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ORIGINAL ARTICLE

Antioxidative/oxidative effects of strontium-doped bioactive glass as bone graft. *In vivo* assays in ovariectomised rats

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Summary
Recently, oxidative stress has been identified as a pivotal pathological factor inducing bone osteoporosis. This phenomenon is responsible for low bone density. It alters bone quality and generates bone fractures. Strontium is found to induce osteoblast activity by stimulating bone formation and reducing bone resorption by restraining osteoclasts. Bioglass (BG) has been used to repair bone defects, and, in combination with strontium (BG-Sr), offers an opportunity to treat this disease. This study investigated the potential role of BG-Sr in improving antioxidant activity and regenerative bone capacity, The effects of both BG-Sr and BG were tested on osteoblast SaOS2 and endothelial EAhy926 cell proliferation *in vitro*. *In vivo*, BG-Sr and BG were implanted in the femoral condyles of Wistar rats and compared to that of control groups. Cell proliferation increased significantly by 120% at SaOS2 and 127% at EAhy926. Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) were significantly enhanced in BG-Sr treated rats compared to other groups. Moreover, a significant decrease of thiobarbituric acid-reactive substances (TBARs) was observed. The Ca/P ratio increase improved progressive bone mineralization. According to these results, BG-Sr ameliorated cell proliferation and developed an antioxidative defense against ROS. The histological findings highlight the BG-Sr implications in the osteoporosis treatment confirmed by bone construction. The development of BG-Sr as a therapeutic biomaterial protecting against oxidative stress might make an effective choice for application in tissue engineering.

Key words: oxidative stress; osteoporosis; strontium-substituted bioactive glasses; free radical; bone regeneration

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INTRODUCTION

When bone defects occur, a remarkable yield of Reactive Oxygen Species (ROS) is generated by the damaged tissues and implicates oxidative stress. This mechanism is defined as an imbalance between excessive ROS generation and insufficient antioxidant defense mechanisms (Sánchez-Rodríguez et al. 2007).

The enhanced osteoclastic activity observed in bone disorders may be responsible for increased ROS production, and severe metabolic malfunctions (Hensley et al. 2000) especially in post-menopausal women.

In rodents, ovariectomy induces the post-menopausal period and serves as an osteoporotic model (Chesnut et al. 2000, Saito et al. 2002). For the treatment and prevention of osteoporosis, strontium ranelate (SrR) has been shown to reduce the number of hip and vertebral fractures in post-menopausal women (Meunier et al. 2004). The drug combines two atoms of stable Sr with the organic moiety ranelic acid. The cellular mode of SrR action appears to be through the Sr cations themselves, which have been shown to work by stimulating osteoblasts to make new bone and preventing osteoclasts from resorbing bone (Gentleman et al. 2010).

Because of its chemical and physical similarities to calcium (Ca), Sr is also a natural bone-seeking element and about 98% of the total body Sr content is localised in bone tissues (Effah Kaufmann et al. 2000, Jell et al. 2008). Sr has been shown to promote osteoblast replication, differentiation and survival (Hamdy 2009), and is regarded as a bone-forming agent due to its stimulation of the osteoprogenitor cells replication and collagen synthesis. Moreover, Sr can not only stimulate non-collagenic protein synthesis in osteoblasts but *in vitro* also can inhibit the release of Ca from pre-labelled bone (Caverzasio and Thouverey 2011). Nevertheless, the action of the molecular mechanisms of Sr on bone forming cells are still under investigation, although in fact, a limited amount of research has indicated the dual effects of Sr delivery by BG in the promotion of bone formation and reduction of bone resorption.

BG forms a strong bond with living tissues via the formation of an hydroxyapatite layer on their surface (Hench et al.1971) and has been used to repair hard tissues in a variety of craniofacial, maxillofacial, and periodontal applications (Hojjatie and Anusavice 1990). New potential bioactive glass possessing a composition similar to that of Hench's 45S5 – called 46S6 in the SiO_2-CaO-Na_2O-$P2O_5$ system – has been investigated *in vivo*. and, in view of its potential clinical application, we investigated whether it could stimulate cultured cells to proliferate.

The aim of this work was to clarify whether 46S6 glass actually exerts toxic effects, and whether Sr, which it releases, might play a role in raising such cytotoxicity. In order to assess the possibility that an *in vitro* model can mimic what may happen *in vivo*, indices of oxidative stress are followed in an osteoporotic rat model. While the response of bone to oxidative stress in ovariectomised rats has been well studied, the effects of implanted Sr doped bioactive glass on the ROS balance have not yet been addressed in an osteoporotic model. Therefore, the present study aims to investigate the antioxidant activity of novel Sr-doped bioactive glass against free radicals induced by estrogen deficiency.

MATERIAL AND METHODS

Bioactive glass synthesis

The first material studied was pure 46S6 possessing a composition close to that of Hench's 45S5 (Hench 1998) whichwas used as a reference to validate our experimental procedure. Then, 0.1% wt of Sr was introduced into the 46S6 bioglass; a similar content of Sr to that of bone. Appropriate amounts of calcium metasilicate, sodium metasilicate, sodium metaphosphate, and magnesium oxide were weighed and mixed for 45 min using a planetary mixer. The powdered mixture was heated in a platinum crucible at 1300 °C for 3 h. The molten material was then poured into preheated brass molds to form cylinders of 13 mm in diameter and 10 mm in height. The prepared samples were annealed for 4 h at the appropriate temperature, corresponding to the phase transition temperature of the glass composition (about 560 °C), in a regulated muffle furnace, which was left to cool to room temperature at a rate of 1°C min^{-1}. After elaboration, the powder particles, sized between 40–63 μm, were compressed in a perfectly isostatic manner. The prepared implants were sterilized by γ-irradiation from a ^{60}Co source gamma irradiation at a dose of 25 Gy (Equinox, UK) using standard procedures for medical devices.

Cells culture

The human osteosarcoma cell line Saos-2, and the endothelial cell line Eahy926, were cultured under standard conditions (37 °C, 5% CO_2/95% air, 100% humidity) in DMEM (Sigma Chemical Co, St. Louis, MO) containing an antibiotic (1% Penicillin 100 μg/ml) (Gibco Laboratories) and supplemented with 10% (v/v) foetal bovine serum (FBS) and 2 mM L-glutamine, Gibco Laboratories).

SulfoRhodamin B (SRB) assay

The SulfoRhodamin B (SRB) assay (Voigt 2005) was used to measure cell proliferation based on the total cellular protein. Measurements were made by a SRB assay (Sulfo-rhodamine B). The Saos-2 and EAhy926 cells were fixed with 10% (w/v) cold trichloroacetic acid for 1 h, then washed, dried, and stained for 30 min with 300 μl of 0.4% SRB (w/v in 1% acetic

acid) under mechanical agitation. Unbound dye was removed by four washes. With 1% acetic acid, protein-bound dye was extracted with 200 µl of Tris base (10 mM, pH 10.5), for 5 min under mechanical agitation. Finally, 100 µl of each well was used for the determination of the optical density at 492 nm.

Animal model

Female Wistar rats (16–19 weeks of age), obtained from the central pharmacy, Tunisia, and bred in the central animal house, were used in this study. The rats were acclimatized to their new environment for 7 days before the beginning of the study and were fed on a pellet diet (Sicco, Sfax, Tunisia) and water *ad libitum*. All the animals were kept under climate-controlled conditions (25 °C; 55% humidity; 12 h of light alternating with 12 h of darkness). The handling of the animals was approved by the Tunisian ethical committee for the care and use of laboratory animals. All rats were randomly divided into five groups (5 animals per group), the first group (I) used as negative control (T). Sixty days after bilateral ovariectomy, Groups II, III, IV and V used respectively as positive control (OVX), were implanted with BG (OVX-BG), BG-Sr (OVX-BG-Sr); the last one presented empty defects (OVX-NI).

Surgical and postoperative protocol

All surgical interventions were performed under general anaesthesia in aseptic conditions. Anaesthesia was induced with xylazine [7 to 10 mg/kg (i.P) ROMPUN® 2%] and ketamine [70 to 100 mg/kg (i.m) imalgene®] depending on the body weight. The pre-operative preparation of the surgical sites was routinely carried out by cleaning with 96% alcohol and antiseptic solutions (PROLABO; AnalaR Normapur®, France). The resulting bone defects were irrigated profusely with physiological saline solution (0.9 wt. % NaCl; Ref.091214; Siphal, Tunisia) to eliminate bone debris. A drilled hole, 3-mm diameter and 4-mm deep, was created on the lateral aspect of the femoral condyle using a refrigerated drill to avoid necrosis. The drill-hole was filled with 10mg of BG-Sr in OVX-BG-Sr group and with 10 mg of BG in OVX-BG group. The filling was done carefully in a retrograde fashion to ensure both minimal inclusion of air bubbles and direct implant – bone contact. The closure of the wounds was performed in layers (i.e. fascias and the subcutaneous tissue), using resorbable material (Vicryl 3/0; Ethicon, Germany) in a continuous fashion. After the surgical operation, all rats received subcutaneous analgesiea (Carprofen 10 mg/kg I CRimadyl®) for three postoperative days and they were allowed unrestricted mobility: during this period, they were checked daily for clinical lameness or other complications. On days 4, 7, 15, 30 and 60 after implant insertion, all rats were sacrificed and specimens were harvested for biological and physico-chemical evaluation.

Haematological and biochemical assays

White blood cell (WBC) counts, red blood cell (RBC) counts, the mean corpuscular volume (MCV), haemoglobin concentration (Hb), haematocrit (Ht) and platelet counts were measured by the electronic automate coulter MAXM (Beckman Coulter, Inc., Fullerton, USA). Calcium (Ca), phosphorus (P), and total alkaline phosphatase (ALP) were examined using commercial Biomaghreb kits (Tunisia).

Tissue preparation

The implanted femoral condyles of all groups were minced and homogenized (100 mg/ml) at 4 °C in 0.1 mol/l Tris-HCl buffer pH 7.4 and centrifuged at 3,000G for 10 min.

Oxidative stress measurements

Lipid peroxidation was measured by the quantification of thiobarbituric acid-reactive substances (TBARS) determined by the method of Buege and Aust (1984). The activity of SOD was assayed by the spectrophotometric method of Marklund (Marklund and Marklund 1975). The GPx activity was measured by the method described by Pagila and Valentine (1967). CAT was assayed calorimetrically at 240 nm and expressed as moles of H_2O_2 consumed per minute per milligram of protein, as described by Aebi (1984). The level of total protein was determined by the method of Lowry and al. using bovine serum albumin as the standard at 660 nm (Lowry et al. 1951).

Physico-chemical analyses

Femoral condyles were dried for 24 h at 65 °C weighed accurately and placed in 25 ml tubes with 2 ml of added nitric acid. One milliliter of 30% H_2O_2 was placed in the tube after 10 min. The volume of the mixture was made up to 500 ml with distilled water. Standard solutions of Ca, P, Sr, Na, K and Zn, were used to prepare the working standard solution, and a blank solution. The element concentrations were detected using inductively coupled plasma optical emission spectrometry ICP-OES (Ciros; Spectro Analytical Instrument, Germany). Scanning electron microscopy (SEM) (Jeol JSM 6301F) was used to identify morphological changes between bone, BG-Sr and BG. The collected samples were prefixed with 2.5% glutaraldehyde solution (phosphate buffer solution, pH 7.4) overnight, and

then washed with a phosphate buffer solution (pH 7.4), before being post fixed with a 2% osmic acid solution (phosphate buffer solution, pH 7.4) for 90 min and dehydrated with an alcohol evaporating system. The samples were carried out with a freeze-dryer (JFD-300 Electron Optic Laboratory) and a vapour deposition system (JFC-1200).

Histological studies

After 15 days the implanted femoral condyles were harvested from each rat and fixed in Burdack, (formalin). The time delay was selected to assess the performance of the biomaterials on bone formation before degradation. Samples were included in a mixture of methylmethacrylate (MMA) and glycolmthacrylate (GMA) without prior decalcification. Sections 6 to 7 µm thick were debited along a transverse plane using a sliding microtome (Reichert-Jung).

Statistical analysis

The statistical analysis of the data was carried out using the Student's t-test. The determinations were performed from 5 animals per group. All values were expressed as means ± SE at the significance level $2\alpha=0.05$.

RESULTS

In vitro study

Cell proliferation at SulfoRhodamin B (SRB) assay

As illustrated in Fig. 1A, cell proliferation studies in the presence of BG extracts showed that Saos-2 osteoblast and Eahy 926 endothelial were enhanced by 112% compared to those of the controls. After contact of BG-Sr, cell proliferation (Fig. 1B) showed an increase of 120% at SaOS2 osteoblast and 127% at EAhy926 when compared to those of the controls in 6 days of incubation and in culture media diluted to 0.2%. However, after 3 days of incubation, in almost all experiments, a decrease in the cell number was noted. This led to a renewed conditioned medium after 72 h.

In vivo study

WBC, RBC, MCV, Hb, Ht, platelet counts and serum Ca and P showed no significant difference in all treated animals as compared to the controls. Moreover, the OVX group exhibited a significant decrease in serum ALP 4.24±1.51 when compared to that of the controls 6.39±1.04 (Table 1). In the other treated groups, ALP demonstrated no significant variations as compared to those of OVX and controls 60 days after surgery.

Table 1. **Alkaline phosphatase (ALP) activity in ovariectomised female Wistar rats** presented empty defects (OVX-NI), received strontium-doped bioglass (OVX-BG-Sr) and bioglass (OVX-BG). Animals were given free access to food and tap water and were allowed unrestricted mobility. After 60 days post operation, blood samples were taken by cardiac puncture under light diethyl-ether anesthesia. Other experimental conditions are described in the text.

	T	OVX	OVX-NI	OVX-Bg-Sr	OVX-BG
ALP (µkat/l)	6.39±1.04	4.24±1.51*	4.15±1.65	4.43±1.39	4.17±1.64

Data represent mean ± standard deviation to the mean value of controls. * Significant as compared with control rats.

Measured oxidative stress in bone

As illustrated in Fig. 2A, B, C, the data on the CAT, SOD and GPX activities in the femoral condyle of OVX rats showed a highly significant decrease when compared to those of control rats. Also, ovariectomy significantly elevated the MDA levels (Fig. 3). In the same way, after 4,7,15 days, OVX-BG-Sr, OVX-BG rats exhibited a highly significant decrease of SOD, GPX and CAT activities as compared to OVX rats. Oxidative stress increased MDA levels in OVX-NI, OVX-BG and OVX-BG-Sr rats by 180% 165% and 164% respectively. After 60 days, OVX-BG-Sr and OVX-BG showed a particular increase in SOD, GPX, CAT activities when compared to other groups and a significant decrease of MDA. This positive action was more pronounced in OVX-BG-Sr than in OVX-BG. In fact, after comparison with OVX-BG groups, an increase of OVX-BG-Sr in the SOD, CAT and GPX activities of 13.7%, 8.13% and 14% respectively, was shown at the end of the experiment. Moreover, a decrease in MDA by 5% was detected in OVX-BG-Sr as compared to the OVX-BG groups.

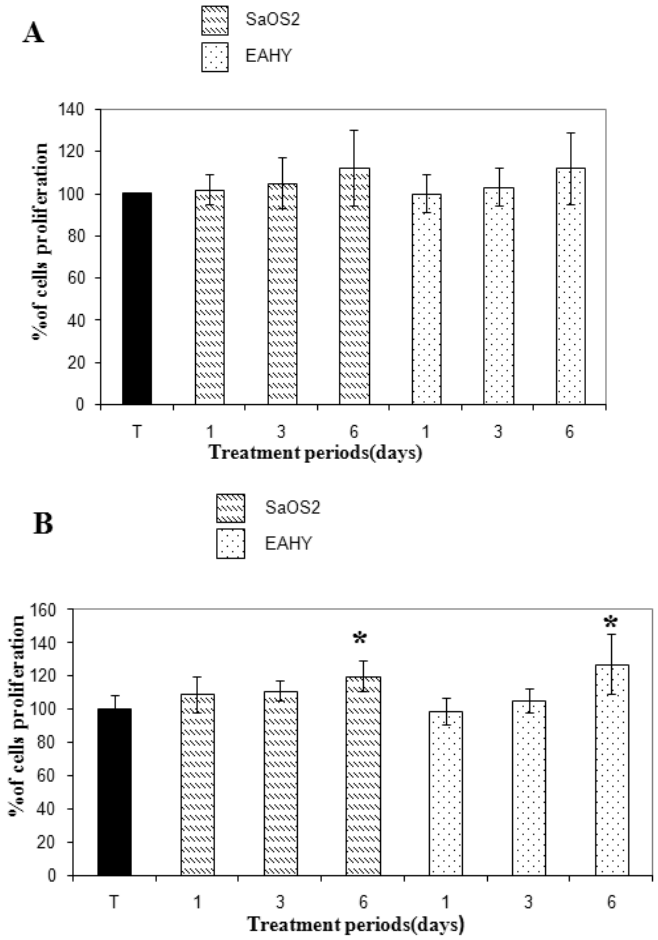


Fig. 1. **SulfoRhodamin B (SRB) assay of Saos-2 human osteosarcoma and Eahy 926 endothelial cells** treated with dissolution ions from bioglass (BG) *(A)* and strontium-doped bioglass (BG-Sr) *(B)*. Data represent mean ± standard deviation to the mean value of controls after 24 h, 3 and 6 days in culture media diluted to 0.2%. * Significant as compared with controls rats.

A significant enhancement of enzymatic defenses was observed especially in comparison with OVX groups. A pronounced increase of the SOD, CAT and GPX activities by 169%, 154% and 82% respectively was shown. Moreover a very important decrease in MDA by 28% was detected in OVX-BG-Sr when compared to the OVX groups. Nevertheless, the antioxidative activities did not attain the normal value of the control rats.

ICP-OES of newly-formed bone

The P and Na concentrations changed somewhat but the variations were not very important over 60 days. As shown in Fig. 4A, the Ca concentrations in both BG-Sr and BG matrix increased gradually in the cancellous bone tissues. On the other hand, Fig. 4B which gives the Ca/P value of the newly-formed bone for each period, shows that the ratio value was close to 1.30 when the glass matrix presented poor degradation and near to that of biological apatite ≈1.66 after 30 and 60 days of implantation. The increase in the Ca/P ratio over time is an indication of progressive mineralization. In addition, as shown in Fig. 4C Sr was slightly detected at about 60.04 ug/g in the treated BG rats. Sr was barely taken up by osteoid tissue or by bone cells or marrow cells in the first period, but 60 days after surgery, it exhibited 144 ug/g when the control bone amount was 154.30 ug/g. As an overall consequence of BG-Sr biodegradation, the release of Sr into the extracellular fluid as a result of erosion surface showed that Sr presented 152 ug/g by the end of treatment. The Sr/Ca ratio could clarify the progression of Sr deposition in the newly-formed bone. As shown in Fig. 4D, a higher Sr/Ca ratio, equivalent to 6.20, was observed in the new bone of the BG-Sr groups 60 days after

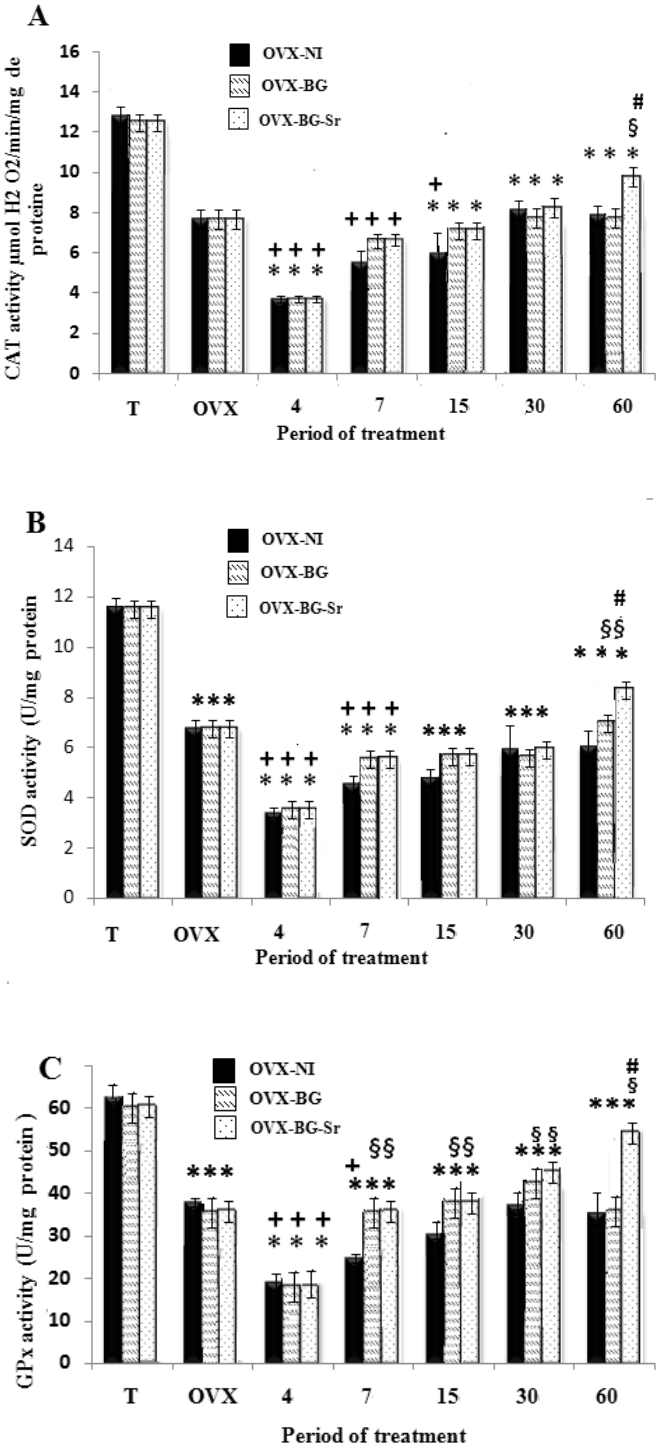


Fig. 2. **Effects of bioglass** (BG) **and strontium-doped bioglass** (BG-Sr) **on catalase** (CAT) *(A)*, **superoxide dismutase** (SOD) *(B)* **and glutathione peroxidase** (GPx) *(C)* activities in femoral condyle cells of ovariectomised female Wistar rats for 4, 7, 15, 30 and 60 days. Values are given as mean ± S.E. * Significantly less enzymatic activity in the indicated group versus the control group, $^{+}$ versus the ovariectomised group, § versus the ovariectomised group with empty defects and $^{\#}$ versus the ovariectomised group and implanted with bioglass group.

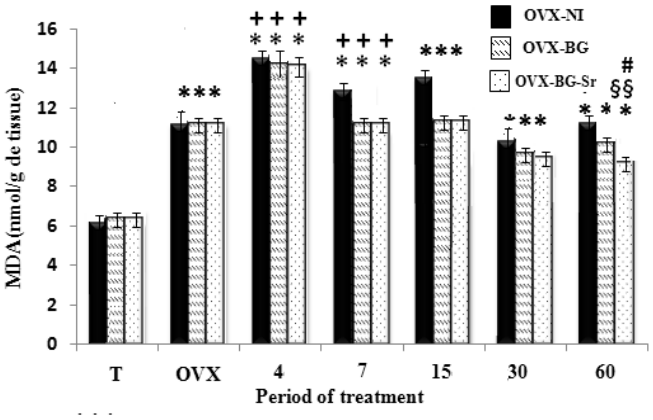


Fig. 3. **Effects of bioglass** (BG) **and strontium-doped bioglass** (BG-Sr) **on malondialdehyde** (MDA) **level.** * Significantly higher level in the indicated group as compared with the control group, $^{+}$ as compared with the ovariectomised group, § lesser as compared with the ovariectomised group with empty defects and $^{\#}$ significant as compared with the group implanted with bioglass.

surgery. However, the ratio didn't seem to be in the same order of magnitude as those of controls which were about 6.33. The absolute value of the Sr/Ca ratio is low, because Sr is a trace element. On the other hand, as shown in Fig. 4E, a small quantity of zinc (Zn) which the bioactive glass did not contain was detected in the newly-deposited bone tissue at about 0.12 mg/g after 30 days and this quantity represented only about half that of the control (0.25 mg/g). Zn appeared throughout the bioactive glass implant area and increased progressively during the implantation period to reach about 0.20 mg/g after 60 days, but never reached the amount found in bone. Finally, Potassium (K) disturbance was revealed in BG-Sr as well as in BG 1.06 and 1.05 mg/g respectively (Fig. 4F). These levels are normally found in the control rat bone and represent about 1.14 mg/g.

SEM morphological analysis

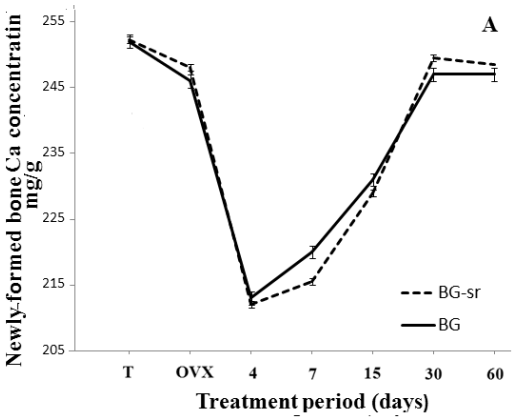
SEM images showed cells anchored tightly on the surface of BG-Sr (Fig. 5A, C, D), and exhibited osteoblast morphology, indicating that cells had finished attachment and were in the process of spreading. Cells could spontaneously attach, spread and proliferate well on the BG-Sr surface. However, we noted an unstimulated layer of cells on the BG surface (Fig. 5B). The apatite nucleation was extensive on the BG-Sr surface (Fig. 6) and the spread antennae of cells partially covered the apatite nucleation. As a result, bone was formed by the mineralization of an organic matrix including cells, osteoid and proteins by the apatite nucleation and growth. This structure indicates that Sr-incorporated glass could not only stimulate apatite formation, but also induce the adhesion and proliferation of osteoblast cells, which might be the template for new bone formation. In fact, the comparison with non implanted and osteoporotic bone (Fig. 7A) showed that the formation and mineralisation of bone matrix implanted by BG-Sr was very accentuated (Fig. 7B, C).

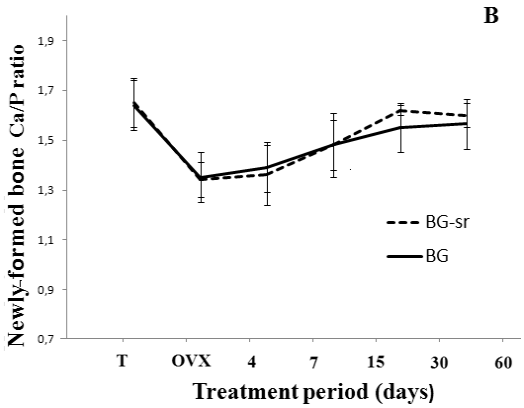
Histological evaluation of BG and BG-Sr implantation

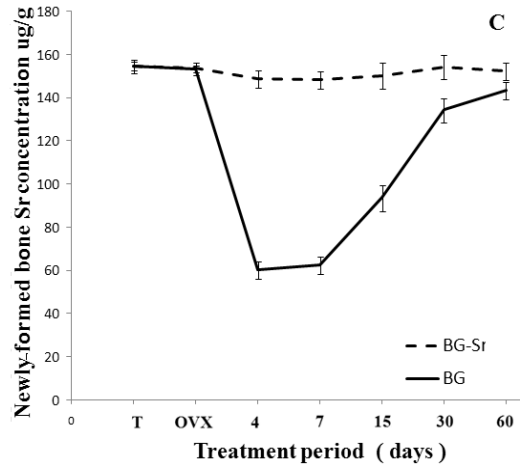
New bone formation in all implanted defects was observed in close contact with BG and BG-Sr (Fig. 8A, B). Both biomaterials were replaced by a large amount of newly-formed bone. However, at the same time clear differences were observed: the more sporadic ingrowth within the periphery of the BG implant showed a sparser osteoid deposition; the bone surrounding BG-Sr presented a highly cellular layer, more advanced ossification and a much larger stimulation of osteoregeneration than those of the BG group (Fig. 8C, D). This bone regeneration paralleled the significant enhancements of the antioxidative enzyme in the OVX-BG-Sr.

DISCUSSION

Recently, great interest has been focused on Sr as a potential anti-osteoporotic factor (Collette et al. 2010). We hypothesise that the local presence of 0.1% Sr^{2+} at bone-to- bioactive glass implant interface would be an excellent candidate for protective surface treatment. Sr may contribute to the control of ROS


A
Newly-formed bone Ca concentratin mg/g
255
245
235
225
215
205
BG-sr
BG
T
OVX
4
7
15
30
60
Treatment period (days)


B
Newly-formed bone Ca/P ratio
1,9
1,7
1,5
1,3
1,1
0,9
0,7
BG-sr
BG
T
OVX
4
7
15
30
60
Treatment period (days)


C
Newly-formed bone Sr concentration ug/g
180
160
140
120
100
80
60
40
20
0
BG-Sr
BG
0
T
OVX
4
7
15
30
60
Treatment period (days)

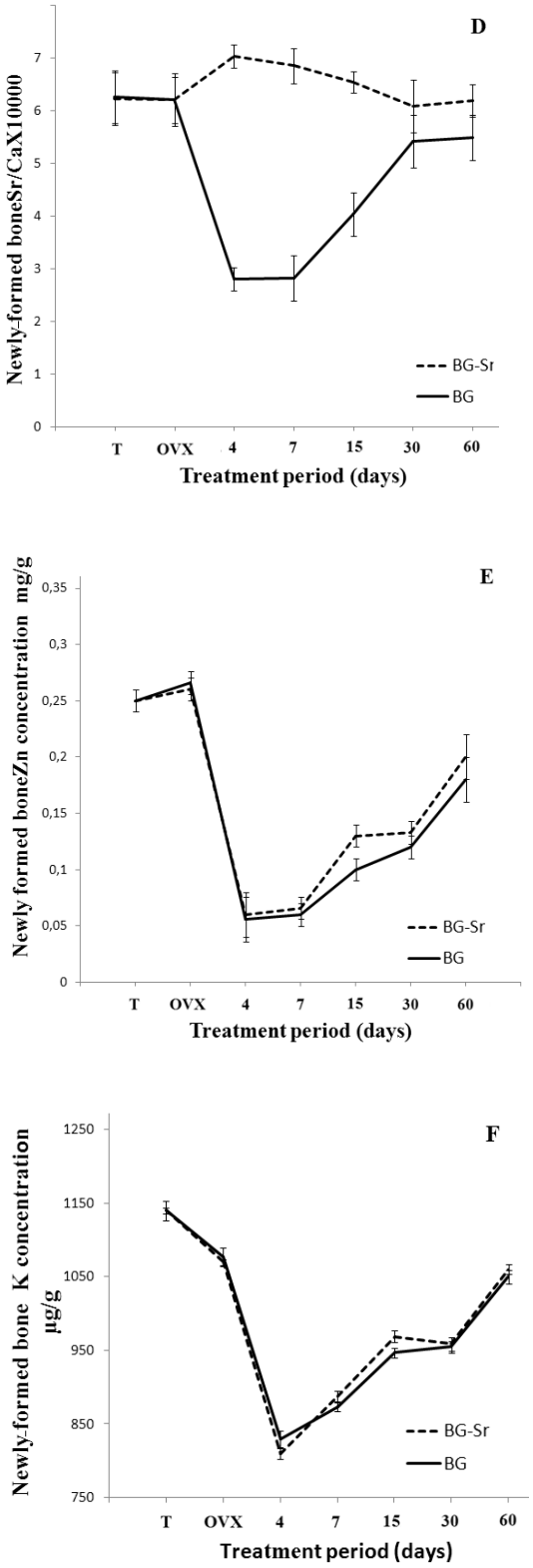


Fig. 4. **Distribution of Ca** *(A)*, **Ca/P** *(B)*, **Sr** *(C)*, **Sr/Ca** *(D)*,Zn *(E)* **and** K *(F)* in newly-formed bone of ovariectomised female Wistar rats implanted with bioglass (BG) and strontium-doped bioglass (BG-Sr) for 4, 7, 15, 30 and 60 days.

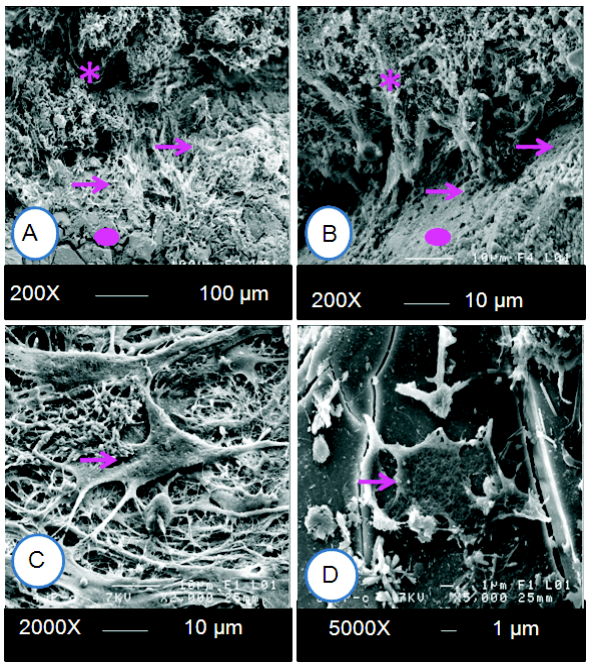


Fig. 5. **SEM image *in vivo* showed increase of osteoblast proliferation** on 0.1% strontium-doped bioglass 15 days post surgery *(A)*, unstimulated osteoblast proliferation with contact of bioglass (BG) *(B)*. A large osteoblast attachment on 0.1% strontium-doped bioglass (BG-Sr) *(C, D)*. Arrows indicate osteoblasts and bioglass osseointegration and * bone. Circles indicate bioglass.

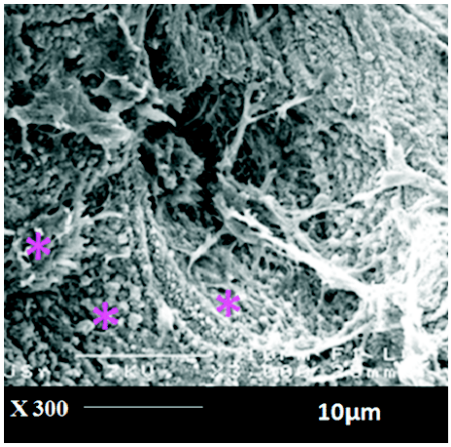


Fig. 6. **Mineralized nodule formation** on 0.1% strontium-doped bioglass (BG-Sr) and development of bioactive calcium phosphate layer over the surface of strontium-doped bioglass (BG-Sr).

imbalance during hormonal deficiency and healing response. To our knowledge, there is no previous report of a comprehensive study of antioxidant defense systems in osteoporotic animals receiving BG-Sr and BG.

In previous work, the link between the toxic effects of Sr-containing bioactive glasses and the release of Sr^{2+} clarify was clarified (Oudadesse et al. 2011). After immersion in simulated body fluid (SBF), the kinetic reaction between BG-Sr and the surrounding synthetic fluids influences the formation and the evolution of the newly-formed layers. However, the intensity of these effects depends on the content of Sr introduced in the glass matrix. Starting from this observation, our *in vitro* study was aimed at clarifying whether BG actually exerted toxic effects

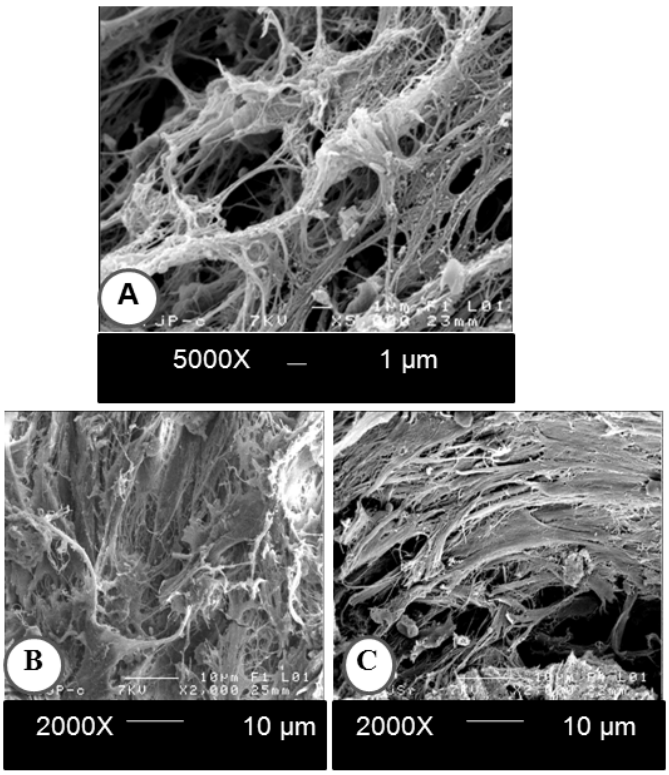


Fig. 7. **SEM image of non implanted osteoporotic bone** *(A)*, **reparative events of bioglass** (BG) **showed the regenerated bone** *(B)*. Regenerated bone surrounding strontium-doped bioglass (BG-Sr) (60 days post surgery) *(C)*.

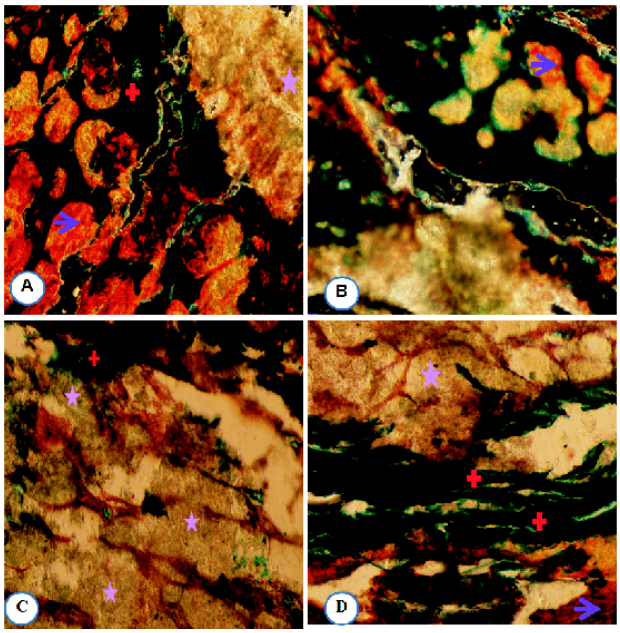


Fig. 8. **Goldner's trichrome staining** (10× objective). Histological sections of the interface between bioglass (BG) and bone *(A, C)*, strontium-doped bioglass (BG-Sr) and bone *(B, D)* showed osteostimulative properties and acceleration of new bone formation surrounded strontium-doped bioglass (BG-Sr). Arrows indicate unmineralized bone (osteoid tissue). * Bioglass/strontium-doped bioglass; $^{+}$ mineralized tissue at the same period (15 days post surgery).

on cells, and whether Sr might play a role in such cytotoxicity.

In this study an SRB assay showed a better enhancing effect on cell proliferation with Sr than when cells were cultured in media with no Sr. The response was optimal after 6 days of contact. The need for several days of incubation was necessary for the dissolution of bioactive glasses products and for the expression of genes regulating the cell cycle (Jell and Stevens 2006). It has a dual role, indirectly promoting the adhesion of osteoblasts to the hydroxyapatite layer (Anselme 2000) and directly stimulating osteogenesis through Runx2 expression in preosteoblastic cells (Zhu et al. 2007) or through the expression of three key genes: Colla 1, Colla 2, and Runx 2.33. The *in vitro* results pointed to the biocompatibility and relative bioactivity of BG-Sr and BG, confirming their ability to support the invasion of cells (osteoblasts). However, after 3 days of incubation, in almost all experiments, a decrease in the cell numbers was recorded, which led to renewal of the conditioned medium after 72 h. The mechanism by which the bioglass modifies cell physiology is not fully understood; the kinetics of release, the pH, the synergy between the different chemical entities, and the role of trace elements all remain hypotheses.

In the *in vivo* study, no adverse effects of BG-Sr, or BG were observed in clinical examinations because of the stability of the haematologic parameter, and the serum levels of Ca and P. In contrast, the high level of alkaline phosphatase activity in OVX-BG-Sr, OVX-BG, amd OVX-NI when compared to the OVX group reflected osteoblastic activity (Isomura et al. 2004). This might be due to a difference of bone turnover, especially bone formation. A high ion intensity of Ca, and P was detected in cancellous bone shown by the high dissolution rate of BG-Sr. As the glass matrix dissolved, various elements dispersed in the glass and combined with elements in the bioactive glass. The results clearly showed that during the dissolution of the bioactive glass the different species of ions were released selectively. Sodium was rapidly exchanged with the surrounding tissues. Si, Ca and Sr were released more slowly; P was released later (Zreiqat et al. 2010). A series of physico-chemical reactions at the material periphery led to the precipitation of a Ca-P rich film on top of a pure Si-layer through diffusive phenomena (Greenspan and Hench 1976). In this study, 0.1% of Sr derived from BG-Sr was taken up in the apatitic environments of the crystals and was rapidly and easily exchanged with Ca. After 30 days of exposure, the uptake of Sr was observed in new cancellous bone tissues and seemed more marked in newly-formed bone BG-Sr than that of BG-bone. This can be explained by higher Sr levels in the bone environment as a result of bioglass matrix degradation. Most of the Sr amount found inside the degraded layer of glass could prove that this element was released slowly into the biological environment, thus stimulating osteogenic processes (Xue et al. 2006, Peng et al. 2009, Tsigkou et al. 2009). Sr was an essential element associated with bone growth and bone activity. With enough time (8 weeks), new bone with higher Sr content was transformed into mature bone.

The increase of Sr/Ca ratio in bone might be explained by the fact that, in newly- formed bone, Sr was not only incorporated into the crystals (heteroionic substitutions), but also taken up onto their surface (adsorption and exchange) (Grynpas et al. 1996). The Ca/P ratio increased progressively but never reached the amount found in the control bone. This might be due to the replacement of Ca by Sr in the newly- formed apatite crystals.

In addition, as reported by (Jallot et al. 2000) the Si layer might be composed with Si (OH) groups which induce heterogeneous nucleation of the apatite. An appreciable amount of silicate ion was, however, released from the bioglass. This was due to the breaking of the Si-O-Si bonds such as dissolution which led to an increase of Si at the glass periphery and possibly to the formation of Si-OH and Si $(OH)_4$ groups at the glass/bone interface. On the other hand, the accumulation of Na or K might increase locally the degree of bone environmental supersaturation. This rapid bone clearance of Na revealed that the glass became low in sodium as the bioactive glass underwent degradation.

By the end of treatment, the low rate of Na^+ could be explained by the fact that the depositions of hydroxyapatite on the bioactive glass surface (Izquierdo-Barba et al. 2008) might well reduce the rate of Na^+ leaching but certainly did not eliminate it. The presence of potassium was due to ion exchange between the glass and the biological fluids (Jones et al. 2007). As explanation of the increase level of Zn contents during bone consolidation, authors (Ovesen et al. 2009) demonstrated that collagen forming cells invaded the blood clot and produced a specialised form of collagen which wrapped itself around the hole.

Slowly, the bone forming cells moved into the collagen tissue called a callus. These cells laid down the calcium, making the bone regain its strength. The enzymes responsible for laying down the bone callus were activated by Zn, and Osteon and Osteoid structures are known to have a high Zn content (Lusvardi et al. 2009). Our study suggested that BG-Sr implantation into the rat cancellous bone could

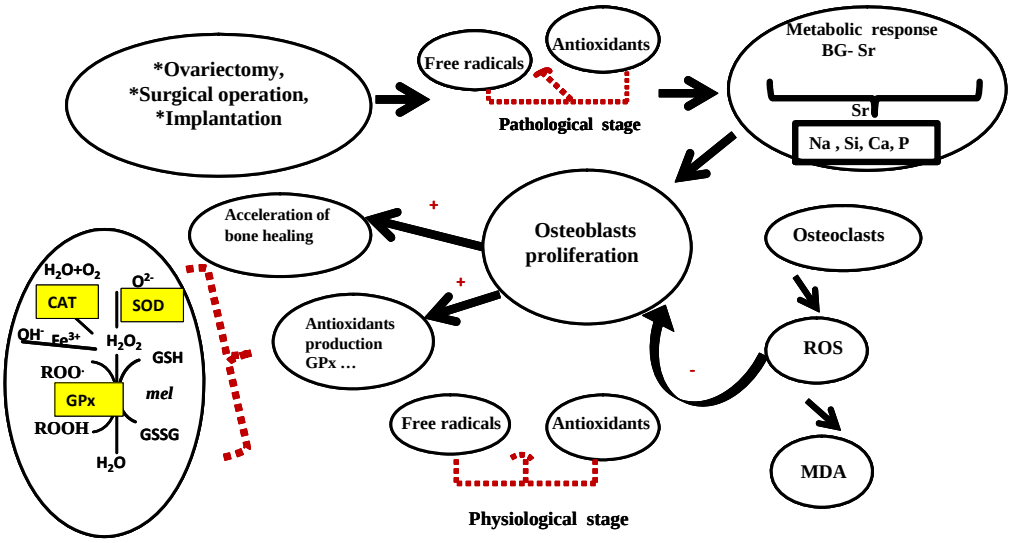


Fig. 9. **Diagram of hypothetical mechanism of strontium-doped bioglass** (BG-Sr) **biomedical material on oxidative stress balance** described in femoral condyle of ovariectomised female Wistar rats.

increase the amount of osteoblasts and osteoid layer, and stimulate new bone formation as well.

Likewise, the present study emphasized the impact of BG-Sr on the antioxidant system in ovariectomised rats. As an effect of ovariectomy, we reported a significant decrease in the antioxidant levels in the femoral condyle of OVX rats, OVX BG-Sr, and OVX-BG. These findings suggest that a deficiency in ovarian hormones fails to combat oxidative stress, and also that during tissue regeneration, endothelial cells, by secreting cytokines and expressing surface adhesion, play an important role in the development of complications in biomaterial implant through free radical-induction.

In fact, interactions at the material-macrophage interface trigger macrophage activation including the production of pro-inflammatory species (cytokines, leukotrienes, etc.). Further GPx was found to suppress osteoclastic differentiation (Lean et al. 2005). Similarly, when GPx failed to eliminate H_2O_2 from the cell. The accumulated H_2O_2 has been shown to cause an inactivation of SOD and a decrease in GPx in OVX animals (Sinet and Garber 1981) supports the potential significance of our findings. The decrease in SOD, CAT activities may result in more accumulation of O^{2-}, which was shown to inhibit other antioxidant enzymes (Kono and Fridovich 1982).

The induction of oxidative stress in the period of 4, 7, 15 days could be explained in two ways. Cells are exposed to two types of stress: i) imbalance between production and neutralization of ROS due to hormonal insufficiency ii) stress due to the post surgery production of pro-inflammatory species. The observation time of a 60-day treatment by bioactive glass limited the increase in the level of MDA and increased antioxidative acivities. These data suggest that the healing response did not lead to failure of the materials.

Regarding previous studies (Anderson et al. 2008), with biocompatible materials, the early resolution of the acute and chronic inflammatory responses occurred, with the chronic inflammatory response usually lasting no longer than two weeks. Besides, the persistence of the acute and/or inflammatory responses beyond a three-week period usually indicates an infection. The excellent tolerability of BG-Sr showed that this chronic inflammatory response was usually of short duration.

These results agree with our findings. The results obtained after 60 days could be explained in three ways, as described in Fig. 9: i) ions released from BG-Sr works by helping resident cells in the bone to restore the normal bone remodelling and enhance metabolic activity in osteoblasts. These cells produce antioxidants such as GPx to protect against ROS (Sadeghi et al. 2008). ii) Sr was reported to be an effective scavenger for oxidative radicals and this antioxidant activity protects from damage and death (Kaviarasan et al. 2008). iii) Sr released following the BG-Sr dissolution might act synergistically with other

ions like silica, calcium, phosphate, and sodium ions that contribute to a balanced oxidative status. That's why BG-Sr was more efficient than BG.

In vitro studies agreed with *in vivo* findings and showed that BG-Sr and BG stimulated osteoprogenitors to differentiate into mature osteoblasts producing bone-like nodules. Similarly, in vivo studies showed the gradual degradation of implanted bioglass with subsequent formation of new bone at the implant surface and especially the stabilization of oxidative status at a level close to that of normal animals, suggesting antioxidative properties of BG-Sr. Thus, this study might be the first report indicating that BG-Sr could improve bone regeneration and oxidative Status stabilization in OVX rats.

In conclusion, it is important to note that BG-Sr stimulated *in vitro* proliferation of SaOS-2, Eahy 926 endothelial cells. *In vivo*, BG-Sr had good bioactivity, bone-bonding ability and oxidative balance stability shown 60 days after surgery. The incorporation of 0.1% of Sr in BG may be an effective strategy for creating materials for bone repair/antioxidation/ regeneration therapies. Although promising results suggest possible clinical use of bioactive bone cement for revision bone replacement procedure, further investigations of long-term outcomes are necessary before this bioactive bone cement can be applied in human orthopedic surgery.

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