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In vitro and *in vivo* release of cefuroxime axetil from bioactive glass as an implantable delivery system in experimental osteomyelitis

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Abstract

The aim of this study was to evaluate the characterisation, *in vitro* and *in vivo* biocompatibility and antimicrobial activity of bioactive glass (BG) impregnated with an antibiotic. The BG was prepared by normal glass melting procedures as a controlled release device to treat experimental osteomyelitis. The study design was for prospective *in vivo* experimental study. Two sets of porous bioactive glass ceramic blocks (9 mm × 4 mm × 4 mm and 20 mm × 9 mm × 9 mm) were fabricated using bioactive glass powder and subsequently antibiotic cefuroxime axetil (CFA) (55 and 125 mg on an average) was impregnated in these two sets of blocks, respectively. Osteomyelitis was produced in the right tibia of the rabbits according to the model of Norden. After thorough *in vitro* characterization of the porous blocks [including X-ray diffraction (XRD), Fourier-transformed infra-red spectroscopy (FTIR), thorough chemical analysis by inductively coupled plasma-atomic emission spectra (ICP-AES) and field-emission scanning electron microscopy (FESEM)] and *in vitro* elution of the said drug, *in vivo* test was carried out with rabbit species split into two groups: (a) animals treated with CFA impregnated bioactive glass and (b) parenteral [intra muscular (IM)] administration of CFA. Histological, radiological and drug concentration in bone and serum (measured by HPLC) in both groups were carried out. HPLC technique was used for determination of concentration both *in vitro* and *in vivo*. Fabricated porous struts showed amorphous microstructure without formation of any crystallite. The elution of said drug was stopped after 6 days *in vitro*. Histological studies at 3 and 6 weeks revealed formation of well-developed lamellar bone and havarsian canal. Radiological evaluation pointed out disappearance of sequestrum and existence of newly formed bony specules. Concentration of cefuroxime axetil in bone and serum showed highest value on day 21 which reduced marginally by day 42 and these values were higher than minimum inhibitory concentration (MIC) against *Staphylococcus aureus* (known pathogen for chronic osteomyelitis). It could be concluded that the biodegradable antibiotic carrier system developed in this study proved to be an effective therapeutic approach toward an experimental model of osteomyelitis. Based particularly on the *in vivo* results of the study, this cefuroxime axetil incorporated bioactive glass blocks can be successfully used in clinical cases of osteomyelitis in veterinary as well as human orthopaedic surgery.

Keywords: Osteomyelitis; Sustained release; Bioactive glass; *In vitro*; *In vivo*

1. Introduction

Despite continuous advances in the surgical and antimicrobial armamentarium, the treatment of osteomyelitis poses a significant challenge even in specialized centres with a team approach involving clinicians of different subspecialties. Osteomyelitis is refractory because of the characteristics of bone. The soft tissues of bone are surrounded by hard walls, and inflammation of the contained tissues cause circulatory disturbances which can readily lead to necrosis of various parts of the bone. These anatomical features provide an environment suited for the localization and colonization by bacteria [1]. The current aggressive treatment protocols for chronic osteomyelitis constitute both prolonged IM (intramuscular) antimicrobial therapy and radical surgical debridement of all dead bone [1,2]. However, these strategies still carry

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significant relapse rates. Even if all necrotic tissues have been thoroughly surgically debrided; the bone bed has to be considered contaminated with the pathogens [3]. Obviously, systemic antibiotic therapy has a limited efficacy in this poorly perfused bone bed and alternative strategies of antimicrobial delivery should be explored. Even after successful eradication of the infection, there is a remaining problem related to the reconstitution of bone continuity. Radical debridement leaves inevitably a major bony defect, which generally requires bone grafting or some other bone reconstruction procedure at a second stage [4].

Antibiotic-impregnated poly-methyl methacrylate (PMMA) beads, the drug delivery systems which have been used successfully [5], provided a simple method to treat such chronic infection but the disadvantages include low biocompatibility, a very low release ratio and possible thermal damage to the antibiotics. Attention has therefore, been focused on biodegradable antibiotic delivery systems which provide high, effective concentrations at the site of infection with no systemic effects [6]. To attain the desired therapeutic effect without the side effects, it is necessary that (i) initial release of an active drug should exceed the minimum effective concentration (MEC) in the systemic circulation but should be less than the minimum toxic concentration (MTC), (ii) after that, the drug release should be at a constant or near-constant rate, according to zero-order kinetics, resulting in a constant, non-fluctuating plasma drug concentration in the prescribed therapeutic range and (iii) the duration of drug release should be prolonged, e.g. 12 h to 1 year [7]. Osteomyelitis could be treated following the above parameters using a bioceramic based drug delivery system since compatibility with the body fluids and the physical characteristics of them are well suited to the body environments. Moreover, these are porous, biocompatible, non-immunogenic and eventually biodegradable [7]. Impregnation of antimicrobial agents within osteoconductive biomaterials like calcium sulphate, different calcium phosphates like hydroxyapatite or tri-calcium phosphate have been proposed for local treatment of osteomyelitis and to aid dead space management [8,9]. As a common feature, these implants show a rapid release of the antibiotic in a more or less controlled manner [10].

It has been known that bioactive glass (BG) as a synthetic bone graft material prove to be promising [11,12]. For example, BG may be superior to other graft materials to regenerate osseous tissue loss from periodontal disease and maintenance of the alveolar ridge after dental extraction. It is assumed that 'bioactive nature' of BG helps bone formation [13] and osteoblasts exploit the BG as an osteoconductive template. Moreover, bone forms within the BG matrix concurrently with BG biodegradation. Bonding to bone was first demonstrated for a certain compositional range of these bioactive glasses, which contained SiO_2, Na_2O, CaO and P_2O_5 in specific proportions [14,15]. However, the following three key compositional features are essential to impose bioactivity within the material: (i) <60 mol% SiO_2; (ii) high Na_2O and CaO content; and (iii) high CaO/P_2O_5 ratio. These compositional features make the surface highly reactive when exposed to a respective aqueous medium.

Owing to its ability to bond to bone strongly, we now report the development of an antibiotic cefuroxime axetil impregnated bioactive glass composite by simple vacuum infiltration technique and describe its release profile *in vitro* and *in vivo* and outline its ability to repair osteomyelitis at the site of the tibia of rabbits. In this regard, it may be noted that cefuroxime axetil has broad spectrum antimicrobial activity against Gram negative and Gram positive organisms. Most of the major causative bacteria of osteomyelitis are sensitive to cefuroxime axetil. In a preliminary study, it has been observed that this drug impregnated cement was proved to be effective in the prevention of early to intermediate deep infection after primary total knee arthroplasty [16].

2. Materials and methods

2.1. Bacterial isolate

Clinical isolate of *Staphylococcus aureus* (coagulase positive) from the abscess of a rabbit with chronic osteomyelitis was used. Pure cultures of the bacteria were obtained on blood agar at 37 °C. Standardized suspensions (3×10^6 CFU/mL) were prepared in saline and kept on ice throughout the surgical procedure. The samples (1 mL) were directly delivered into the medullary cavity of rabbit tibiae. The swab specimens for culture were taken from cavity of infected bone in order to confirm the clinical success of the induction of *Staphylococcus aureus* based on Manitol salt agar test.

2.2. Preparation and characterization of a bioactive glass composition

The bioactive glass was prepared through a conventional glass melting procedure [17]. The appropriate amounts of reagents/raw materials silica (SiO_2), calcium carbonate ($CaCO_3$), dry soda ash (Na_2CO_3), decahydrated borax ($Na_2B_4O_7{\cdot}10H_2O$), TiO_2, di-ammonium hydrogen ortho-phosphate (all chemicals were analytical grade from M/s S.D. Fine-Chem Limited, India) were mixed homogeneously in water. The batch composition of the glass is shown in Table 1, while Fig. 1 shows the schematic representation of the procedures followed to prepare the final glass composition.

X-ray diffraction (XRD) analysis (X'Pert Pro, Phillips Analytical, Netherlands) using 35 milliamps, and 40 kV current, with a monochromatic Cu Kα1 radiation ($\lambda = 1.5406$ Å) with scanning range from $2\theta = 10$–$80°$, was performed on the formed powders to check the compositional nature, Fourier-transformed infra-red (FTIR) spectroscopy studied for confirmation of the functional groups present. These were measured at room temperature (~20 °C) in the wavenumber range of 4000–400 cm^{-1} at resolution 2 cm^{-1} using Spectrum 100 (PerkinElmer Instruments, USA). The samples were pulverized into fine powder, and then mixed with potassium bromide powder, a weight ratio of 1:100 (0.002 g:0.2 g, samples:KBr, respectively). The mixture was subjected to a load of 15 T cm^{-2} in an evacuable die for 5 min to produce clear homogenous discs. The spectra were measured

Table 1
Batch composition of raw materials.

Raw material	% by wt.
SiO_2	43–44
$Na_2B_4O_7{\cdot}10H_2O$	6–7
Na_2CO_3	11–12
$CaCO_3$	29–30
$(NH_4)_2HPO_4$	8–9
TiO_2	1–2

Table 2
Final composition of the bioactive glass.

Composition	% by wt.
SiO_2	43.70
CaO	19.20
P_2O_5	5.46
B_2O_3	9.40
Na_2O	22.24

immediately after preparing the discs to avoid moisture attack. Inductively coupled plasma-atomic emission spectral (ICP-AES) (Spectroflame Modula, Spectro Analytical Instruments, Germany) analysis was performed to obtain the final composition, which was used subsequently for fabrication**.** The final composition thus obtained is given in Table 2.

2.3. Preparation of antibiotic loaded porous bioactive glass block

Two sets of porous bioactive glass scaffolds measuring 9 mm × 4 mm × 4 mm and 20 mm × 9 mm × 9 mm dimensions were fabricated using the above powders. The powder at first mixed with naphthalene of requisite quantity (both the powders and the naphthalene were of particle sizes ranging between 10 and 200 μm) and pressed to a cylindrical block by a cold-isostatic press (EPSI, Belgium) at 150 MPa with 40 s holding time. Subsequently the naphthalene was driven off very slowly up to a maximum temperature of 60 °C and finally fired at about 725 °C for 6 min in a Pt–Rh crucible. The temperature selected for firing the green block was to ensure the incipient of melting together with retention of mechanical strength for subsequent processing. The porosity of the blocks were measured by water displacement method (Archimedes' principle) and found to be about 60–68%. XRD analysis was again performed on the fabricated specimens for the variation of compositional nature, if any and the microstructures of the specimen was observed through FESEM (field-emission scanning electron microscopy) (Supra 35 VP, Carl-Zeiss, Germany).

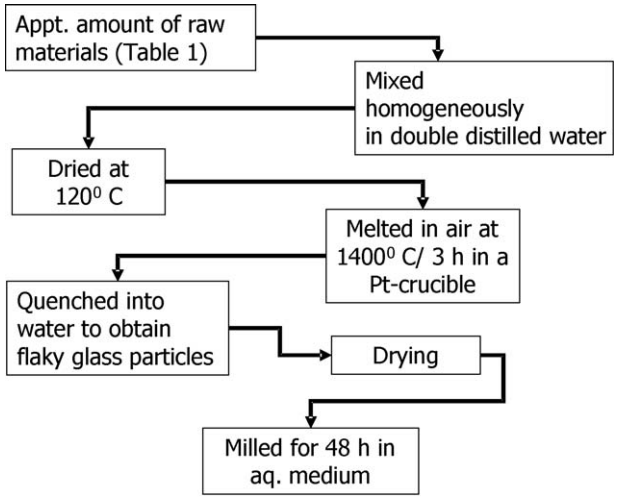

Fig. 1. Schematic representation of the procedures followed to prepare bioactive glass.

Highly concentrated solution (250 mg/mL) of Cefuroxime axetil (Zuventus Pharmaceuticals Limited, India) in acetone (SDFine-Chem Limited, India) was used to infiltrate the porous samples by vacuum infiltration method. Samples were kept in vacuum (10^{-3} torr) unless all the solution containing pre-determined amount of drug went into scaffold and found to be 30 min for all the cases. After evaporation of acetone at room temperature and calculating the initial and final weight of each blocks, the final drug entrapment was calculated. Smaller struts were used for *in vivo* trial while the bigger ones were used for *in vitro* studies. On an average, it has been found that there was ∼125 and 55 mg of antibiotic resided in each bioactive glass scaffolds for the subsequent *in vitro* and *in vivo* trial.

2.4. In vitro elution study

Each porous and antibiotic loaded *in vitro* sample was first placed in a 3 mL of phosphate buffer saline (PBS) at pH 7.2 at a temperature of 37 °C. Elution fluid was collected after each 24 h for continuously up to 21 days, '0' days being the buffer only. Fresh buffer was added every 24 h after the collection of the eluted fluid from the samples and preserved at −20 °C until assayed. Eluted fluids were analyzed by using a high performance liquid chromatography (HPLC) instrument (SPD-M10A, Shimadzu Company, Kyoto, Japan). The column RPC_{18} (Phenomenex, Japan) was used as a stationary phase in which column length was ∼250 mm, diameter 4.6 mm with particle sizes ∼5 μm). The mobile phase consisted of a mixture of 50:50 volumes of HPLC grade acetonitrile and 0.05 M AR grade ammonium di-hydrogen phosphate (both the chemicals Merck, Germany). The flow rate was 1 mL/min. and the experiments were carried out at room temperature. The detection wavelength was 278 nm.

2.5. In vivo studies

Osteomyelitis was produced in the right tibia of 18 (eighteen) nos. of New Zealand white rabbits (2.5–3 kg body weight) according to the model of Norden [18]. After anaesthesia with Nembutal 0.5 mg/kg intravenous (IV) (Thiopentone sodium, Thiosol, Neonlab, Mumbai, India), the proximal part of the tibia was exposed anteriorly and a hole drilled through the cortex into the medullary cavity using a 1.2 mm diameter dental burr. About 1 mL of bacterial suspension containing approximately 3×10^6 CFU/mL of *Staphylococcus aureus* were inserted into the medullary cavity.

The hole was covered with bone wax to prevent bacterial leakage into the soft tissues. The skin was closed and the animals were then allowed to free move in their cages. Infection was allowed to develop for 3 weeks and thereafter, the animals underwent second-stage surgery. All the animal experimentations were carried out following the procedures conforming to the standards of the Institutions Animal Ethical Committee of the West Bengal University of Animal and Fishery Sciences, India. Now by using the previous surgical approach, the area was exposed and bone defects were created. The swab specimens for culture were taken from each animal species to confirm the clinical development of the induction of *Staphylococcus aureus*. Antibiotic impregnated bioactive glass blocks were then impacted into the medullary space of defect area to treat the infection. The wound was closed in layers. The animals were monitored after surgery. All the animals received standard postoperative pain medication (Carprofen; 4 mg/kg of body weight) for 3 days.

2.6. Design of experiment and in vivo elution study

All the 18 animals were divided into three Groups hereinafter would be designated as Group I, II and III. The details of the experimentation with these animals are tabulated (Table 3). Histological examinations were done from decalcified cross sections of the infected bone at 3 and 6 weeks after induction of osteomyelitis. The implanted bone/antibiotic impregnated ceramic implants were collected and sections were cut (3–4 mm thick). The bone pieces were washed thoroughly with normal saline and were fixed in 10% formalin for 7 days. Subsequently bones were decalcified in Goodling and Stewart's fluid containing formic acid 15 mL, formalin 5 mL and distilled water 80 mL solution and it was stirred daily and changed once in 3 days. The sections were checked regularly for the status of decalcification. They were considered as completely decalcified when sections become flexible, transparent and easily penetrable by pin. The decalcified tissues were processed in a routine manner and 4 micron sections were cut and stained with haematoxyline and eosin. Radiographic images from the infected bones were taken by direct radiographic magnification. Anterio-posterior views were taken. Estimation of concentrations of antibiotic (Cefuroxime axetil) in bone samples and serum were made by high performance liquid chromatography (HPLC) technique with the same conditions mentioned earlier. Blood samples were taken from the ear vein of rabbits for determination of the level of cefuroxime axetil in serum. After coagulation and centrifugation, the serum was stored at $-20\ ^{\circ}C$ until assay. In case of bone, after removal of soft tissue and bone marrow, the cancellous and cortical tibia bone was pulverized, homogenized with phosphate buffer and centrifuged. The supernatant fluid was collected and stored at $-20\ ^{\circ}C$ for evaluation of concentration of cefuroxime axetil. The results were expressed as means $\pm$ standard errors. Statistical analyses were performed using the simple one way ANOVA method. The levels of significance were taken $P \leq 0.01$ and $P \leq 0.05$.

3. Results and discussions

3.1. Characterization of the powders and blocks

XRD of both powders formed after rapid quenching from 1400 °C together with the porous blocks fabricated in a Pt–Rh crucible at 725 °C are given in Fig. 2, while FTIR of the same are given in Fig. 3. It has been found that the relative positions of the hump as seen from the XRD pattern were unchanged, although amorphous natures of respective pattern were also noticed. There is no incipient of formation of crystals, which was desirable for actual *in vivo* application.

FTIR spectra (Fig. 3) showed well-defined transmission bands characteristic of the samples prepared at 1400 °C and fabricated at 725 °C/6 min with sharp split bands. All the transmission spectra showed a broad band at around 3455 cm^{-1} and another broad band at about 1627 cm^{-1}. The transmission spectra of the samples showed the same bands at around 416, 776 and 1080 cm^{-1}.

FESEM photomicrographs of the porous specimen is given in Fig. 4, showing amorphous nature of the microstructures.

Table 3
Design of experiment.

Group	No. of animals	Implant	Days of experiment	Experiment
Group I	6	Not given	After 3 weeks	Six animals were sacrificed for histological, radiographic and microbiological examination to confirm development of osteomyelitis.
Group II	6	Cefuroxime axetil injection parenterally (15 mg/kg, bid) twice daily for 6 weeks	After 3 weeks	Three animals were sacrificed for histological and estimation of drug concentration in bone and serum.
			After 6 weeks	Three animals were sacrificed for histological and estimation of drug concentration in bone and serum.
Group III	6	Cefuroxime axetil impregnated bioglass beads	After 3 weeks	Three animals were sacrificed for histological and estimation of drug concentration in bone and serum.
			After 6 weeks	Three animals were sacrificed for histological, radiographic, and estimation of drug concentration in bone and serum.

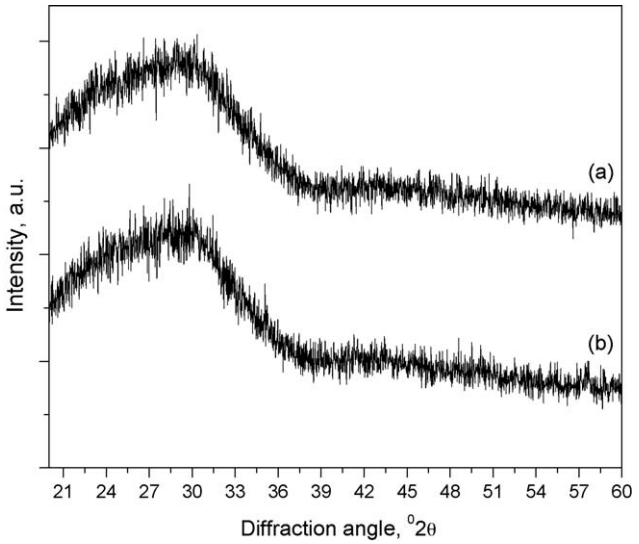

Fig. 2. X-ray diffraction of powders prepared at 1400 °C (a) and porous blocks fired at 725 °C.

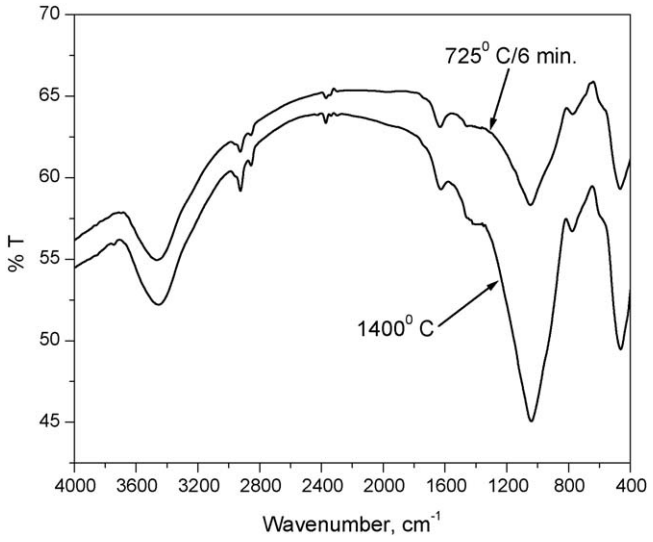

Fig. 3. FTIR of powders prepared at 1400 °C and porous blocks fired at 725 °C.

There were no as such grain boundary between particles and micron to nanosize level of the porous matrix could be observed. Macropores were restricted on the surface with pore closures could be seen on the subsurface. The pores were moderately interconnected and there was no as such geometry of the pore morphology resembling the escape of naphthalene while dried. In the microstructure, nanosized pores having size ranges between 100 and 400 nm could also be observed.

3.2. In vitro elution study

The concentration of the drug determined from each assay, starting from day '0' up to 21 day is given in Fig. 5. It was observed that the elution was stopped right after 6 days and no further drug was eluted from the samples. In addition, the initial release of drug was high and continued only up to day 3.

3.3. In vivo study

3.3.1. Bacterial colony counts at various sampling points

The swab specimen was collected from the infected site of bone after 21 days of post-inoculation from animals of all groups and was streaked on mannitol 10% salt agar slant and incubated at 37 °C for over night. Characteristic bacterial growth was observed. From single colony bacterial growth was collected and stained by Gram's staining method. The organisms were Gram positive coccid and arranged in single or diploid similar to the organism inoculated. At the time that the animals were sacrificed (3- and 6-week after IM treatment and post-implanted antibiotic impregnated bioactive glass blocks), swab specimen was collected from the implanted site of bone and inoculated to Mannitol 10% salt agar and incubated at 37 °C for over night. No bacterial growth of *Staphylococcus aureus* was found.

3.3.2. Histopathological examination

After surgery, no postoperative complications were developed. The bone marrow showed changes of haemopoigenesis centre, consisting of degenerative fat cells (adipose tissue) along with mild fibrovascular proliferation of connective tissues. The peripheral region of the bone marrow showed infiltration with mononuclear cells, osteoclast etc. indicating development of osteomyelitis (Fig. 6). Photomicrograph on day 21 after treatment with cefuroxime axetil impregnated

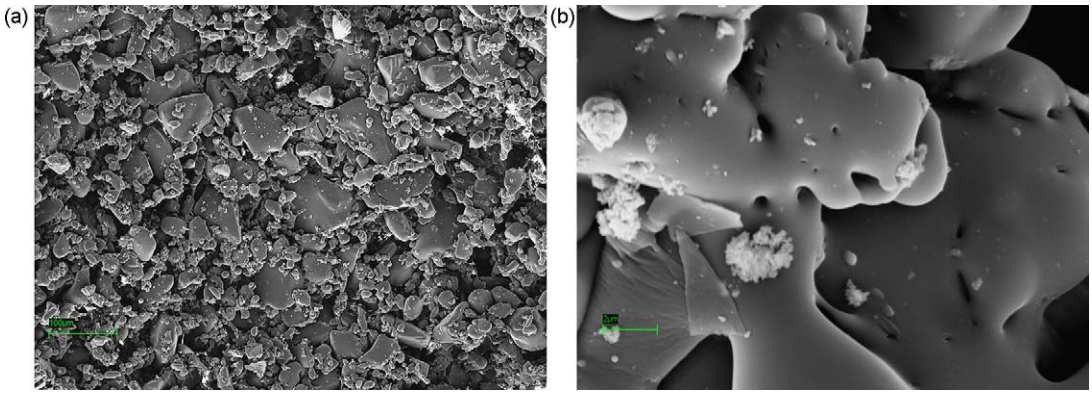

Fig. 4. FESEM photomicrograph of porous struts fired at 725 °C.

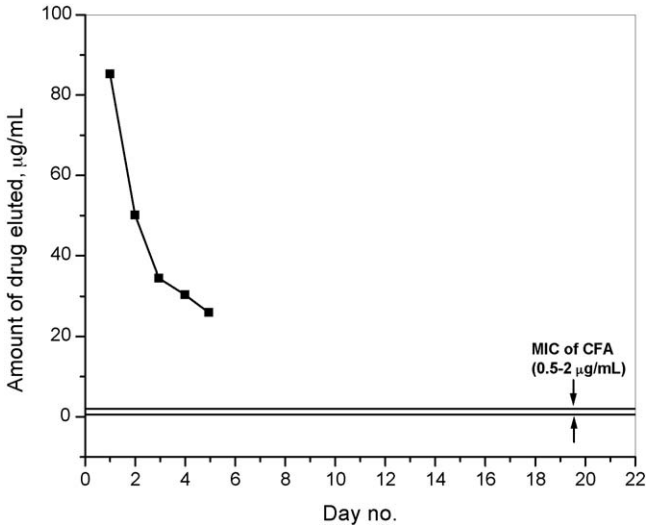

Fig. 5. *In vitro* elution of the drug cerfuroxime axetil (CFA) from day '0' up to 21st day.

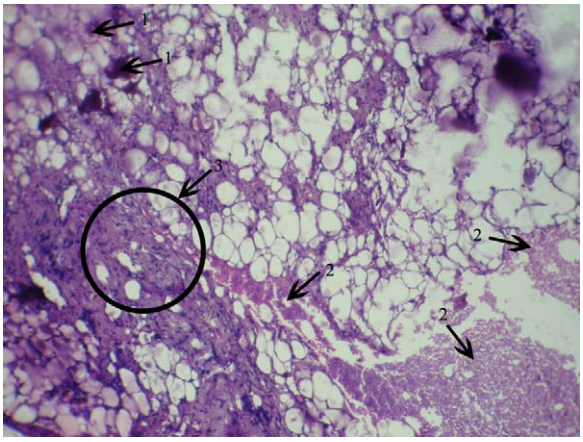

Fig. 7. Histopathology after 21 days for bioglass impregnated with the drug (Group III animal) (HE × 10). (1) Bits of foreign particle within Haversian canal, (2) well developed angiogenic tissue, and (3) well developed lamellar bone with presence of Haversian canal.

bioactive glass showed well-developed lamellar bone with havarsian canal. A few of havarsian canal showed presence of small bits of foreign materials (Fig. 7). But, on day 42, it exhibited well-developed lamellar bone along with havarsian spaces containing marrow materials. A few marrow spaces showed presence of small bits of bioactive glass particles surrounded by angiogenic tissue (Fig. 8). IM treatment with cefuroxime axetil on day 21 and 42 showed only moderate lamellar bone formation with havarsian canal and marrow material (Figs. 9 and 10).

3.3.3. Radiological examination

In the present study, osteomyelitis was inducted successfully in animals of all groups by inoculating *Staphylococcus aureus* as supported by radiographic zone of cortical lysis, formation of sequestrum and radially oriented radiodense bony tissue at the site (Fig. 11). In animals of Group III which were treated with antibiotic impregnated bioactive glass showed rectangular shaped radiodense unaltered antibiotic impregnated bioactive glass implant. Metaphyseal region showed complete disappearance of radiodense hard tissue aggregation as observed in the early days of osteomyelitis. Radiodensity of medullary cavity as well as cortical bone was homogenous to that of unaffected diaphyseal bony tissue (Fig. 12).

3.3.4. Drug concentration

Maximum concentration of cefuroxime axetil in bone and serum of animals (Group II) after intramuscular injection is presented in Table 4. Values for concentrations were expressed as micrograms of antibiotic per gram of bone (wet average). *In vivo,* the release of cefuroxime axetil from bioactive glass implants in bone and serum is presented in Table 5. It has been observed from the figure that there was an initial high release of cefuroxime axetil in bioactive glass implant in bone on day 21, the release was continued up to day 42 which was further high compared to that of corresponding day 42 observation of Group II animals. In case of serum, the reverse tendency was observed.

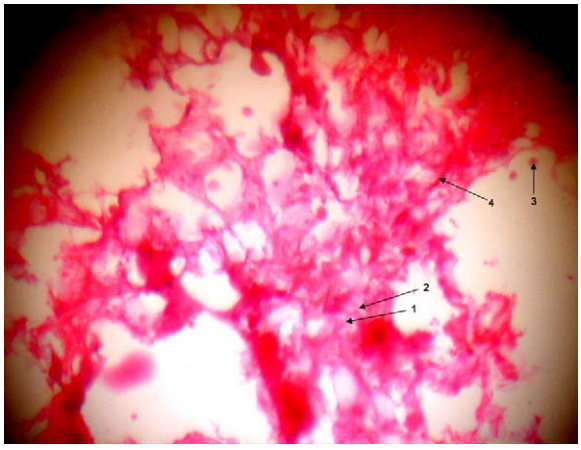

Fig. 6. Photomicrograph showing degenerative osteoblast indicating osteomyelitis (HE × 10) after 21 days. (1) Bony matrix, (2) osteocyte, (3) osteoclast, and (4) immature bony osteoid.

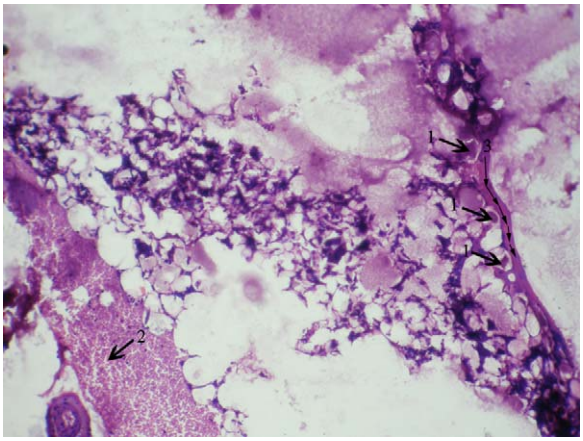

Fig. 8. Histopathology after 42 days for bioglass impregnated with the drug (Group III animal) (HE × 10). (1) Presence of foreign particles within havarsian canal, (2) well developed angiogenic tissue, and (3) well developed lamellar bone.

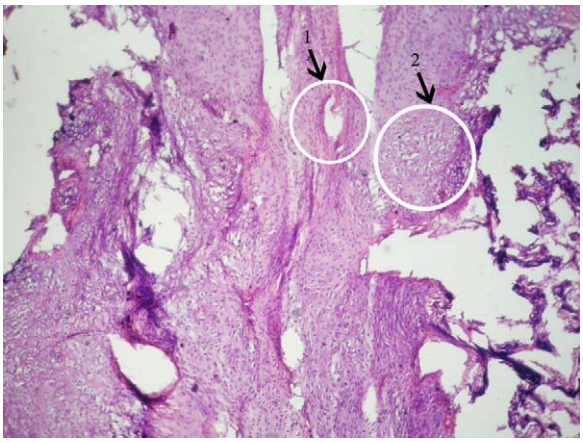

Fig. 9. Histopathology after 21 days for IM treatment of the drug (Group II animal) (HE × 10). (1) Moderately developed Haversian system and (2) well developed cartilaginous tissue.

The drug released through serum was very high in case of Group II animals in day 21 and 42 and the tendency for drug release was even more after day 42. In case of BG blocks same tendency was observed i.e. drug released through serum was more in day 42 than day 21, but quantity released was very much less than that of Group II and more uniform.

The XRD technique was to assess the phase purity and crystallographic changes, if any. Bioactivity of bioactive glass depends on crystallographic changes, thus, it is necessary to study its nature of phase before intending for in vitro and in vivo trials. In the present study, there is no incipient crystallization, since it could be correlated with the presence of phosphate and silicate network and the possible phase separation even in microscale. The system SiO_2–CaO–Na_2O–P_2O_5 has the tendency to form sodium calcium silicate phase of the formula $Na_2Ca_2Si_3O_9$ as the main phase. This tendency is confirmed in all the samples where the $Na_2Ca_2Si_3O_9$ phase is detected by X-ray diffraction with amorphous nature. This observation could also be related with the microstructure by FESEM, where residual glassy phase is considerably high. This may be due to higher silica content as seen in Table 2. These results are in compliance with the previous results of Hench [19,20] who

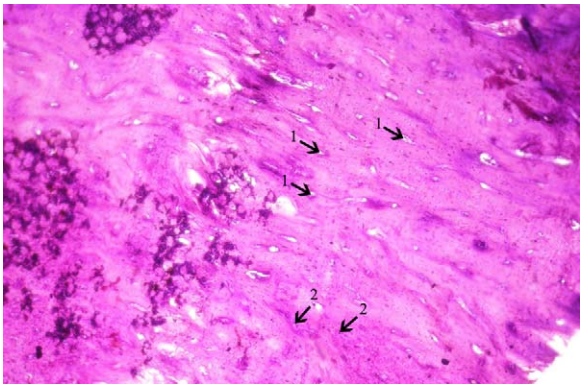

Fig. 10. Histopathology after 42 days for IM treatment of the drug (Group II animal) (HE × 10). (1) Moderately developed Haversian system and (2) scanty cartilaginous cells.

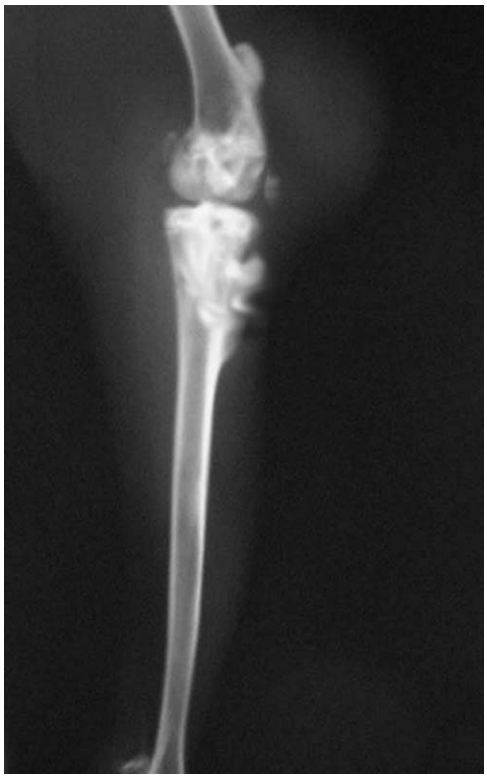

Fig. 11. Radiograph showing periosteal reaction in the epiphysis with presence of secondary sequestrum in the medullary cavity indicating development of osteomyelitis.

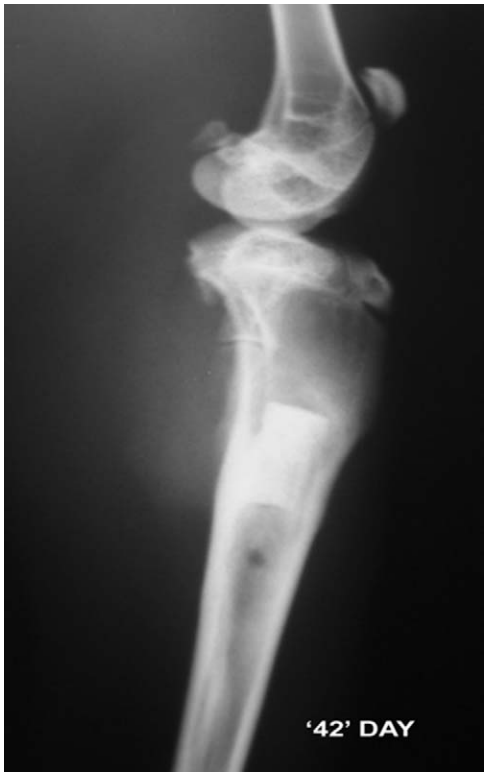

Fig. 12. Radiograph at day 42 with antibiotic incorporated bioactive glass showing recovery from the osteomyelitis changes at the adjacent area of implant.

Table 4
Concentration of cefuroxime (mean ± S.E.) in serum and bone in parenteral injection (Group II).

Treatment	21-day		42-day	
	Bone (μg/g)	Serum (μg/mL)	Bone (μg/g)	Serum (μg/mL)
Cefuroxime axetil injection parenterally (15 mg/kg, bid) twice daily for 6 weeks	4.54 ± 0.6^{a}	22.04 ± 0.58^{a}	7.27 ± 0.42^{b}	28.49 ± 0.83^{b}

Means with different superscripts (a and b) within rows varied significantly ($P < 0.0001$).

Table 5
Concentration of cefuroxime (mean ± S.E.) in serum and bone following implantation of antibiotic impregnated bioactive glass beads on tibia (Group III).

Treatment	21-day		42-day	
	Bone (μg/g)	Serum (μg/mL)	Bone (μg/g)	Serum (μg/mL)
Bioactive glass (Group III)	11.59 ± 0.51^{b}	8.94 ± 1.18^{b}	23.72 ± 1.71^{a}	16.22 ± 0.96^{a}

Means with different superscripts (a and b) within rows varied significantly ($P < 0.05$).

inferred about the most favourable bioactive glass for maximum bioactivity and ease of crystallization. However, increasing the SiO_2 content will eventually enhance retardation of the bioactivity of the glass. The surface physiochemical properties play an important function in the bone bonding mechanism [21] because the physical properties and the crystal chemistry of the surface together with the chemical composition of the surrounding extracellular fluid determines the nature of the new surface to be formed on the material.

The IR transmission spectra show well-defined transmission bands characteristic of the Si–O–Si stretching and bending modes which are associated with the phases of the samples (Fig. 3). The weak inflection at 1627 cm^{-1} can be assigned to the molecular water. The broad band centered at 3455 cm^{-1} can be assigned to hydroxyl group (–OH) or silanol group (SiO–H). There were bands at around 1094, 776 and 416 cm^{-1} which might due to Si–O–Si asymmetric stretching of bridging oxygen atoms within the tetrahedra, Si–O–Si symmetric stretching of bridging oxygen atoms within the tetrahedral and Si–O–Si bending, respectively. This observation could be correlated to the observation of Hench [19]. The degree of bioactivity of this bioactive glass in terms with the degree of crystallinity could be inferred from the IR spectrum. Since the material was drug intercalated and further *in vivo* trial was made, it was interesting to note about the crystallization of this bioactivity in presence of physiological fluid. However, El-Ghannam [22] reported that adsorption of serum protein on bioactive glass surface is influenced by the crystallization process which further alters the corrosion behaviour and increases the surface charge negativity, that subsequently decrease the above adsorption.

In the *in vitro* trial, elution of the drug cefuroxime axetil was stopped after 6 days. This may be attributed for many reasons. When glass with a low ratio of network former and alkali ion content is implanted in tissue or in PBS (as in this case with pH of 7.2 and constant temperature of 37 °C), a sequence of reactions occurs, starts with surface dissolution, together with deposition of calcium phosphates and eventually all the alkali ions leach out, yielding the formation of a silicon-rich layer, on top of which a Ca–P-rich layer is formed. Both diffusion from the glass as well as deposition from the surrounding fluids around the sample create this Ca–P-rich layer [23–26]. Now, porosity in the sample also had an important role to play. Since the surface of the porous struts were mainly macroporous, the elution was very fast for first 3 days, the rate of which dropped significantly after that and stopped ultimately. As seen from the microstructure subsurface pores below the top surface may be closed due to heterogeneous nucleation of Ca–P phases after dissolution. Mineralization of bone however involves a concentration enhancement of silicon at the mineralization front. This biologically active SiO_2 in combination with apatite is responsible for inhibiting the proliferation of fibroblasts at a bioactive implant interface. It is reported that presence of silicon at a critical concentration is an essential prerequisite to trigger bone formation [23]. On the other hand in bioactive glass the concurrence of SiO_2 hydrolysis and condensation with biological hydroxyapatite mineralization promotes bone growth, which is also the natural process of bone repair [24]. Porosity within the sample has an effect on bone bonding ability *in vivo* due to the following reasons: (a) it has got a large surface area resulting in a high tendency to bioresorb, and induce high bioactivity, (b) interconnected pores can provide a framework for bone growth into the matrix of the implant, and thus anchor them with the surrounding bone, preventing micro-motion that in turn increases further bone growth, (c) interconnected porosity acts like an organization of vascular canals, which can ensure the blood and nutrition supply for the bone. Although there is no optimal ''porosity database'' that can ensure rapid osteo-integration in bioactive glass, a broad guidelines exist regarding the level of total porosity (>50–60%), minimal interconnection size (>50–100 μm channels) and level of strut porosity (>20% with an upper limit typical for resorbable materials).

Recent studies on *in vitro* and *in vivo* have shown that not only macroporosity of the implants has an influence on integration and volume of regenerated bone but microporosity also has an influence on biological sensitivity for bone formation [27–29]. This is assumed to be through mediation

of cell attachment and/or selective sequestering and binding of adhesion proteins and growth factors (GF) [30]. Studies on the influence of bioactive glass microporosity on the rate and quality of bone healing *in vivo* demonstrated that faster apposition in microporous scaffolds with microporosity levels of >20% was linked to the rate of development of the vascular network.

Histological observations provide more detailed knowledge about the cellular events during incorporation of different types of ceramic implants. Besides, best evidence of the efficiency of treatment of osteomyelitis could be observed in histopathological and microbiological findings [31]. In the present study, well developed lamellar bone with havarsian canal was formed on day 21 and 42 after local implantation of cefuroxime axetil impregnated bioactive glass in osteomyelitis of rabbit tibia. Havarsian canal also contained foreign unabsorbed material and with well developed vascularization. This might be due to the fact that cefuroxime axetil was released at an adequate concentration in the osteomyelitis site and controlled the infection which subsequently helped in new bone formation. The presence of particles of unabsorbed bioactive glass materials suggested that the materials were not resorbed within this time. The histological findings also suggest that there was only a minimal reaction towards biomaterial and gradual new bone formation in the area. In similar findings, Dominguesa et al. [32] observed that the histological examination of tissue at the site of implantation showed moderate inflammatory reactions in all groups after 72 h in tetracycline impregnated bioactive glass implant. Similar findings were also observed by Itokazu et al. [33] and Sanchez et al. [34] in osteomyelitis model.

The treatment of osteomyelitis in orthopaedic surgery poses a great challenge due to improper and unsustained drug concentration at the site for prolonged time. Though the microbial organisms responsible for causing osteomyelitis are very much sensitive to different routinely used antibiotics *in vitro* but due to inherent characteristic of bony tissue, the success of treatment with such infections are very limited. In the present study, osteomyelitis was induced successfully in animals of all groups by inoculating *Staphylococcus aureus* as supported by radiographic zone of cortical lysis, formation of sequestrum and radially oriented radiodense bony tissue at the site. Inflammation with hyperaemia is the squeale of bacterial contamination of bone which ultimately leads to formation of abscess [35]. The abscess continues to expand, with further production of purulent exudates [36]. The exudate enters the cortical bone and spreads across it via Haversian and Volkmann's canals and ultimately leading to necrosis of fragments of bone sequestrum and lysis [36,37]. In animals of Group III which were treated with antibiotic impregnated bioactive glass showed more or less equally controlled infection. After a certain period of time, the lysis and sequestrum so far observed in the pre-treatment period could not be ascertained to that extent due to effect of antibiotic in desirable concentration at the site which might have controlled antimicrobial activity. Normal phenomenon of bone healing are formation of osteon in the phase of remodelling which aggregate in the acentric manner as any site of fracture area during the remodelling phase. In the present study, the desired level of antibiotic concentration at the site might have controlled the infection which in turn accelerated the bone healing and remodelling as supported by newly grown periosteal bone. In a similar finding, Korkusuz et al. [38] also reported radiographically mild periosteal elevation, architectural deformation, widening of bone shaft, new bone formation and soft tissue deformation after 6 weeks of implantation with Duocid and Sulperazone-loaded PHBV rods in rabbits.

Several biomaterials have been used as vehicles for the transport and sustained release of antibiotics. The pharmacokinetics of the composites *in vivo* showed that therapeutic concentration of antibiotic was maintained at the site of implantation which was adequate to provide antimicrobial activity. The reason for the efficacy of bioactive glass in the treatment of osteomyelitis is probably the advantageous pharmacokinetics at the site of infection. The minimum inhibitory concentration (MIC) of cefuroxime axetil against *Staphylococcus aureus* is 0.5–2 μg/mL [39]. Concentrations exceeding the MIC were obtained around the implant in all bone tissues. The above results suggest that the composites in the present study fulfil this condition. The administration of antibiotics for 4–6 weeks is usually recommended for the treatment of chronic osteomyelitis [40,41]. The efficacy of systemically applied antibiotic for precluding osteomyelitis seems to be very poor due to impermeability of this antibiotic in attaining desirable concentration at the target site due to blood–bone barrier [42]. Juxtaposely same phenomenon is also applicable for poor concentration of antibiotic in serum from bone tissue while applied by drug delivery system. The local antibiotic treatment may use the blood–bone barrier effectively as a protection of the body against a very high local antibiotic concentration without systemic side effects [43]. In the present study also, wide difference of antibiotic concentration between bony tissue and serum irrespective of two groups in corresponding days were noticed which might be due to presence of such blood–bone barrier.

4. Conclusions

The biodegradable antibiotic carrier system developed in this study proved to be an effective therapeutic approach toward an experimental model of osteomyelitis. Further studies on the prolongation of the drug release duration and more bone ingrowth into the pores are needed before clinical studies on human subjects can be undertaken.

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