BIOCOMPATIBILITY OF ALENDRONATE-LOADED ACRYLIC CEMENT FOR VERTEBROPLASTY

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Abstract

This paper reports a biological evaluation of a non-resorbable acrylic cement loaded with alendronate for the treatment of osteoporotic vertebral compression fractures. The cement formulation was based on polymethyl methacrylate and acrylic monomers; one of these had covalently linked vitamin E residues. The same cement in the absence of alendronate was used as a control. The setting of the charged cement presented a maximum polymerization temperature of 44ºC, a setting time of 24 min, a residual monomer content lower than 3 wt.%, a compressive strength of 99±10 MPa and an elastic modulus of 1.2±0.2 GPa. Cytotoxicity studies using human osteoblast cultures revealed that the leachable substances of the alendronate loaded cement collected between 1 and 7 days decreased cell viability to values lower than 80%. However, morphological changes and cellular damage in cells produced by the extracts decreased with the leak time. Cell adhesion and growth on charged cement was significantly lower than on the control. Implantation of the cement paste in the intra-femoral cavity of rabbits showed that initially the osteogenic activity was evident for the cement charged with alendronate, and the osteosynthesis process took place mainly in the trabeculae and was manifested by the presence of a non-mineralised osseous spicule. The interface between material and adjacent bone tissue was initially characterized by a variable fibrous response that in many cases it appeared reduced to thin connective tissue after a 24-week-period.

Keywords: Injectables, biocompatibility (*in vivo*), cytocompatibility (*in vitro*), vertebroplasty.

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Introduction

Osteoporotic vertebral compression fractures (VCFs) constitute an important public health concern. During the last decade, two new treatment methods for VCFs have gained considerable credibility: percutaneous vertebroplasty (PV) and balloon kyphoplasty (KP) (Liu *et al.*, 2010). Both techniques involve injection of acrylic cement under fluoroscopic control in the fractured vertebra using a percutaneous approach. The most commonly used cement is poly(methyl methacrylate (PMMA) (Boger *et al.*, 2009) and its function is to immobilize the fracture and increase the strength of the vertebra. One of the commercial cements, different from PMMA and recently approved for osteoporotic VCFs, is Cortoss® (Sun *et al.*, 2008). This cement, a glass-ceramic reinforced composite based on the Bowen molecule diluted with triethylene glycol dimethacrylate (TEGDMA) has given good results in the spine area for both pedicle screw augmentation and the treatment of painful compression fractures (Smit *et al.*, 2008). However, as far as we know, none of the latest formulations of injectable acrylic cements for the treatment of osteoporotic vertebral compression fractures (VCFs) contain bisphosphonates. This type of formulation, besides providing adequate reinforcement of the vertebral body, would release locally the drug preventing the subsequent resorption and contributing to a better stabilization of the spine in the long term. The mixing of a bisphosphonate with polymethyl methacrylate bone cement has been reported for other applications, mainly those dedicated to prevent aseptic loosening of orthopaedic implant devices (Simpson, 1996; Yu *et al.*, 2008).

Previously, (Rodriguez-Lorenzo *et al.*, 2007) our research team has developed a non-resorbable self-curing acrylic formulation based on methyl methacrylate (MMA), vitamin E methacrylate (MVE), and TEGDMA charged with alendronate (ALN) to be applied in the treatment of osteoporotic vertebral bodies in minimally invasive surgery. The presence of vitamin E anchored to the matrix would diminish the adverse effects of the free radicals still remaining after the setting of the cement (Mendez *et al.*, 2002) and the bifunctional monomer TEGDMA would produce a crosslinked network improving adhesion to the bone surface (Deb *et al.*, 1997). Alendronate sodium (Fosamax™, Merck, Rahway, NJ, USA) is a member of the nitrogen-containing bisphosphonates that inhibits the resorptive activity of mature osteoclasts and causes

osteoclast apoptosis (Hughes *et al.*, 1995). This drug is one of the currently most frequently used bisphosphonates that is administered orally to treat osteoporosis and osteoporosis-related fractures in postmenopausal women (Liberman *et al.*, 1995; Bone *et al.*, 1997). Women with low bone mineral density (BMD) and pre-existing vertebral fractures who received oral treatment of ALN had a lower incidence of several types of fractures than women who received placebo (Black *et al.*, 1996). In animal models it has also been demonstrated that continuous local infusion of alendronate in a distraction osteogenesis in rabbits increased bone mineral content (Abbaspour *et al.*, 2009).

The present paper reports a biological study of the alendronate charged acrylic formulation described previously. The biocompatibility of the cement and the effects of alendronate released *in vitro* were studied on human osteoblast cultures and the effects produced *in vivo* on bone attachment and new bone formation to cement surface were analysed in rabbits in which a femur defect was filled with the cement paste.

Materials and Methods

Preparation and characterization of bioactive formulations

Acrylic formulations were synthesized by mixing solid and liquid phases using a solid to liquid ratio (s:l) of 1.8:1. The liquid phase consisted of 69 wt.% of MMA (Acros Organics, Thermo Fisher Scientific, Geel, Belgium), 20 wt% of MVE, synthesised as reported previously (Mendez *et al.*, 2002), 10 wt% of TEGDMA (Fluka, Sigma-Aldrich, St. Louis, MO, USA) and 1 wt.% 4,4'-bis-dimethylamino benzydrol (BZN) (Sigma), as an activator of reduced toxicity (De la Torre *et al.*, 2003). The solid phase was composed of 33 μm of average diameter PMMA beads (Industrias Quirúrgicas de Levante, Valencia, Spain), 10 wt% zirconium dioxide (ZrO_2) of 14 μm average diameter (Riba Commercial, S.A.) and 1.5 wt% benzoyl peroxide (BPO) previously purified from fractional re-crystallization from methanol, mp=104ºC (Fluka). The formulation charged with the bisphosphonate ALN (alendronic acid monosodium trihydrated salt) (Merck, Darmstadt, Germany) contained 1.5 wt% ALN in the solid phase. This amount was established according to the doses employed in the literature (Simpson, 1996; Sabokbar *et al.*, 1998). This formulation will be called the experimental (EXP) cement or group. The formulation prepared in the absence of ALN was used as control and will be hereafter called the CTR cement or group. Cements were prepared by hand-mixing solid and liquid phases with a spatula at room temperature to give a fluid paste, which became a solid due to the radical polymerization of the monomeric phase. The exothermic polymerization temperature-time profiles were registered using a type J thermocouple connected to a high sensitivity thermistor (Testoterm, Testo, Sparta, NJ, USA) positioned within its junction in the centre of the mould at a height of 3 mm in the internal cavity. The mould (10 mm in diameter and 15 mm high) was placed in a thermostatic bath at 25ºC. Curing parameters were calculated according to the international standard specification ISO 5833 (ISO5833, 2002). Dough time (t_{dough}) was taken as the time elapsed between the beginning of mixing until the mixture was able to separate cleanly from a gloved finger, maximum temperature (T_{max}), was obtained from the maximum of the exotherm, which corresponds to the maximum temperature attained by the bulk during polymerisation and the setting time ($t_{setting}$) was calculated as the time to reach a temperature midway between ambient and maximum. Compression testing was carried out in a universal testing machine (Instron 4301; Instron, High Wycombe, Bucks, UK) with a crosshead speed of 20 mm/min. Cylinder specimens of 12 mm high and 6 mm in diameter were tested after 1 month of storage in synthetic body fluid prepared according to Kokubo's recipe (Ebisawa *et al.*, 1990). A minimum of five specimens was tested for each cement. Residual monomer was analyzed gravimetrically according to a protocol described for crosslinked resins (Ferracane and Condon, 1990). Rectangular films (10 x 30 mm and 1 mm thickness) were introduced in a mixture of ethanol/water 75:25 v/v (10 mL) and were kept at 37ºC for 1 week. Then, the films were washed and dried at 60ºC until constant weight. The amount of residual monomer (R.M.) was calculated from eqn. (1):

$$\% \text{ R.M.} = 100 \times (W_i - W_f)/W_i \qquad (1)$$

where W_i refers to the initial weight of the sample before analysis, W_f refers to the final weight of the sample after 1 week of immersion in the solution, dried in an oven until constant weight. Three measurements were carried out for each group and the results were averaged. The exudates were isolated and analyzed by proton NMR spectroscopy with a Varian (Palo Alto, CA, USA) XL300 spectrophotometer. Three samples of each type were dissolved in deuterated chloroform (5 % w/v) using tetramethylsilane as internal standard.

Cell cultures

Specimens and cell culture medium

Cell culture experiments were performed following the recommendations of ISO 10993-5 (ISO10993-5, 1999) using human osteoblasts (hOB) (Pharmakine, Derio (Bizkaia), Spain; DPK-BOBC-H). Thermanox® (TMX) control discs (Nunc, Roskilde, Denmark) were used as negative control. The positive control was Triton X-100 (Sigma-Aldrich). Discs of 10 mm diameter and 1 mm thickness of the cured cements were used for direct and indirect biocompatibility experiments. All specimens were sterilized with ethylene oxide. Cultures were maintained at 37ºC in humidified air with 5% CO_2. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM)/ Nutrient Mixture F-12 Ham with L-glutamine, 15 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and sodium bicarbonate (DMEM-F12) (Sigma) and supplemented with 15% foetal bovine serum (FBS) (Gibco, Invitrogen, Paisley, U.K.), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (complete medium) (Sigma). The culture medium was changed every two days with care to cause little disturbance to culture conditions.

Cytotoxicity of monomers and drug

The MTT (3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay was used to evaluate the cytotoxicity of monomers and drug (Mosmann, 1983). For MVE, a solution of the surfactant Tween 80 in serum-free medium (0.66 wt%) was first prepared in which the monomer was dispersed at a concentration of 2 wt%. Because of their hydrophilic character, TEGDMA and the drug ALN were directly dissolved (2 wt%) in serum free medium. All the solutions were successively diluted with serum-free medium. Separately, cells were seeded at a density of 11 x 10^4 cells mL^{-1} in complete medium in a sterile 96-well culture plate and they were incubated to confluency. After 24 h of incubation the medium was replaced with the corresponding dilution and incubated at 37ºC in humidified air with 5% CO_2 for 24 h. A solution of MTT (Sigma) was prepared in warm phosphate buffered saline (PBS) (Sigma-Aldrich) (0.5 mg mL^{-1}) and the plates were incubated at 37ºC for 4 h. Excess medium and MTT were removed and 100 µL dimethylsulphoxide were added to all wells in order to dissolve the MTT taken up by the cells. This was mixed for 10 min and the absorbance was measured with a Biotek (Winooski, VT, USA) ELX808IU spectrometer using a test wave length of 570 nm and a reference wave length of 630 nm. The relative cell viability (R.C.V.) was calculated from eqn (2):

$$\% \text{ R.C.V.} = 100 \text{ x } (OD_S - OD_B)/ OD_C - OD_B) \quad (2)$$

where OD_S, OD_B and OD_C are the optical density of formazan production for the sample, blank (medium without cells) and the corresponding control, respectively.

Cytotoxicity of cements

The MTT assay was used to evaluate the cytotoxicity of the cements derived from the release of low molecular substances. For that, discs of TMX and cements were first set up in 5 mL of complete medium without FBS and placed on a roller mixer at 37ºC. The medium was removed at different periods of time (1, 2 and 7 days) and replaced with 5 mL of fresh medium. All the extracts were obtained under sterile conditions. Separately, cells were seeded at a density of 11 x 10^4 cells mL^{-1} in complete medium in a sterile 96-well culture plate and were incubated to confluency. After 24 h of incubation the medium was replaced with the corresponding extract and incubated at 37ºC in humidified air with 5% CO_2 for 24 h. The rest of the assay was similar to that explained in the previous subsection.

To observe the morphological changes produced in the cells cultured in the presence of the extracts, cells were seeded at a density of 14 x 10^4 cells mL^{-1} in complete medium and incubated at 37ºC for 24 h. The culture medium was replaced with the corresponding extract and incubated again at 37ºC for 24 h and 96 h. Then, the cells were fixed, using 10% formaldehyde solution, and stained using Harris haematoxylin and eosin. The obtained samples, all of them in duplicated, were observed and photographed in a Nikon Eclipse E600 optical microscope with a Nikon DXM 1200 camera.

The LDH (lactate dehydrogenase) assay was used to evaluate the cytotoxicity of the cements when they were cultured directly with osteoblasts. This test measures cell lysis through the LDH activity that is released from the cytoplasm of the cells cultured on the cements. The LDH released is quantified through a reaction in which a tetrazolium salt is converted into a red formazan product (Legrand *et al.*, 1992). Cells were seeded at a density of 14 x 10^4 cell mL^{-1} over the testing dry specimens placed in 24-well culture plate for 1 and 7 days. Following these periods, the content of the wells was recovered and conserved at -20ºC. After collecting all the samples, 25 µl of the medium were added in each well of a 96-well plate (n=8). Next, 50 µl of the reaction mixture (equal amounts of LDH assay, substrate, cofactor and dye solutions) (Sigma) were also added in each well and the plates were incubated at room temperature in the dark for 30 min, when 10 µL of 1N hydrochloric acid were added in each well to stop the reaction. Finally the absorbance was measured at 490 nm, with a reference wavelength of 630 nm, with a Biotek ELX808IU spectrometer. Cell death was expressed as arbitrary absorbance values, after subtracting the values measured for the blank from those obtained for the samples and control TMX. These values were normalized with respect to the number of viable cells attached on the surface of each formulation to obtain the effective mortality that occurs in each type of cements evaluated.

Cellular adhesion and proliferation

The Alamar Blue (AB) assay was used in these experiments. Alamar Blue dye is based on a redox reaction by the metabolic activity of the cells resulting in a chemical reduction of the surrounding environment (Nociari *et al.*, 1998). Cells were seeded at a density of 14 x 10^4 cell mL^{-1} over the testing dry specimens placed in 24-well culture plate for 24 h. After that, 1 mL of Alamar Blue dye (Serotec, Oxford, UK) (10% Alamar Blue solution in phenol red free culture medium) was added to each specimen. After 4 h of incubation 100 µL (n=4) of culture medium for each test sample was transferred to a 96-well plate, and the absorbance was measured at 570 nm, with a reference wavelength of 630 nm on a Biotek ELX808IU spectrometer. The specimens were washed twice with PBS to remove remnants of the reagent, and 1 mL of culture medium was added to monitor the cells over the materials. This step was done at 3, 7, 14 and 21 days.

Statistical analysis

Analysis of variance (ANOVA) was performed by using Statistica 6.0 software (Statsoft, Tulsa, OK, USA). The statistical analysis of the biological results (MTT, AB and LDH) was done by the application of one-way ANOVA, comparing the results of EXP or CTR groups with TMX separately, and the results of EXP group with those of CTR group. Three different significance levels were considered: $p<0.05$, 0.01 and 0.001.

Animal experiments in rabbits

Experimental model

In vivo experiments were performed following the recommendations of ISO 10993-6 (ISO10993-6, 1994)

using *Oryctolagus cuniculus* female New Zealand rabbits of average weight 3.820 kg (3.450-4.260 kg) and 3 months old at the beginning of the study. The model applied consisted of the intraosseous implantation of the formulation paste in the femur of rabbits. The rabbits were pre-medicated with atropine sulphate (0.3 mg kg^{-1}, intramuscular (IM)) and chlorpromazine (10 mg kg^{-1}, IM). General anaesthesia was given intramuscularly by injection of ketamine hydrochloride (50 mg kg^{-1}, IM) and fentanyl (0.17 mg kg^{-1}, IM). Antibiotic prophylaxis was given intramuscularly by injection of penicillin (240.000 UI) and streptomycin (300 mg). After shaving the skin, the surgical field was cleaned with iodine and a longitudinal incision was made. Two critical size defects were created manually in the femur, one of them in the femoral condyle, epiphysis/methaphysis, and the other one in diaphysis. The defect was 5 mm in diameter and 8 mm depth. The corresponding formulation paste was prepared at the moment of the surgical act and then, injected and cured inside the femur of the animal. The muscle was sewn with a suture of vicryl (3/0) and the skin with interrupted silk. After surgery, the animals were allowed to move freely in their cages without joint immobilization and they were sacrificed by an intravenous injection of Pentotal® (90 mg kg^{-1}, IV). A total of 24 rabbits (48 legs) were used for these experiments. 12 Animals were used for each group, with three of them each being killed at 2, 4, 8 and 24 weeks after operation.

Sample extraction

When the animals were sacrificed at the established periods, the surgical scar was cleaned with iodine and a skin incision was made at the site of the surgical intervention to reach the femoral bone. This bone was disarticulated from the knee to the hip. Once the femur was extracted and the surrounding tissue removed, it was fixed in a saline solution of formaldehyde (10 v/v%).

Histopathological analysis

The femoral condyle was cut longitudinally (Woodworker band saw 9 inches, Lombarte & Aluju, S.L., Sabadell, Spain) and was then embedded in methacrylate resin. This method allows the bone to be processed without decalcification. Five µm sections were obtained with a microtome (Microm-HM 350 S; Thermo Fisher Scientific, Invitrogen). The sections were stained with Von Kossa stain for bone mineral and Goldner's trichrome technique for assessing the bone tissue around the cement and examined with a Nikon (Tokyo, Japan) Eclipse E600 light microscope with a Nikon DXM 1200 camera.

Results

Preparation and characterization of cements

The polymerization process of the liquid phase slowed down in comparison to that of pristine MMA, giving values of setting times around 25 min and maximum temperature around 45ºC for both EXP and CTR formulations. This change in the curing parameters compared to those of PMMA cements, is attributed to the presence of the high molecular weight monomer MVE, as demonstrated previously (Mendez *et al.*, 2002), and it would favour the application of this formulation as injectable system. Polymerization conversion was high for both formulations giving values of residual monomer lower than 3 wt%, that is, they were in the order of those reported for the conventional PMMA cement (Hernandez *et al.*, 2009). The chemical analysis of the residual monomers showed the presence of MMA, MVE and TEGDMA in a MVE:TEGDMA:MMA mole ratio of 1:0.27:0.06. The presence of alendronate in the EXP group did not have any influence on the curing parameters nor residual monomer content with respect to the CTR group. Compressive strengths of the cured cements were nearly 100 MPa and compressive moduli close to 1.3 GPa, that is, they were in the same order of magnitude as those of PMMA cements, indicating that neither the modification of the liquid phase nor the presence of the drug in the solid phase have influence on the compressive behaviour. Properties of these formulations are shown in Table 1.

Table 1. Values of dough time (t_{dough}), setting time ($t_{setting}$), maximum temperature (T_{max}) during the exothermic reaction, residual monomer (R. M.), compressive strength (σ_c) and elastic modulus (E_c) of formulations prepared in this work. Standard deviations are in brackets.

Formulation	t_{dough} (min)	$t_{setting}$ (min)	T_{max} (ºC)	R. M. (%)	σ_c (MPa)	E_c (GPa)
CTR group	9.7 [0.3]	26.0 [1.1]	44.0 [0.1]	2.3 [0.1]	99 [6]	1.3 [0.1]
EXP group	8.0 [0.1]	24.1 [1.1]	44.8 [1.5]	2.0 [0.2]	99 [10]	1.2 [0.2]

In vitro biocompatibility

The cytotoxicity of MVE, TEGDMA and ALN analyzed by the MTT assay using human osteoblasts showed a dose-response curve of R.C.V. for each compound. From the linear portions of these curves, the median inhibitory concentration (IC_{50}) values were determined as the concentration that depressed MTT-formazan production by 50%. Results summarized in Table 2 clearly indicate that the most cytotoxic compound of the three analyzed was the cross linking agent TEGDMA.

Cytotoxicity of cements coming from the release of low molecular residuals was assessed by the MTT test and results showed a significant decrease of the R.C.V. in the presence of the substances leaking from the CTR and EXP cements with respect to TMX ($F_{1,94} = 27.97$, $p<0.001$ for

Table 2. Values of the median inhibitory concentration (IC_{50}) for the monomers vitamin E methacrylate (MVE), triethylene glycol dimethacrylate (TEGDMA) and the drug alendronate (ALN) determined by the MTT assay.

Cell culture	IC_{50} (mM)		
	MVE	TEGDMA	ALN
Human Osteoblasts	14.02±0.87	1.94±0.09	11.61±0.61
Human Fibroblasts*	14.1	1.70	9.9

*Rodríguez-Lorenzo *et al.*, 2007

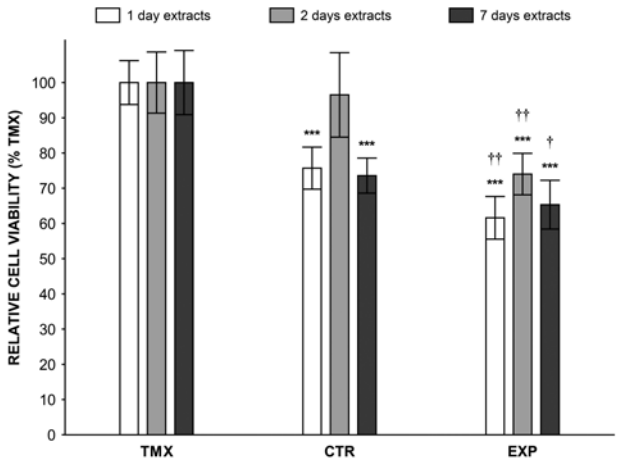

Figure 1. MTT results. ANOVA for CTR and EXP groups was performed with respect to TMX (***: $p<0.001$); and for EXP group with respect to CTR group (†: $p<0.05$, ††: $p<0.01$).

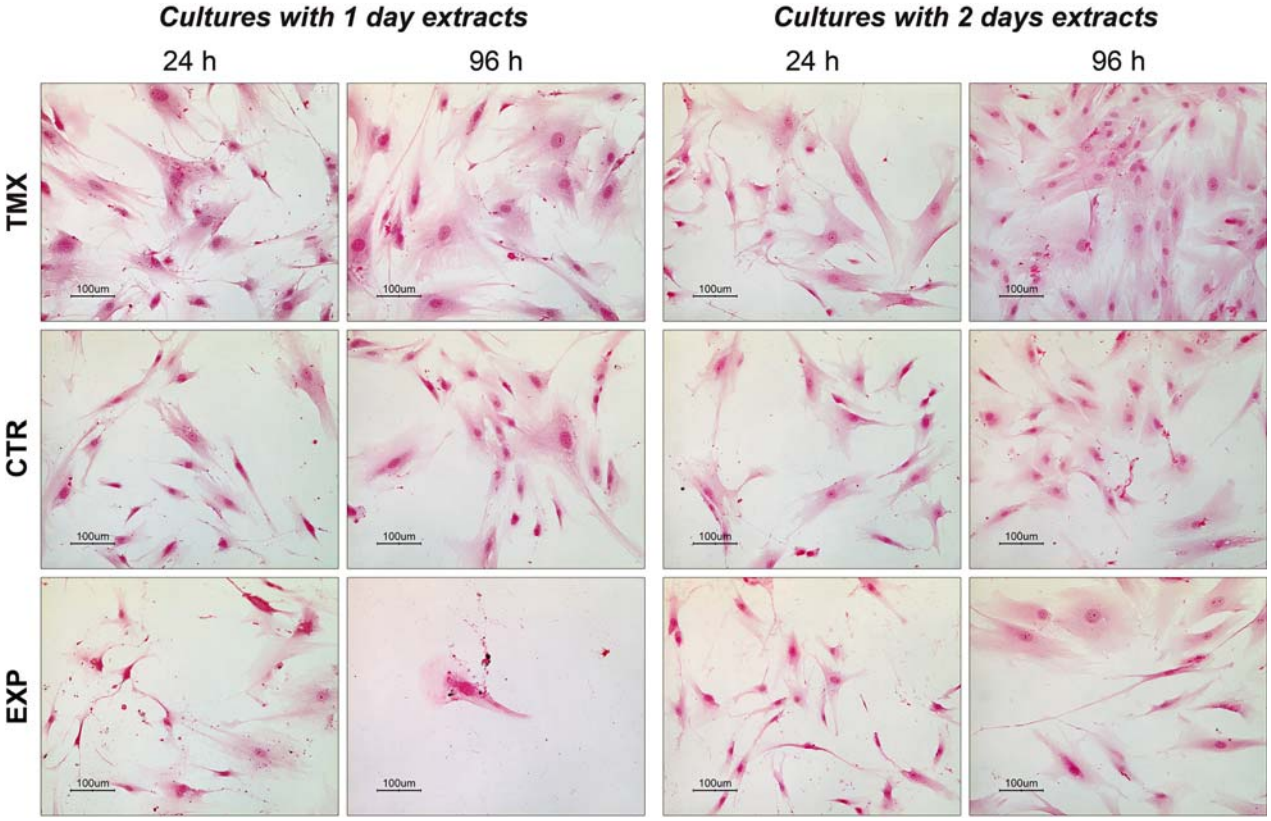

Figure 2. Optical micrographs (200X, haematoxylin and eosin stain) of human osteoblasts cultured for 24 and 96 h in presence of the extracts collected at 1 day (left) and 2 days (right) of TMX and EXP and CTR cements eluted in culture medium.

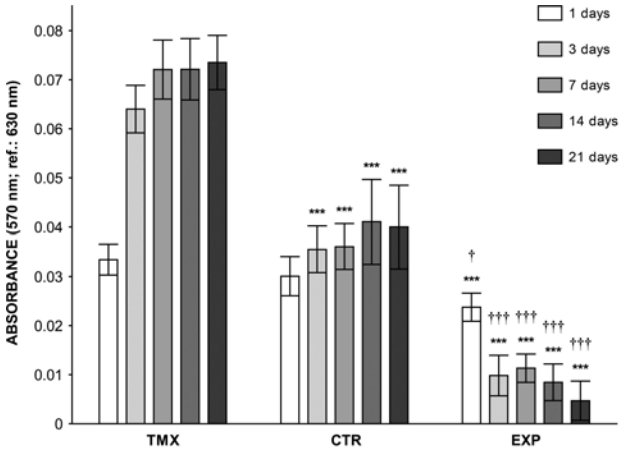

Figure 3. Alamar Blue results of osteoblasts cultures seeded on the surface of TMX and CTR and EXP cements over a period of 21 days. Results are the mean ± 95% confidence interval for the mean (n=16). ANOVA for CTR and EXP groups was performed with respect to TMX (***: $p<0.001$); and for EXP group with respect to CTR group (†: $p<0.05$, †††: $p<0.001$).

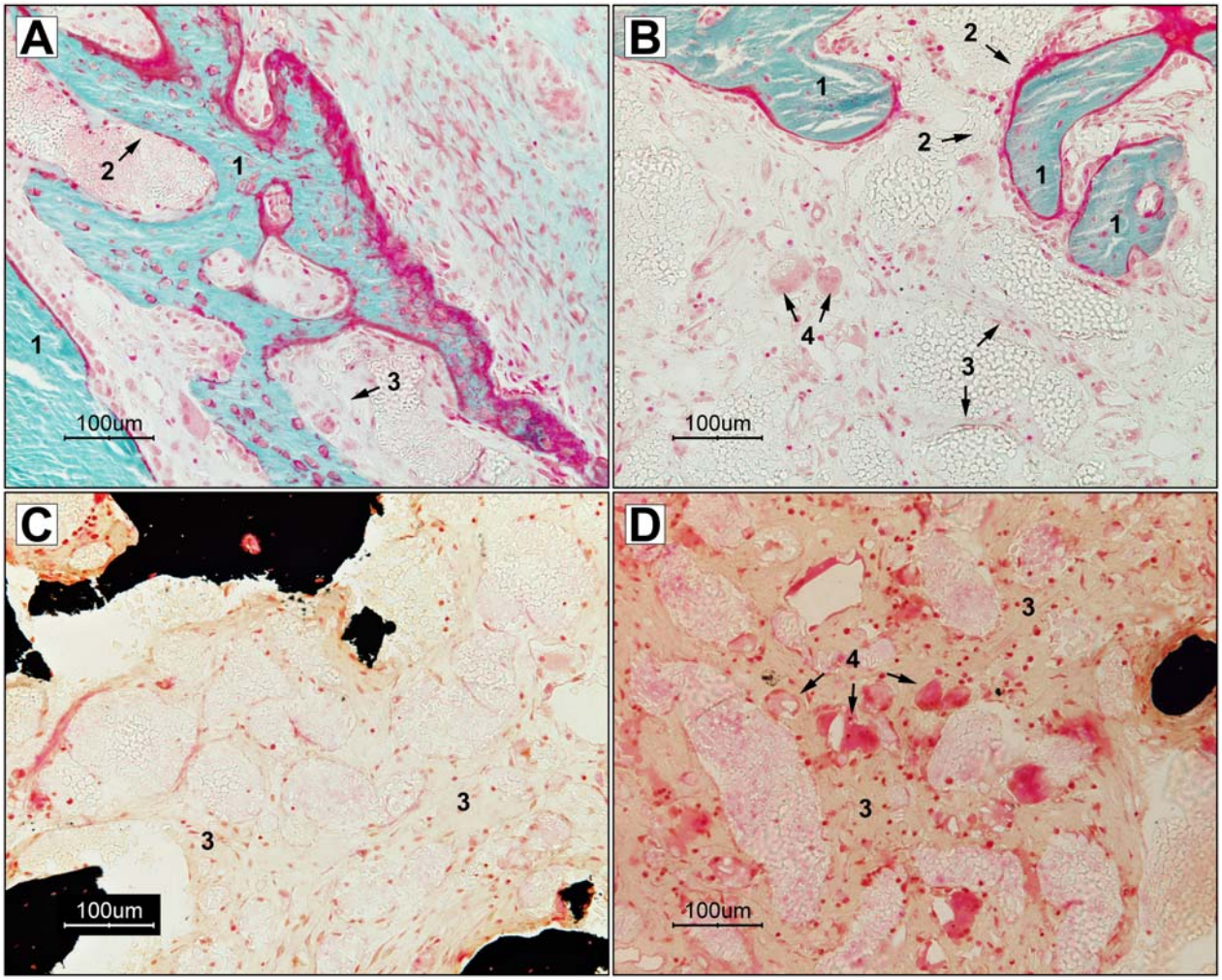

Figure 4. Optical micrographs of CTR cement 2 weeks post-implantation (200X. A and B: Goldner trichromic's stain. C and D: Von Kossa's stain). Numbers in the images indicate, 1: active bone spicules, 2: interface without evident fibrosis, 3: fibrous interface, 4: FBGCs.

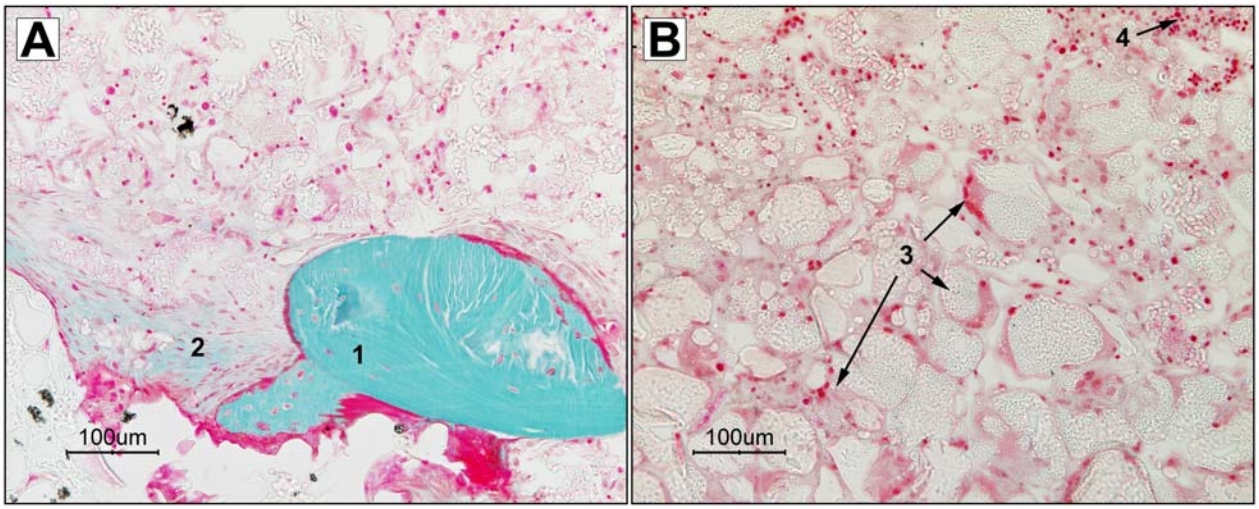

Figure 5. Optical micrographs of CTR cement after 4 weeks of implantation (200X, Goldner trichromic's stain). Numbers in the images indicate, 1: active bone spicule, 2: fibrous interface, 3: foreign body cells, 4: chronic inflammatory cells.

the CTR group and $F_{1,94} = 136.51$, $p<0.001$ for the EXP group), this decrease was higher in the presence of extracts of EXP cement collected at 1 and 2 days ($R.C.V._{CTR} = 81.93\pm5.94\%$; $R.C.V._{EXP} = 66.98\pm3.69\%$) (Fig. 1). Also, significant differences were found in the ANOVAs performed comparing the R. C.V of osteoblasts cultures maintained with the substances leaking from the CTR and EXP groups obtained at 1 ($F_{1,30} = 12.48$, $p<0.01$), 2 ($F_{1,30} = 12.88$, $p<0.01$) and 7 days ($F_{1,30} = 4.26$, $p<0.05$).

The effect of the substances leaking from the cements on the cellular morphology is shown in the optical microscope images (Fig. 2). The substances leaking from

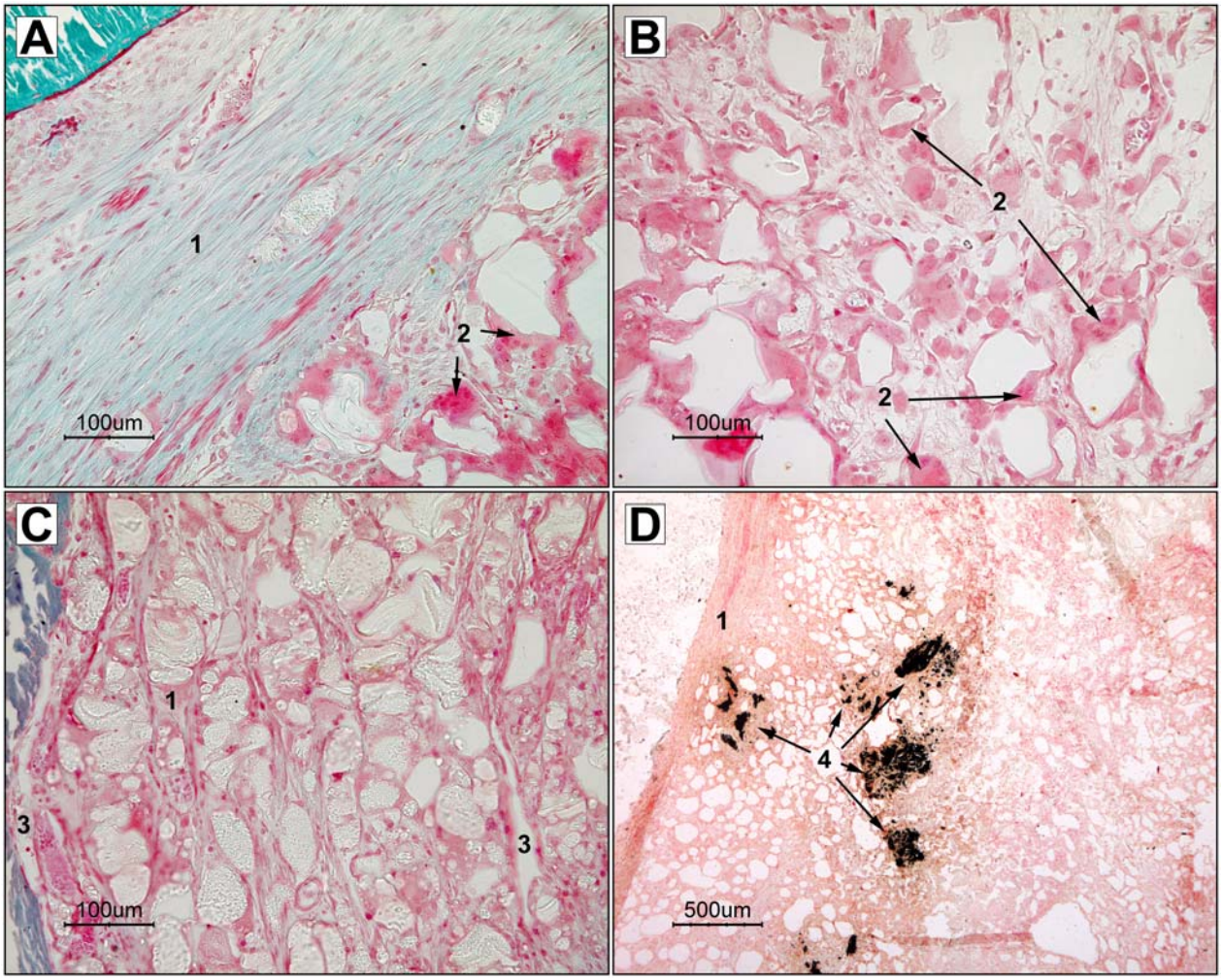

Figure 6. Optical micrographs of CTR cement after 8 weeks of implantation (A, B and C: 200X, Goldner trichromic's stain. D: 40X, Von Kossa's stain). Numbers in the images indicate, 1: fibrous interface, 2: FBGCs, 3: capillaries arranged longitudinally in the direction of bone spicules, 4: calcified deposits.

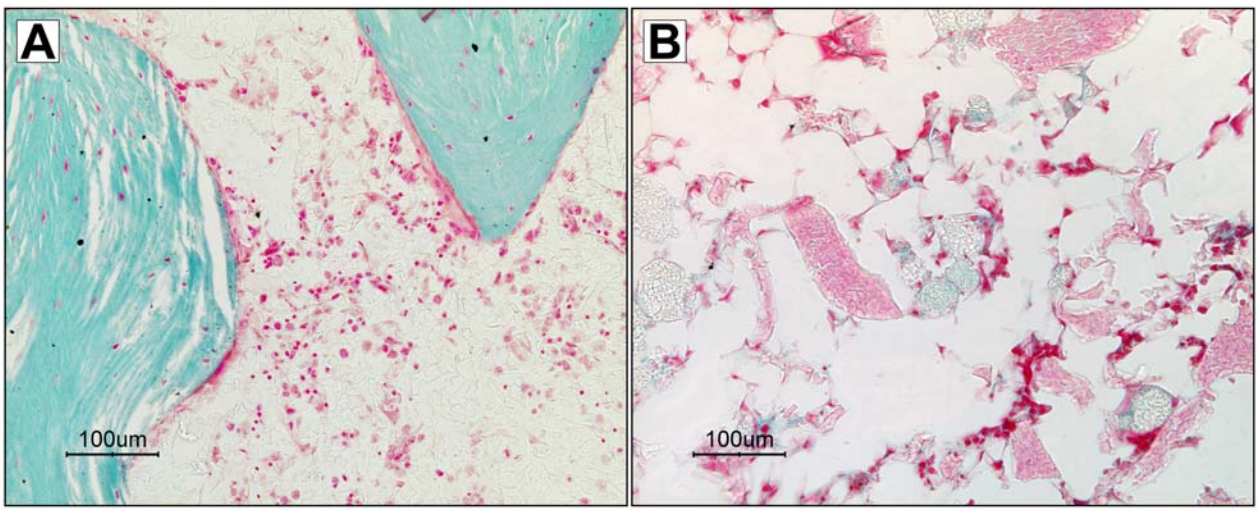

Figure 7. Optical micrographs of CTR cement 24 weeks post-implantation (200X, Goldner trichromic's stain).

the CTR cement collected at 1 day produced differences in the cellular density and cellular size of osteoblasts after 24 h of culture compared with TMX results. In some zones, phagocytosis of cellular remnants was observed, which was indicative of cellular death. However, these phenomena were not present after 96 h of culture showing a density of osteoblasts rather similar to TMX. The leachable of EXP cement at 1 day added to osteoblast cultures, also produced cellular death, signs of cellular atrophy and other cellular pathologies after 24 h of culture and the effects persisted after 96 h. However, when osteoblasts were cultured with the substances leaking from

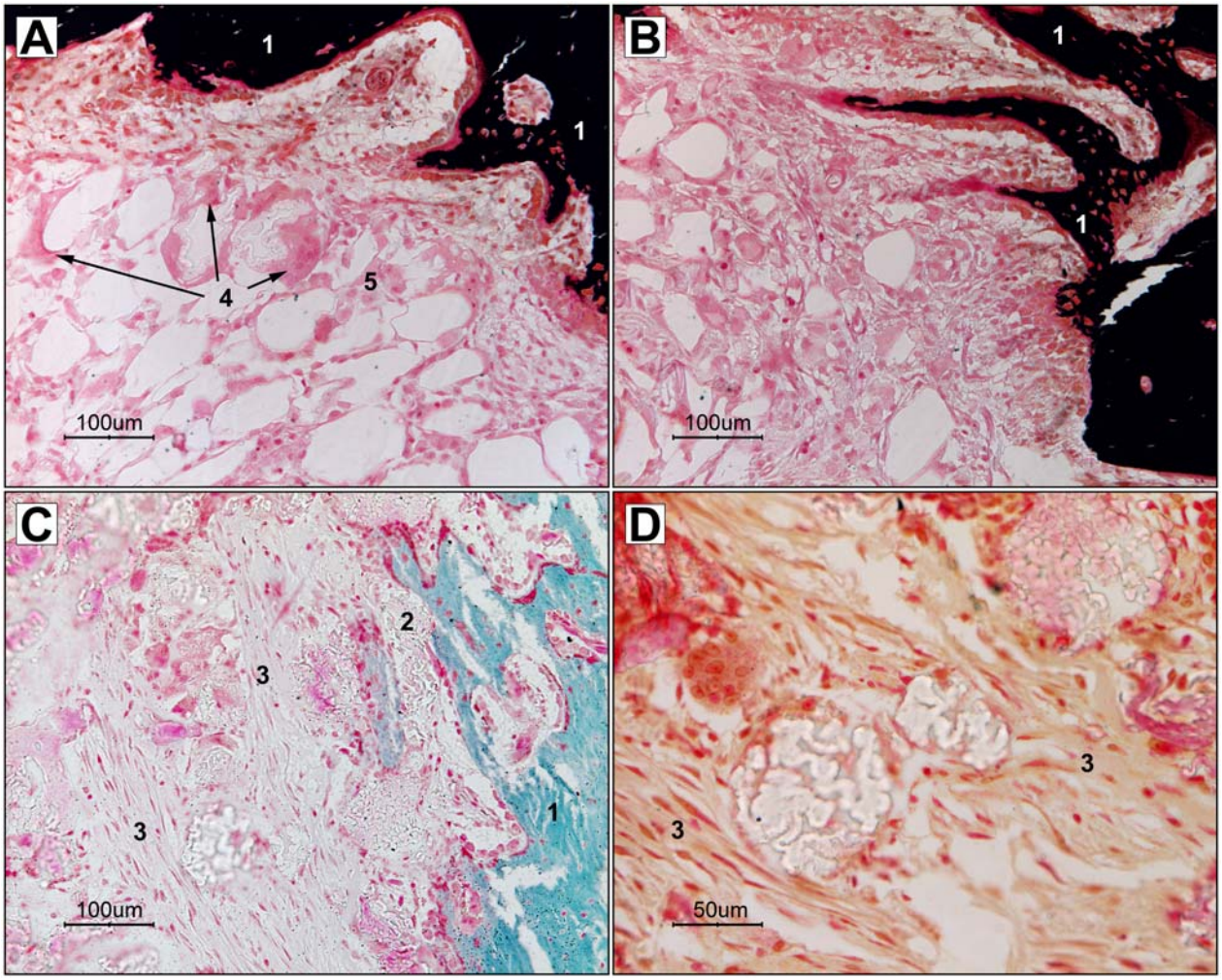

Figure 8. Optical micrographs of EXP cement after 2 weeks of implantation (A and B: 200X, Von Kossa's stain. C: 200X, Goldner trichromic's stain. D: 400X, Von Kossa's stain). Numbers in the images indicate, 1: active bone spicules, 2: interface without evident fibrosis, 3: fibrous interface, 4: FBGCs.

the CTR or EXP cements collected at 2 days, cellular population after 24 h was comparable with that of TMX and it increased with time of culture.

Cell death was also evaluated after seeding the materials directly with osteoblasts and quantified with the LDH test. After normalizing the values obtained with respect to the number of viable cells attached on the surface of each formulation after 1 and 7 days of incubation, significantly lower values of effective mortality were obtained compared to those of TMX for both CTR cement (after 1 day: $F_{1,30}$ = 198.72, p<0.001; after 7 days: $F_{1,30}$ = 1017.44, p<0.001) and EXP cement (after 1 day: $F_{1,30}$ = 289.92, p<0.001; after 7 days: $F_{1,30}$ = 880.78, p<0.001). In addition, significantly higher values of effective death in cultures of EXP cement were obtained at 1 day ($F_{1,30}$ = 20.72, p<0.001) and 7 days ($F_{1,30}$ = 11.65, p<0.01) compared with those of CTR cement.

Cellular adhesion and proliferation on the cements was evaluated through the Alamar Blue test (Fig. 3). Cement without drug (CTR group) showed a similar pattern in cell growth as that of TMX, with no significant differences at 1 day, indicating that osteoblasts were able to adhere on this cement in the same way as on TMX discs. However, between 3 and 21 days proliferation on the CTR group was significantly inferior with respect to TMX. For cement containing the bisphosphonate an opposite trend was observed. Maximum adherence was reached at 1 day and it drastically decreased afterwards, with a reduction in the range 85-95%. Comparison of results between CTR and EXP groups showed significant differences with a marked reduction on the cellular proliferation on the cement charged with ALN.

In vivo biocompatibility

The *in vivo* effects produced by the implantation of CTR and EXP groups on the osseous tissue were evaluated after direct injection of the cement paste in a critical size defect created in the femoral cavity of rabbits. Both cements persisted at the implantation site throughout the studied period as it corresponds for non-resorbable formulations. The histological response at the bone-cement interface was analyzed in terms of inflammatory response, formation of fibrous membrane, and new bone formation.

The histopathological analysis of CTR cement samples after 2 weeks of implantation is shown in Fig. 4. The result of the biological interaction between tissue and injected cement was variable: whereas in some areas no fibrous interface was seen, in other areas a fibrous response of

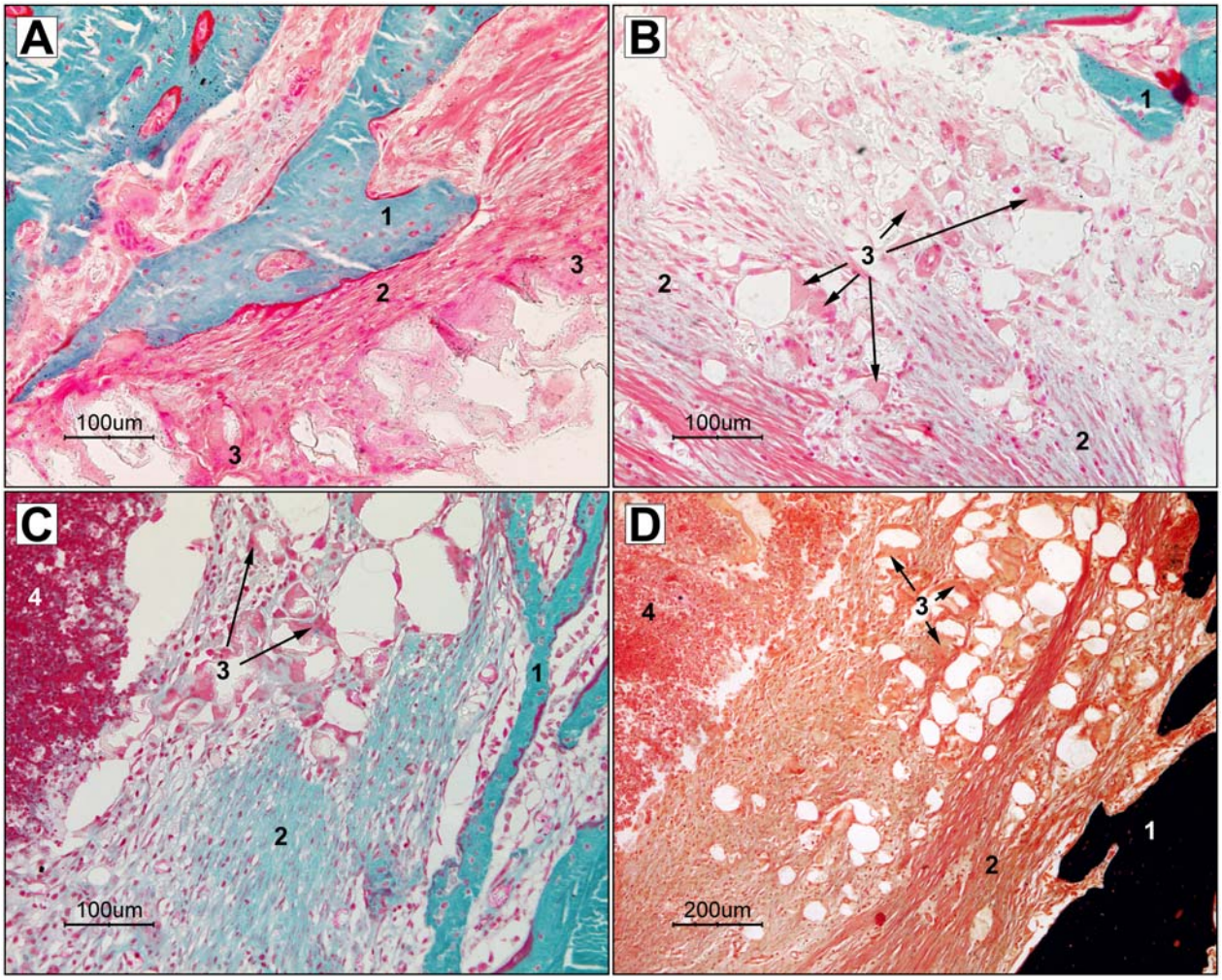

Figure 9. Optical micrographs of EXP cement 4 weeks post-implantation (A, B and C: 200X, Goldner trichromic's stain. D: 100X, Von Kossa's stain). Numbers in the images indicate 1: active bone spicules, 2: fibrous interface, 3: FBGCs, 4: necrotic focus.

highly variable thickness was detected (Fig.4A,B). This response, in which the fibrous tissue encapsulated the injected material, was associated with a foreign body reaction with the presence of typical foreign body giant cells (FBGCs) (Fig. 4B,D). In general, the material appeared dissociated by connective tissue of varying thickness (Fig. 4A-C). In the analyzed samples of this period, lymphocytic round cells were also observed usually associated with the foreign body inflammatory response. In addition, some necrotic areas were observed and, occasionally, small dystrophic calcification foci. However, active bone spicules were observed at the site of implantation by this time (Fig. 4A,B). Four weeks post implantation, similar observations regarding fibrosis, fragmentation, foreign body reaction and presence of lymphocytes were found (Fig. 5). After 8 weeks, foreign body reaction and fibrosis were maintained (Fig. 6A,B), processes that are associated to the angiogenesis phenomenon (Kanczler and Oreffo, 2008). As can be seen in Fig. 6C, the fibrous tissue contained highly developed capillaries, which were arranged longitudinally in the direction of bone spicules. Moreover, amorphous calcified deposits of different sizes, often with powdery aspect, were seen. These deposits were positive to the Von Kossa technique, consistent with a phenomenon of dystrophic calcification (Fig. 6D). The histopathological examination of the 24 weeks CTR samples was characterized by a moderate foreign body inflammatory response. Bone spicules retained the osteocytes, but the osteogenic components (osteoblasts and osteoclasts) were occasional (Fig. 7A). Likewise, isolated aggregates of material were observed in the bone marrow, which in these areas was hypocellular, with a predominance of adipocytes (Fig. 7B).

With regard to the samples of the cement containing alendronate (EXP group), after 2 weeks of implantation it should be noted first, that the osteogenic activity was generally very evident, irrespective of the relationship between bone tissue and material or the biological response to the implanted material. As shown in Fig. 8A-C, many active bone spicules were observed in the samples of this period. The interface between material and adjacent bone tissue was characterized by a highly variable fibrous response, which in most cases resulted in a fibrous capsule of appreciable thickness (Fig. 8C). In some areas, the fibrous tissue was not so evident, and instead, the foreign body reaction was more outstanding with presence of FBGCs and macrophages (Fig. 8A,B). In other areas, no reaction between the injected material and bone spicules was observed. In the bone marrow, in some areas distant from the bone spicules, foci of FBGCs were also detected,

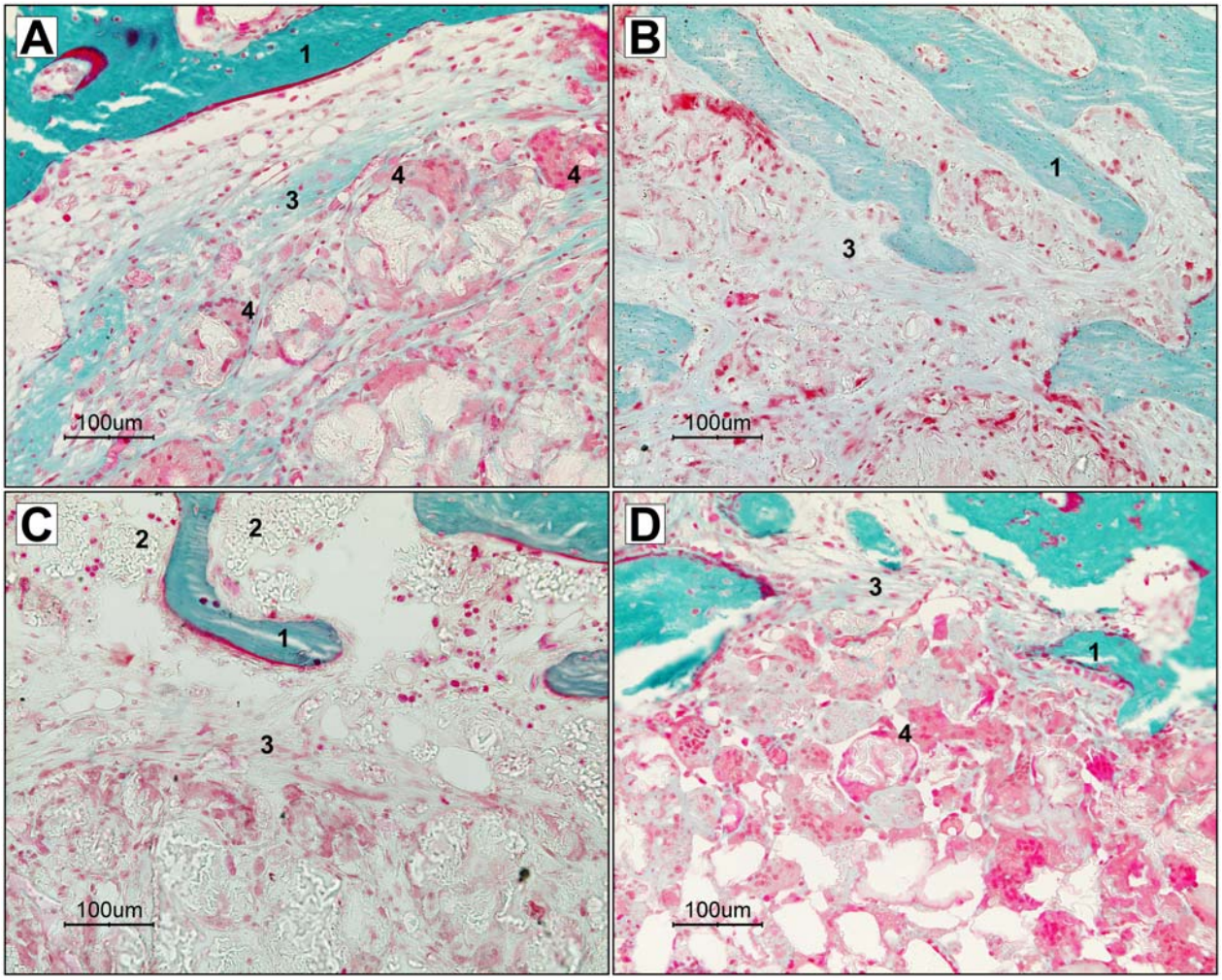

Figure 10. Optical micrographs of EXP cement after 8 weeks of implantation (200X, Goldner trichromic's stain). Numbers in the images indicate, 1: active bone spicules, 2: interface without evident fibrosis, 3: fibrous interface, 4: FBGCs.

along with areas of fibrous tissue that encapsulated fragments of the injected EXP cement (Fig. 8D). After 4 weeks, bone tissue adjacent to the injected cement was characterized by a high osteogenic activity associated with a fibrous and very evident interface in the marrow cavity (Fig. 9A-D). In certain areas, fibrous tissue surrounded aggregates of variable size of fragmentized material. Also, the observations on the samples of this period indicated that the biological response to the material was maintained, as was evident from the presence of multinucleated foreign body cells and macrophages (Fig. 9A-D). Occasionally, small and isolated necrotic foci were observed inside the marrow cavity (Fig. 9C,D). After 8 weeks, the necrotic foci were not detected in EXP samples, and the fibrous response had a less evident cellular component. However, the foreign body reaction was maintained. This response did not appear to interfere with osteogenic phenomena (Fig. 10). Finally, after 24 weeks of implantation, the cement persisted and it frequently appeared surrounded by connective tissue (Fig. 11A-D), although in many cases it appeared reduced to thin connective tissue (Fig. 11A,C). The foreign body reaction phenomena decreased considerably, and the osteogenesis was maintained. Also, normal hematopoietic bone marrow was observed at the site of implantation and consolidated neoformed bone of high density in the proximity of the material was detected (Fig. 11A).

Discussion

A non-resorbable acrylic cement containing vitamin E anchored to the macromolecular chains and charged with alendronate has been proposed for the treatment of osteoporotic VCFs. This formulation has a maximum polymerization temperature of 45ºC and ample setting time to be used as an injectable system (Hernandez *et al*., 2009). The addition of 1.5 wt % of ALN in the form of powder to the solid phase of the cement did not show a significant reduction in the compressive strength as reported by Healy *et al*. for other bisphosphonates (Healey *et al.*, 2003). The main goal of the present work was the biological evaluation of this non-resorbable formulation in order to study its biocompatibility. One of the factors that can compromise biocompatibility of the cement is the possible leaching of unreacted monomers that remain entrapped due to the *in situ* polymerization reaction. In the proposed formulation the monomers MVE, TEGDMA and MMA are able to

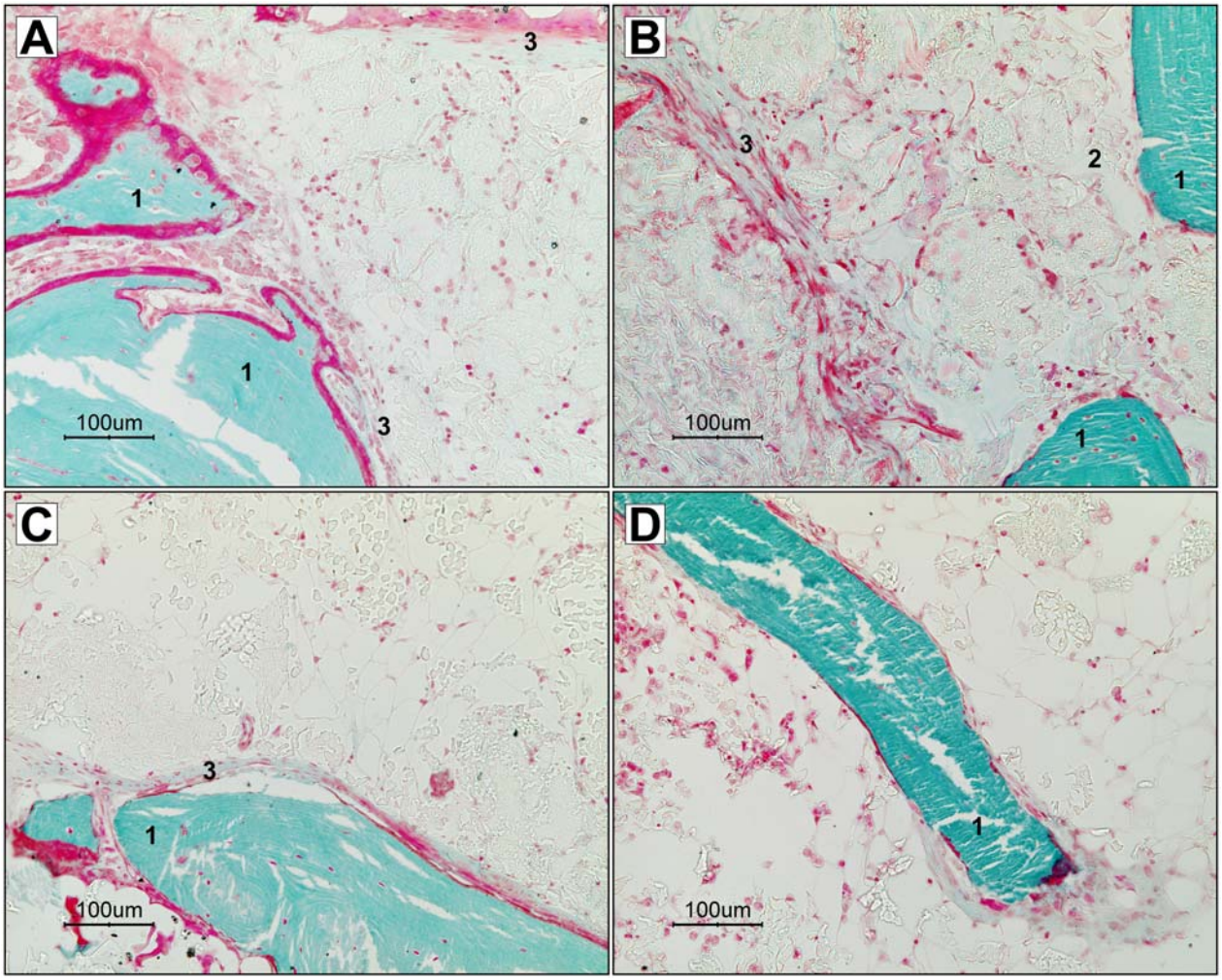

Figure 11. Optical micrographs of EXP cement after 24 weeks of implantation (200X, Goldner trichromic's stain). Numbers in the images indicate, 1: active bone spicules, 2: interface zone without evident fibrosis, 3: thin fibrous interface.

exude to the physiological medium and affect biocompatibility. Cytotoxicity studies of the monomers on osteoblasts indicated that TEGDMA has a median inhibitory concentration (IC_{50}) one order of magnitude lower than MVE. IC_{50} of TEGMA obtained in the present paper approaches values reported for the HeLa S3 cell line (IC_{50}=1.50 mM). The cytotoxicity of MMA has been widely reported in the literature, and it was not studied in this paper. An IC_{50} value of 89.89 mM against the HeLa S3 cell line was reported by Yoshii (Yoshii, 1997); that is, this monomer is less toxic than the other monomeric species of the present formulations and besides, it is present in a lower concentration. For the EXP group we must consider the drug ALN, of which the release in PBS has been shown in previous studies (Rodriguez-Lorenzo *et al.*, 2007). In this study, alendronate in solution was found to decrease osteoblast cell viability at concentrations greater than 7.69 x 10^{-3} M. Some papers in the literature report that bisphosphonates are not always selective for osteoclasts, and that they can affect osteoblast viability depending on the concentration (Bukowski *et al.*, 2005). Particularly, for alendronate (Im *et al.*, 2004), osteoblast proliferation and maturation are promoted at concentrations of 10^{-8} M, but inhibition of cellular growth is obtained at concentrations in the order of 10^{-4} M (Long *et al.*, 2009). However, other studies disagreed and reported that alendronate had no effects on osteoblast differentiation using a similar interval concentration (Itoh *et al.*, 2003). The findings of the present study support the statement of Long *et al.* (2009).

The cytotoxicity of the CTR and EXP cements derived from the residuals was manifested when their leachables collected at different times were added to osteoblast cultures. A decrease in the cellular viability was observed, giving values equal or lower than 80% in presence of the lixiviates. Also, cellular damage was confirmed by optical microscopy, mainly for the lixiviates of 1 day after 24 h of culture, which persisted after 96 h of culture for the EXP cement. However, cellular damage disappeared with increasing time of eluates. Osteoblast adhesion and proliferation on EXP cement was significantly lower compared with the CTR cement, indicating that the effect of the drug can play a role on the cellular recognition of the surface of the cement.

The biological response of the formulation *in vivo* was analyzed considering the evolution of the interaction between the biomaterial and the host tissue. For that we used a surgical model of acrylic bone cement implantation

in the form of paste into the femoral cavity of rabbits to examine the biocompatibility and the effects of the local release of ALN *in situ* at the cement-bone interface and surroundings. The histological response was examined at different time periods up to 24 weeks, time considered sufficient to reach the steady state.

As far as the cement is concerned, no resorption was observed with time of implantation, due to the fact that the acrylic formulation was not biodegradable. In some areas fragmentation of the cement produced some extra soft tissue reaction.

At the beginning of the experiment some necrosis was observed in both cements without appreciable differences among the two formulations. Necrosis phenomena are reported after implantation of self-polymerizing acrylic bone cement in the dough stage (De la Torre *et al*., 2003) and the cause of this necrosis is unclear. Necrosis can be produced by thermal or chemical causes as well as by the surgical procedure itself. Some local bone damage is inevitable with any implantation procedure involving the drilling of bone, even at slow speed, and also insertion of a material that cures *in situ* (Revell *et al.*, 1998). In our formulations thermal necrosis is unlikely in view of the low polymerization temperature. In this initial phase, a typical moderate and unspecific inflammatory response was observed for both EXP and CTR cements and it almost disappeared in the final stages of the study. The release of the monomeric species, mainly MVE and TEGDMA in our formulation, in the physiological medium can be one of the factors responsible for this reaction. TEGDMA has been demonstrated to release *in vivo* conditions (Schweikl *et al.*, 2005; Schweikl *et al.*, 2006) and this monomer was found in the oral cavity where it can penetrate in the tissue.

Regarding fibrosis, a fibrous membrane at the bone-tissue interface was observed from the beginning that was becoming thicker with time of implantation for the CTR cement, and stabilised at the end of the experiment.

In the bone marrow, small necrotic foci were observed in both groups in the initial periods, but they disappeared in the final periods. Hematopoietic bone marrow appeared hypocellular in some samples of the CTR cement corresponding to long periods. Also, in the samples of CTR cement of the final periods, amorphous calcium deposits corresponding to post necrotic calcification were observed.

As far as bone neoformation is concerned, the osteogenesis process was carried out mainly in the trabeculae and it was manifested by the presence of a non-mineralised osseous spicule. Osteogenesis was more evident in the response to the implantation of the cement containing alendronate at short periods of time (2 weeks), as shown in some images of Fig. 8, whereas for the CTR cement, the osseous spicules analyzed looked more diffuse. By this time we can consider that the drug ALN has totally been delivered since prior *in vitro* elution kinetics demonstrated that the charged ALN practically eluted from the cement within a one-week period. Thus with respect to osteogenesis, we can say that the *in situ* release of alendronate of EXP group produced a positive osteogenic reaction at initial periods in contrast to the results obtained in osteoblast cultures in which a reduction in the cellular proliferation by this time was quantified. This can be explained by the fact that when the cement is in an *in vivo* environment, excess drug is removed by the circulatory system whereas in a cell culture plate the drug can accumulate reaching toxic levels.

The effects of BPs on bone neoformation have not been completely elucidated (Boivin and Meunier, 2002). Studies on the *in vivo* effects of alendronate administered subcutaneously on the bone formation after autogenous free bone grafting in rats revealed a higher newly woven and lamellar bone formation compared with the control group, which means that alendronate may act as a bone formation stimulator and potentially has a beneficial influence on the bone formation process (Altundal and Gursoy, 2005). Reports on the *in vivo* effects of BPs after its local release are incipient and scarce. Stadelman *et al.* (Stadelmann *et al.*, 2008) reported that zoledronate delivered locally in osteoporotic sheep bone efficiently increases periprosthetic bone density. Studies on acrylic bone cements reported that etidronate (first generation BP) produced effective inhibition of bone resorption (Simpson, 1996). Local release of zolendronic acid added to the liquid phase of *Antibiotic Simplex*® bone cement did not produced any effect over osseous neoformation, what was related with a low concentration (Yu *et al*., 2008). In our case, as mentioned before, previous *in vitro* release experiments demonstrated that ALN released from EXP cement in approximately 1 week, which can be attributed to the higher permeability of the polymeric matrix, giving an ALN concentration in the order of mM; then, our results regarding good osseous neoformation after implantation of this cement in comparison to CTR group, can be ascribed to the *in situ* action of the bisphosphonate.

Conclusions

A non-resorbable acrylic formulation containing vitamin E residues and charged with alendronate has a reduced maximum polymerization temperature (45ºC) and high setting time as to be used as an injectable system. This formulation when implanted *in vivo* produced a positive osteogenic response characterized by the presence of active osseous spicules in the surrounding of the implanted cement. This formulation can be proposed as an alternative to the currently used formulations based in polymethyl methacrylate for the treatment of osteoporotic vertebral compression fractures and further *in vivo* experiments using more specific vertebroplasty models are required. These studies will be performed in the near future.

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Discussion with Reviewer

Reviewer II: You have mentioned that in some areas fragmentation of the cement produced some extra soft tissue reaction – could this reaction affect the outcome of the treatment eventually?

Authors: We have mentioned that in some areas fragmentation of the cement produced some extra soft tissue reaction, however, this reaction was within the limits reported for acrylic bone cements formulations (De la Torre *et al*., 2003; text reference) and it did not compromise biocompatibility. This phenomenon can be related to the setting of the cement. This formulation was designed specifically for application of the cement in vertebroplasty. Cement formulations for vertebroplasty need to be fluid to be injected through biopsy needles of 11 or 13G, and, therefore, have a high value of setting time. In further studies we plan to use a specific vertebroplasty model in which the outcomes will be directly related with the effectiveness of the treatment. The purpose of the present paper was to study the biological evaluation and biocompatibility of the formulation which is the first step to go forward to future research.