 mL of MolTox LS-9 mix. After incubation, the revertant colonies were counted.

Micronucleus assay

Fifteen specific pathogen-free, male, imprinting control region (ICR) mice were randomly assigned to three groups. The experimental group received the extract liquid of CAPE-loaded PLLA bone-cement described above at a dose of 10 mL/kg by intra-peritoneal injection. The negative control group received normal saline solution of equal volume. Last, the positive control group received mitomycin (MMC) solution at a dose of 2 mg/kg. The experimental animals were sacrificed 24 h after the injection, and bone marrow smears were stained with Wright-Giemsa stain. Under the immersion objective, the marrow smears were examined to determine the number of micronuclei per 1,000 poly-chromatic erythrocytes (PCE), and the chromatophile micronuclei frequency was calculated.

***In vivo* implantation and histological analysis (animal test)**

Four healthy rabbits over 3 kg were used. After clipping the fur the day before implantation, implantation was carried out, and each animal was observed over 12 weeks for clinical signs (mortality and body weight, as well as macroscopic and microscopic lesions, were observed and evaluated). The three control articles were implanted in the right femur at a distance of about 1 cm and the test articles were implanted in the left femur using the same method. The perforation of the femur was conducted with a dental drill (AEU-7000MG implant/Surgery

system), the articles were implanted, and the muscle and skin were sutured. After completion of the test, macroscopic evaluation at the implantation sites was conducted, and the femurs were excised for microscopic evaluation. The macroscopic evaluation of the test- and control-article implantation sites was achieved using a magnifier, and a grade was determined regarding inflammation, encapsulation, haemorrhage, necrosis and discoloration. The histological evaluation of the implantation sites of the test and control articles was evaluated by microscope and by several biological response parameters. All experiments were performed in compliance with the relevant laws and institutional guidelines, and also state the institutional committee(s) that have approved the experiments.

Instrumental analysis

The scanning electron microscope (SEM) measurements were achieved with a JEOL JSM 6700F. Identification and characterization of the PLLA bone-cement and CAPE were carried out using a PerkinElmer Fourier transform-infrared (FT-IR) spectrometer in the range 300 to 4,000 cm^{-1}. Real time RT-PCR was performed using a StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems, USA). RNA samples isolated from bacteria were quantified using a 96 well plate reader (Infinite 200 PRO NanoQuant; Tecan Ltd, Switzerland).

Results and discussion

Formulation and compressive strength of GM/CAPE-loaded PMMA bone-cements

The various antibiotic- and antimicrobial-loaded PMMA bone-cements were prepared in the compositions shown in **Table S1** (Supporting Information). Various concentrations of CA10PE were used (20 to 80 mM) and the GM was 10 mM (maximum loading) to express the antimicrobial effect. During the mixing and polymerization of each component, solid particles of CAPE and GM were dissolved in MMA solution, or were used without being dissolved. Most of the GM-loaded antibiotic bone-cements was prepared using solid particles in the mixing and polymerization process because GM is insoluble in MMA. This property caused several critical problems (e.g., incontrollable and burst release) due to the localization of GM in the PMMA bone-cement. Therefore, a new approach involving homogeneous (dispersed) loading and controlled release is necessary to effectively formulate the antibiotic/antimicrobial bone-cements.

Fig. 1(a) is a schematic diagram of the comparative formulation of PMMA bone-cements for delocalized and localized loading of antimicrobial CAPE and antibiotic GM. The morphology of all formulations of the GM/CAPE-loaded PMMA bone-cements was confirmed using SEM. In these micrographs, the PMMA beads showed a spherical shape with a clean surface and a bimodal particle size distribution (average diameters of 15 and 70 μm, see **Fig. 1(b)**). The surface morphology of PMMA bone-cement without antibiotics or antimicrobials was rough due to polymerization. Moreover, the GM- and CAPE-loaded PMMA bone-cements exhibited different surface morphologies. To confirm the homogeneous or heterogeneous loading of CAPE and GM into PMMA bone-cement matrix, cross-sections were imaged using SEM (**Fig. 1(b**)). GM-loaded bone-cements had a heterogeneous surface due to the incompatibility of gentamicin with hydrophobic MMA. This, heterogeneous surface was of oval and spherical particles with a rugose texture. In

contrast, the CAPE-loaded bone-cement showed homogeneous loading into the PMMA bone-cement matrix. The antimicrobial CAPE was not easily distinguishable from the PMMA matrix because CAPE and MMA are compatible.

To demonstrate the compressive strength of GM/CAPE-loaded PMMA bone-cements as a function of GM and CAPE concentration, the prepared test specimens were aged for 40 hours. The compression strength was determined according to the standards ISO 5833 and ASTM F451. **Fig. 2** shows the compressive strengths of the GM- and CAPE-loaded PMMA bone-cements as a function of the GM and CAPE concentration. The results showed that the CAPE-loaded PMMA bone-cements showed a strong compressive strength (> 130 MPa) rather than that reported previously, in which vancomycin- and gentamycin-loaded PMMA bone-cements had compressive strength of 70 and 50 MPa, respectively.[29] Based on our work, GM-loaded bone-cements showed 80 MPa of maximum compressive strength with 10 mM loaded. This is attributed to the differences of packing density and polymerization of the GM/CAPE-loaded PMMA bone-cements, in which the packing density of CAPE-PMMA is expected to higher than that of GM-loaded PMMA bone-cement due to the homogeneous miscibility.

Sustained release from GM/CAPE-loaded PMMA bone-cements

Cumulative amounts of GM-PMMA and CAPE-PMMA bone-cements were examined *in vitro* for a period of 130 h, and a relationship between the loading concentration of GM (10 mM) and CAPE (20, 30, 50, 70, or 80 mM) and the duration of release, was plotted in **Fig. 3**. The GM-loaded bone-cement showed burst release up to 4 h, and then sustained the release

of GM to 70 h, the CAPE burst release last to 30 h and the sustained release continued to 130 h. The overall release was better controlled in the CAPE-loaded bone-cement than in the GM-loaded bone-cement, because the CAPE was well diffused throughout the PMMA polymeric network and showed a positive squeezing mechanism due to its high delocalization (strongly dispersed). The GM-loaded bone-cement showed a fast, small release within 70 h due to its highly localized loading. The burst release differences between GM- and CAPE-loaded bone-cements with various loaded concentrations, was a remarkable to 25 h (**Fig. S1**).

Antimicrobial evaluation with GM and CAPE-loaded PMMA bone-cements

The antimicrobial activity of GM and CAPE-loaded bone-cements was investigated by the shake flask test against *Staphylococcus aureus.*[30] Various concentrations of GM- and CAPE-loaded PMMA bone-cements (1g) were placed on 10 mL of LB medium containing 100 μL of newly inoculated organisms. One colony, grown overnight, was inoculated into a culture tube containing 2 mL of LB medium and cultured at 30 °C with 200 rpm shaking until reaching the late exponential phase. From this culture, 500 µL was inoculated into a new culture flask containing 50 mL of LB medium and grown to an early exponential phase. After reaching 3 mL of the culture was transferred to new culture tubes containing the different concentrations of CAPE, which were dissolved in an absolute ethanol. The new cultures were all grown until the control culture reached to an optical density of 0.8 at a wavelength of 600 nm. At this cell density, the optical densities of the cultures were measured using a spectrophotometer (Human Corp. Rep. Korea). As a control experiment, the same conditions were used, but without CAPE. All the data were obtained from three independent samples

measured simultaneously for error analysis, and the standard deviations for the results are shown as error bars within the graphs. Antimicrobial efficacy was evaluated as a function of the CAPE concentration, in which the antimicrobial activity was not observed in a control group, but a continual decrease of cell viability was observed with increasing CAPE concentration (**Fig. 4**). In addition, the death rate of the strain was drastically increased to 96% and 92% for 10 mM GM and 80 mM CAPE, respectively.

In vitro bacterial reverse mutation assay and *in vivo* micronucleus assay

An *in vitro* cytotoxicity test and bacterial reverse mutation assay were performed to assess the biocompatibility and mutagenicity of the CAPE-loaded bone-cement. Especially, mutagenicity evaluation was achieved for the safety of the new CAPE-loaded bone-cement. This evaluation was based on histidine dependence and mutations in *S. typhimurium* strains.

These bacterial strains contain a number of gene mutagens that cause them to require special substances such as histidine and tryptophan, for growth.[31-34] Gene mutagens can induce reverse mutation of bacteria yielding revertant colonies, in the absence of histidine or tryptophan. **Table 1** shows a bacterial reverse mutation assay of CAPE-loaded bone-cement for frame-shift and base substitution type mutations. None of the assays, with or without metabolic activation of the bacterial reverse mutation assay showed a mutation factor that was significantly higher in any of the four *S. typhimurium* and one *E. coli* strain. An assessment of the potential genotoxic activity of the CAPE-loaded bone-cement was performed to investigate DNA damage by micronucleus assay using specific pathogen-free male ICR mice. The result showed that the micronuclei percentage in the experimental group

was approximately equal to that of the negative control group. This means that the CAPE-loaded bone-cement has no influence on gene replications. Consequently, CAPE-loaded bone-cements inhibited bacterial genetic reactions and proliferation, which is essential for preventing infectious diseases. Based on these *in vitro* and *in vivo* results, mutagenicity and genotoxicity were not caused by the CAPE-loaded bone-cements.

In vivo implantation effect (animal test)

To investigate the local effect of antimicrobial CAPE-loaded bone-cements on living tissues, 12 of test and control articles were implanted in the femurs of four New Zealand White rabbits. The implantation sites were made by perforation with three control articles and three test articles in the right and left femurs, respectively, of the rabbits (**Fig. 5**). After completion of the test, macroscopic evaluation was conducted at the implantation sites and the femur was excised for microscopic evaluation. For the final result, the mean scores were calculated and added to the total score of the biological response, and then divided by the number of implantation sites. After calculation of the difference in mean scores between the test and control articles, the bioreactivity rating was evaluated as follows: 0.0 - 2.9 (non-irritant), 3.0 - 8.9 (slight irritant), 9.0 - 15.0 (moderate irritant), and > 15 (severe irritant). As a result of 12 weeks of observation of each implantation site using a magnifier, macroscopic lesions specifically related to the test articles were not observed and the evaluation index of the macroscopic findings was “0.0” (**Table S2**). Ten control and ten test articles were observed after necropsy, and they were not degenerated. Furthermore, it was considered that the compact bone adhered to the control and test substances had been generally regenerated,

although there was slight individual differences. Abnormal histopathological lesions were not observed at the implantation sites for either control or test substances (**Table S3**). As a result of observation of the implantation sites and adjacent tissue during 12 weeks, test article-related specific lesions were not observed during macroscopic evaluation, and the microscopic histological evaluation score was "0.0". Therefore, the bio−reactivity rating of the CAPE-loaded PMMA bone-cements was "non-irritant".

Conclusion

New antimicrobial bone-cements with homogeneous (delocalized) loading and highly controlled release of natural antimicrobial CAPE, were prepared. The CAPE- and GM-loaded bone-cements were also evaluated for antimicrobial activity. The obtained CAPE-loaded bone-cements showed greater compressive strength (> 130 MPa) than did the GM-loaded bone-cements. Various *in vitro* and *in vivo* biological assessments, involving a cytotoxicity test, bacterial reverse mutation (Ames) assay, micronucleus assay and *in vivo* implantation test, of the CAPE-loaded antimicrobial bone-cement: showed positive results for all assessments, without any inflammatory response. The present study will be applicable for the controlled release formulation of antimicrobials and antibiotics with all kinds of PMMA bone-cements.

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Table and Figure Captions

Table 1. Results of the bacterial reverse mutation assay of CAPE-loaded bone cement in *S. typhimurium* and *E. coli* strains.

Fig. 1. (a) Schematic diagram of the comparative formulation of PMMA bone-cements for delocalized and localized loading of antimicrobial CAPE and antibiotic GM. (b) SEM images of PMMA beads(left), PMMA bone-cement without antibiotics or antimicrobials, corss-section images of GM- and CAPE-loaded bone cement matrix(right).

Fig. 2. Comparative compressive strengths of the GM- and CAPE-loaded PMMA bone-cements as a function of the GM and CAPE concentration (● : GM-, and ● : CAPE-loaded bone cements).

Fig. 3. Cumulative release profiles of the GM- and CAPE-loaded PMMA bone-cements for 130 h as a function of the GM and CAPE concentration (○: 10 mM GM-, **●:** 20 mM CAPE-, **▼:** 30 mM CAPE-, ▲: 50 mM CAPE-, ■: 70 mM CAPE-, and ◆: 80 mM CAPE- loaded bone cements)

Fig. 4. Comparison of antimicrobial activity of the GM- and CAPE-loaded PMMA bone-cements against *S. aureus.*

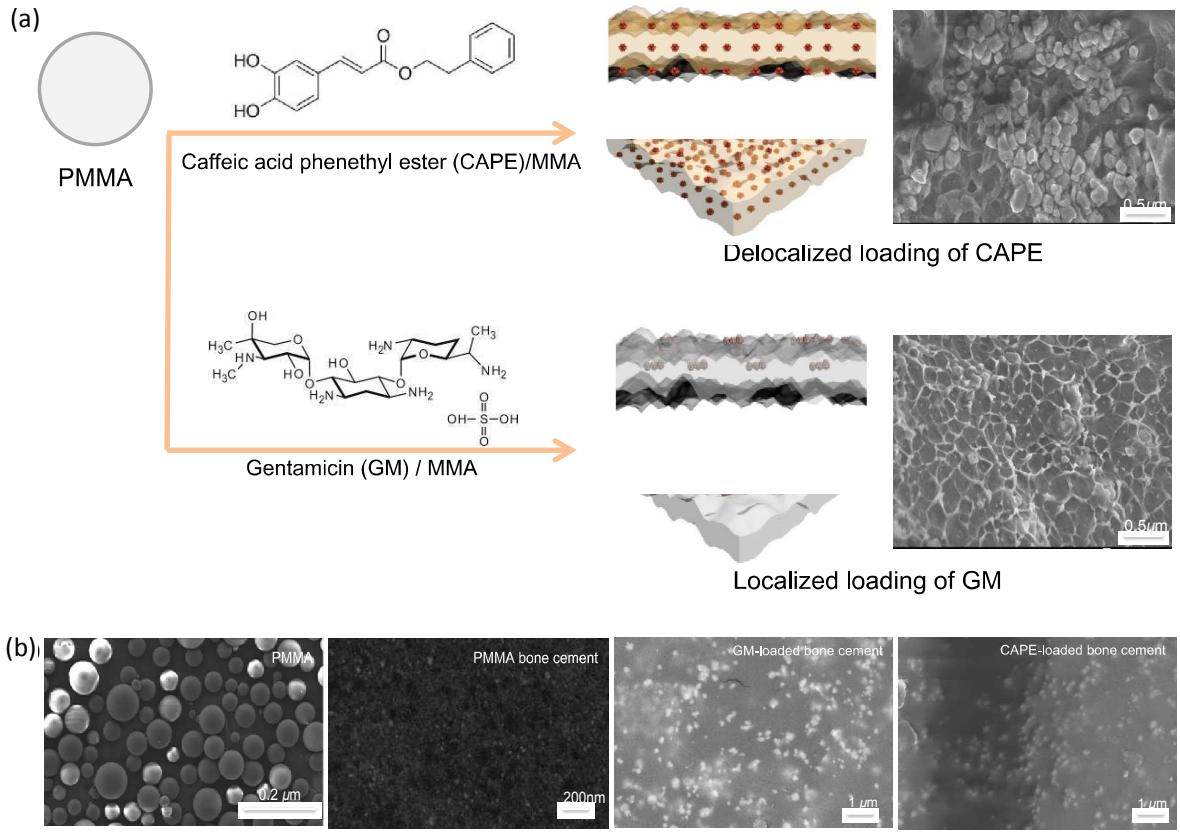
Fig. 5. *In vivo* implantation and microscopic evaluation with hematoxylin–eosin staining for the local effect of antimicrobial CAPE-loaded bone-cements (30 mM) for 3 months.

Table 1. Results of the bacterial reverse mutation assay of CAPE-loaded bone cement in *S. typhimurium* and *E. coli* strains

Test Sample	Mean number of revertant colonies/plate									
	Frame shift type				Base substitution type					
	S. typhimurium TA 98		S. typhimurium TA 1537		S. typhimurium TA 100		S. typhimurium TA 1535		E. coli WP 2uvrA	
	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
CAPE-Bone Cement	21 ± 5	37 ± 3	9 ± 2	21 ± 3	91 ± 9	119 ± 4	9 ± 2	16 ± 6	50 ± 4	61 ± 6
[a]Negative control	22 ± 5	43 ± 4	9 ± 3	18 ± 3	94 ± 9	101 ± 4	17 ± 4	13 ± 3	56 ± 9	62 ± 7
[b]Positive control	516 ± 11	225 ± 14	761 ± 20	203 ± 13	362 ± 20	651 ± 33	315 ± 10	221 ± 12	425 ± 23	423 ± 14

[a]Negative control, saline solution; [b]Positive control, 2-anthramine (1.0 μg/plate) for all strains.

Fig. 1.



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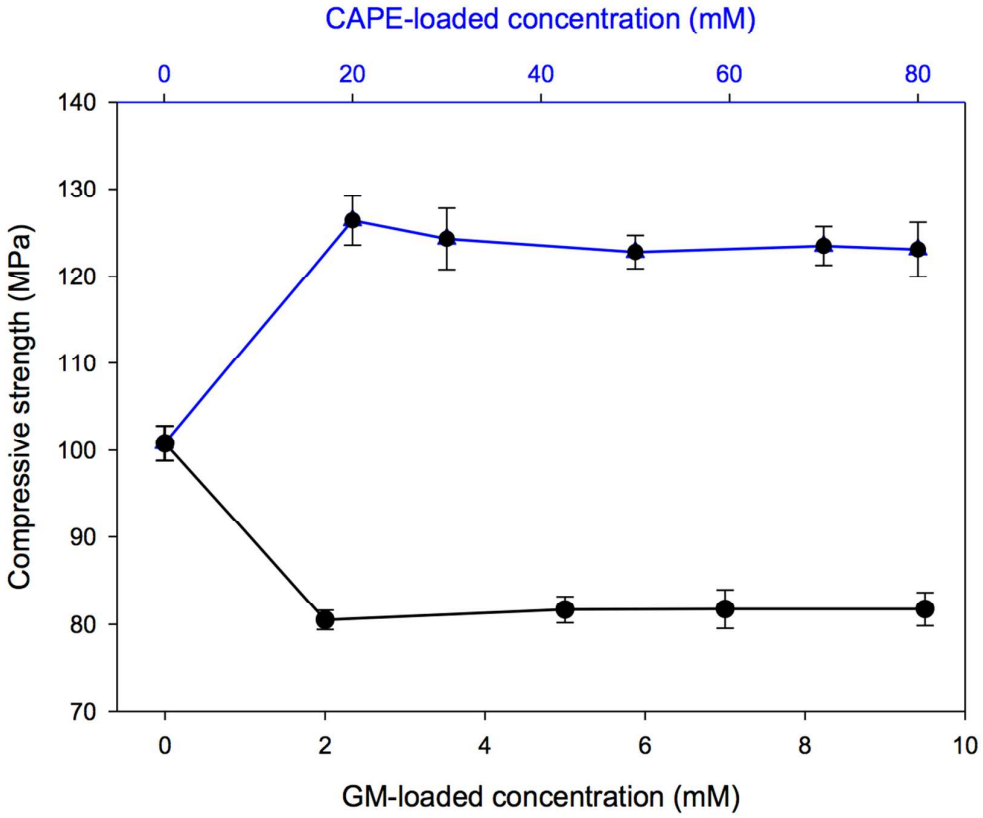
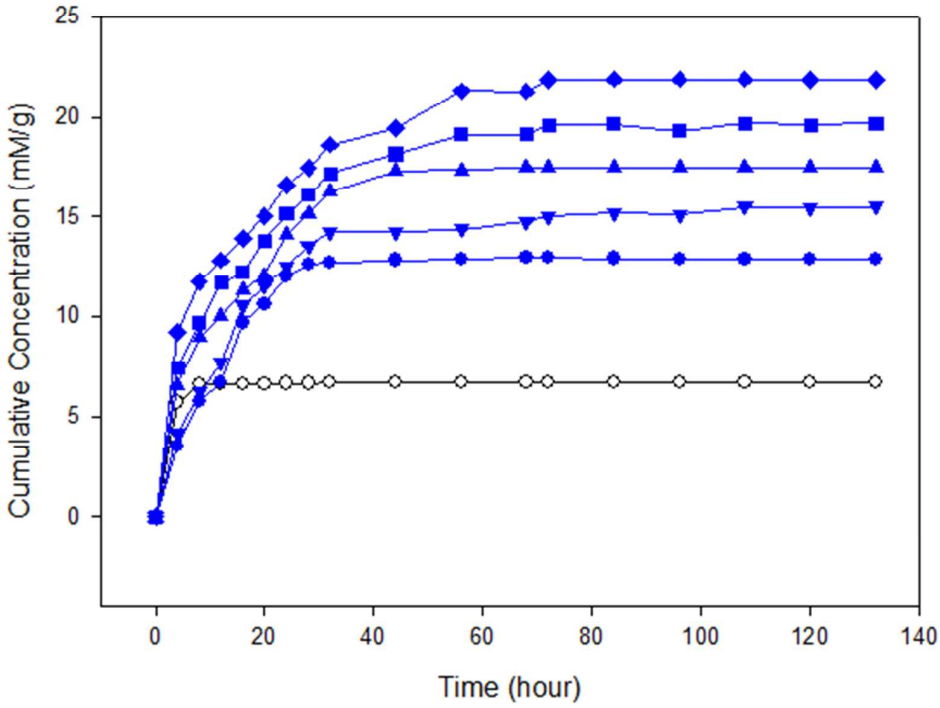


Fig. 3.



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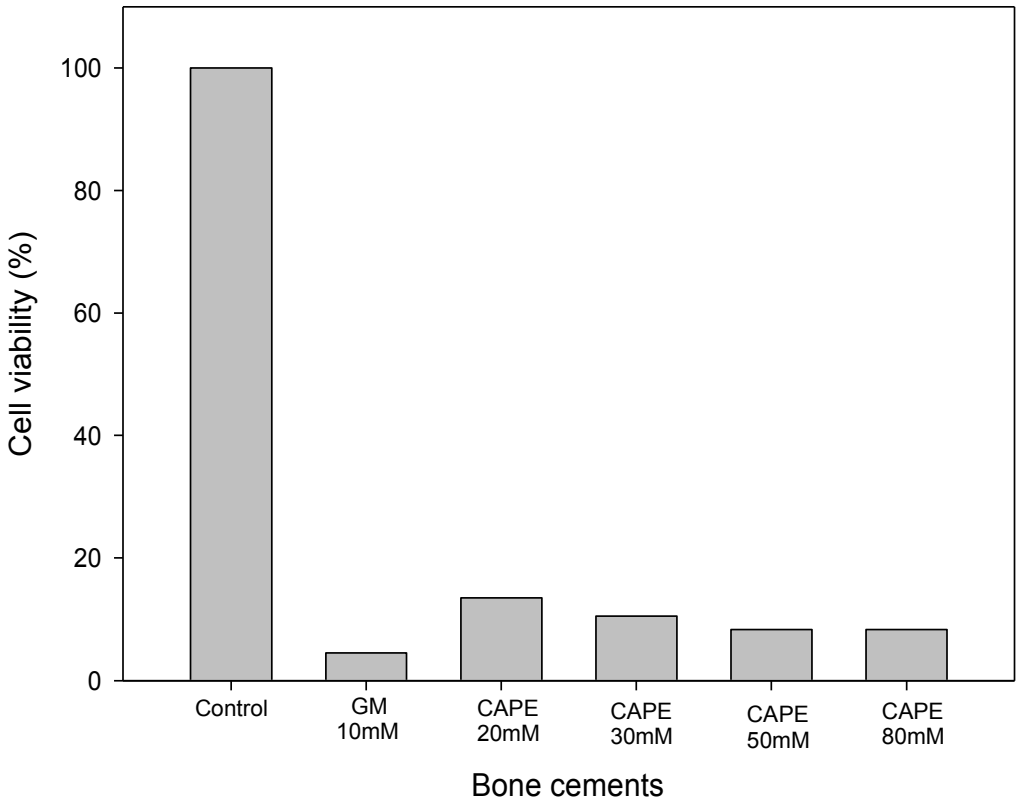

Fig. 5.

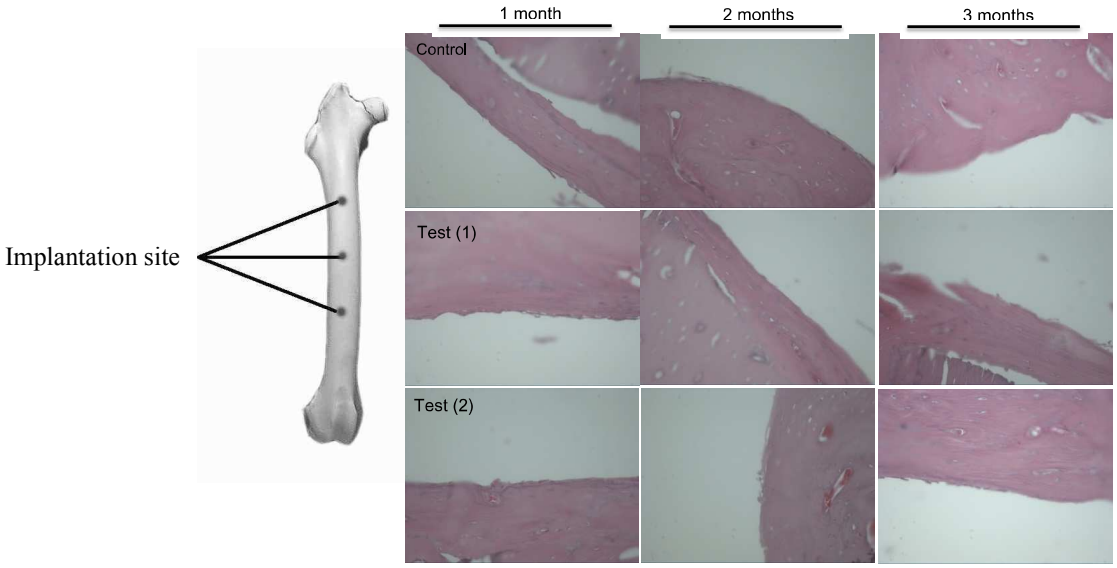

Table of Content