Evaluation of effectiveness of 45S5 bioglass doped with niobium for repairing critical-sized bone defect in *in vitro* and *in vivo* models

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Abstract

Here, we investigated the biocompatibility of a bioactive sodium calcium silicate glass containing 2.6 mol% Nb_2O_5 (denoted BGPN2.6) and compare the results with the archetypal 45S5 bioglass. The glass bioactivity was tested using a range of *in vitro* and *in vivo* experiments to assess its suitability for bone regeneration applications. *In vitro* studies consisted of assessing the cytocompatibility of the BGPN2.6 glass with bone-marrow-derived mesenchymal stem cells (BM-MSCs). Systemic biocompatibility was verified by means of the quantification of biochemical markers and histopathology of liver, kidneys, and muscles. The glass genotoxicity was assessed using the micronucleus test. The regeneration of a calvarial defect was assessed using both qualitative and quantitative analysis of 3D microcomputed tomography images. The BGPN2.6 glass was not cytotoxic to BM-derived MSCs. It is systemically biocompatible causing no signs of damage to high metabolic and excretory organs such as the liver and kidneys. No mutagenic potential was observed in the micronucleus test. MicroCT images showed that BGPN2.6 was able to nearly fully regenerate a critical-sized calvarial defect and was far superior to standard 45S5 Bioglass®. Defects filled with BGPN2.6 glass showed over 90% coverage compare to just 66% for 45S5 Bioglass®. For one animal the defect was completely filled in 8 weeks. These results clearly show that Nb-containing bioactive glasses are a safe and effective biomaterial for bone replacement.

Keywords: Bioactive glass; niobium; bone regeneration; *in vivo;* genotoxicity.

Introduction

Bone fractures are the most widespread large-organ traumatic injury that affects humans.[1-2] The regeneration process after fractures normally achieves a successful healing outcome, yet, up to 10-15% of the patients show an impaired healing, delaying the process or even leading to a non-union.[3] Fractures represent not only a burden for the patient's quality of life, but also the costs of surgery and hospitalization constitutes a considerable cost for socio-economic and health care systems.[3] In fact, it has been reported that the treatment of an established non-union of a bone costs over $10,000 on average.[3] The estimated number of fractures for the year 2000 was 9.0 million worldwide, but, as some fractures cause disability for a period longer than 1 year this number was estimated at approximately 50 million.[4] This high number of fractures stimulates the need to develop better synthetic materials for bone replacement.

In 1971 Larry Hench developed the first bioactive material, the Bioglass® 45S5.[5] This material is a glass composed of 46.1 mol. % SiO_2, 24.4 mol.% Na_2O, 26.9 mol.% CaO and 2.6 mol.% P_2O_5.[6-7] Under physiological conditions the glass slowly dissolves releasing calcium and phosphorous ions into solution. These ions then precipitate to form an amorphous calcium phosphorous layer that then crystalizes to form a layer of hydroxyl carbonate apatite ($Ca_5(PO_4)_3OH$) (HCA) on its surface.[8-9] Due to its micro-/nano-scale complexity this HCA layer supports the adsorption of adhesive proteins that will further anchor integrin proteins in osteogenic cells, which will allow the cells to attach, spread, and produce mineralized bone matrix whilst the biomaterial slowly dissolves, until it is completely substituted by the new-formed bone.[5-6, 8, 10] Many variations of the original Bioglass® 45S5 composition have been designed and investigated in the hope of improving the biological properties.[7-8, 11-12]

Incorporating niobium into biomaterials is of significant interest and has been reported to improve their mechanical and biological properties.[12-23] Niobium has been incorporated into metallic alloys for dental implants where it imparts superior corrosion resistance, low cytotoxicity, and

enhanced wear resistance.[21, 24] Furthermore, some investigations reported that sol-gel-derived niobium oxide gels promote apatite formation within a week of immersion in simulated body fluid which indicates that this element is a good candidate for bioactive glass for bone replacement.[25] Niobium has already been incorporated into calcium phosphate invert glasses[15] and in fluorapatite glass-ceramics[26] showing great biocompatibility and also stimulating osteogenic differentiation of human mesenchymal stem cells (MSCs) and maturation of mouse osteoblast-like cells (MC3T3-E1 cell) by means of direct contactor through its ionic dissolution products. Nb doped glasses are also reported into increase vascularization.[27] However, despite its great potential for biomedical applications niobium has rarely been incorporated in silicon-rich bioactive glass such as Bioglass® 45S5. In view of that, we altered that composition of the original Bioglass® 45S5 replacing P_2O_5 (2.6 mol %) with Nb_2O_5 and investigated the biocompatibility, genotoxicity and the potential for osteointegration of this new Nb-containing glass. The biocompatibility of Nb-containing glass was tested using a series of *in vitro* and *in vivo* experiments. The *in vitro* experiments assessed the cytocompatibility of the Nb-doped glass with Normal Human Osteoblasts (NHOsts) by quantitative MTT analysis and qualitative Live/Dead assay. Systemic biocompatibility was verified by means of quantification of biochemical markers of hepatic (TGO, TGP, and GamaGT), renal (creatinine and urea), and cardiac damage (total CK). In addition, histological sections of liver and kidneys were examined for any sign cellular or tissue damage. The glass genotoxic potential was tested using the micronucleus test. The regeneration of a 5 mm sized calvarial defect was examined by means of both qualitative and quantitative analysis of 3D microcomputed tomography images.

Experimental Section

Preparation of bioactive glasses and conditioned cell culture media. We tested a variation of melt-quench derived Bioglass® 45S5, $(SiO_2)_{46.1}(CaO)_{26.9}(Na_2O)_{24.4}(P_2O_5)_{2.6}$. In this variation 2.6 mol% of P_2O_5 were replaced by 2.6 mol% of niobium pentoxide (Nb_2O_5), resulting the composition named

BGPN2.6. The glass was prepared by thoroughly mixing the precursors oxides SiO_2 (Alfa Aesar, 99.5%), $CaCO_3$ (Alfa Aesar, 99.95-100.5%), Na_2CO_3 (Sigma-Aldrich, ≥ 99.5%), P_2O_5 (Sigma-Aldrich, ≥99.5%), and Nb_2O_5 (Sigma-Aldrich, ≥ 99.99%). The batches were melted at 1400ºC for 90 minutes in platinum crucibles. The melt was then poured into graphite mold which had been preheated to 370°C and annealed at 500 °C overnight before being allowed to cool slowly to room temperature. After cooling down the glasses were ground to powder. Particles with different sizes have different surface areas and therefore different ion release rates, therefore the particle size was standardized between 40 and 63 μm using a series of micro sieves. For experiments with the cells, glass-conditioned media was prepared. For this, powders of BGPN2.6 and 45S5 were added to cell culture medium (Osteoblast Growth Medium Bullet Kit, Lonza, Walkersville, MD) at a concentration of 10 mg·mL, mixed for 24 hours, filtered using an ultrafine filter (0.22μm pore size) and left within the cell incubator for 4 hours (time necessary for pH buffering). In this case filtering the media was important not just for sterilization (bacteria and fungus are bigger than 0.22 μm so they cannot pass through the filter) but also to guarantee no glass particle would be in direct contact with the cells (as all particles were larger than 40 μm), thus only the glass extracts were present in the final cell culture media.

Determining pH behavior of culture media containing bioactive glasses prior cell treatment. Cell culture medium has bicarbonate ions in its composition. These ions ultimately interact with CO_2 in the cell incubator in order to maintain the pH of the medium in an ideal level for cells. Glass dissolution affects the pH of the medium, usually increasing it, and pH directly influences cell behaviour. It well known that the body responds to pH alterations much more efficiently than cell culture medium does, so the increase in pH caused by the presence of bioactive glasses may be rapidly buffered.[28] In view of this, we studied the kinetics of media buffering in the cell incubator over time in order to determine how much time would be necessary to neutralize their pH before adding the cells. For this, 500 μL of each of the following media were added to each well of a 24-well plate, in duplicate: (a) Control medium (Osteoblast Growth Medium Bullet Kit (OGM), Lonza, Walkersville, MD), (b) Osteogenic

medium (OGM containing osteogenic supplements), (c) 45S5 (OGM containing 1% w/v of 45S5), (d) BGPN2.6 (OGM containing 1% w/v of BGPN2.6). The pH was measured prior to incubation and after increasing time intervals up to 72 hours using a pH meter (Accumet®, Fisher Scientific).

Cell Culture. Normal Human Osteoblasts (NHOsts, Lonza, Walkersville, MD) were cultured in growth medium (Osteoblast Growth Medium Bullet Kit, Lonza, Walkersville, MD). Cell flasks were kept in incubator at 37ºC in an atmosphere of 5% of CO_2. The culture medium was changed every other day. All procedures using osteoblasts were carried out following the manufacturer's protocol.

MTT assay. MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) is a soluble tetrazole salt that can be reduced to insoluble strongly pigmented purple formazan by the redox potential in active mammalian cells. 5,000 Normal Human Osteoblasts (NHOsts, Lonza, Walkersville, MD) were seeded in each well of 24-well plates containing 500 µL of osteoblast growth medium per well. After 24 hours the growth medium was replaced by the treatment media (conditioned with glass extracts and negative and positive controls). Cell culture media conditioned with 1% w/v of 45S5 and BGPN2.6 were used to treat the cells for 72 hours. A positive control group of NHOsts was treated with osteoblast growth medium (Osteoblast Growth Medium Bullet Kit (OGM), Lonza, Walkersville, MD). A negative control group of cells was killed by incubation in Etoposide (Sigma-Aldrich). To quantify cell viability 10 µL of MTT (Invitrogen®) (stock solution at the concentration of 5mg·mL) were added to each well (containing 100 µL of phenol red-free culture medium) incubating for 4 hours in the cell incubator at 37°C and 5% CO_2. After the incubation time the formed formazan was solubilized with Dimethyl sulfoxide (DMSO) (Invitrogen®) incubating it for 10 minutes. The absorbance of the converted formazan was measured at the wavelength of 570 nm using a microplate spectrophotometer (Thermo Scientific™ Multiskan™ GO). This experiment was performed in duplicate. One-way ANOVA and the post hoc test Tukey were used to compare the means of the groups.

Live/dead assay. For this assay 10,000 NHOsts were seeded per well in a 24-well plate and treated for 72 h with conditioned media containing the dissolution products of 45S5 and BGPN2.6 glasses at a

concentration of 10 mg/mL. Cells treated with osteoblast growth medium (Lonza, Walkersville, MD) were used as a positive control whereas cells killed with Etoposide (Sigma-Aldrich) served as negative control. In this assay live cells are distinguished by ubiquitous intracellular esterase activity, which is determined by enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. Calcein is retained within live cell's cytoplasm, producing an intense green fluorescence (~495nm).[29] Ethidium Homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells.[29] For this assay all procedures were carried out following the instructions of the manufacturer (ThermoFisher-Scientific®). Briefly, calcein-green at 0.5 μM and EthD-1 at 0.5 μM were combined into one solution and used to treat NHOsts cells. 100 μL of staining solution was added per well and left to incubate for 45 min at room temperature (protected from light). Cells were photographed using a fluorescent microscope at 100× magnification. This experiment was performed in duplicate.

Animals. All experimental protocols were in accordance with the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Committee for Ethics in Animal Use of the University of Campinas – CEUA/UNICAMP (**Protocol Number: 3467-1**). We used 72 adult rats (HanUnib: WH (Wistar), ZFV, Hannover, Germany, 1987) weighing between 350 and 460 g provided by the Central Bioterium of UNICAMP (CEMIB). The rats were maintained in the Bioterium of the Department of Anatomy, in the Institute of Biology (IB), UNICAMP. They stayed in standard boxes in controlled environmental conditions (12 hours' bright/dark cycles) with standard food and water. **Table 1** shows the sample size of the experimental groups and subgroups.

Surgical Procedure. Before the surgery, animals were weighed and transferred into individual boxes. A pre-anaesthetic dose of Tramadol (TRAMAL® - RETARD) (5mg·kg) was applied 15 minutes

before the injection of anaesthetics. Animals were anaesthetized by means of an intraperitoneal injection of a mixture of Xilazin (Xilazin - Syntec) (0.3 mg·kg) and ketamine hydrochloride (0.8 mg·kg). A prophylactic dose of 1 mg·kg of Enrofloxacin (Biofloxacin - Biovet) was applied to prevent bacterial contamination. First, an incision was made starting at the nose bridge, and ending at the base of the skull, using a scalpel. The skin, subcutaneous tissue, temporal muscle, and the periosteum were pulled aside for complete exposure of the parietal bone. A 5 mm round full-thickness calvarial defect was then created in the parietal bone using a 5 mm-diameter tissue punch (Richter®). In the control group, the critical sized defect was left empty whereas in the other groups it was filled with the different glass compositions (45S5 or BGPN2.6) in powder form. The periosteum was sutured using a 6-0 Nylon non-absorbent monofilament (ETHILON®) and the skin was sutured using a 4-0 Nylon non-absorbent monofilament (ETHILON®). In the SHAM group the periosteum and skin were sutured without making the bone defect (**Figure 1**). The sample number of each group is depicted on **table 1**.

Systemic Toxicity. The systemic compatibility of the two compositions of bioactive glasses (45S5 and BGPN2.6) was tested by pathological analysis of histological sections of rats' liver and kidneys and also by comparing the blood serum concentration of biochemical markers of renal, hepatic, and muscular damage of the experimental groups with the SHAM group (group without fracture). For this, following the experimental time (14, 28 or 56 postoperative days) rats were euthanized and their blood, liver, and kidneys were collected. The sample size of each group is showed in **table 1**. The specimens of liver and kidneys were fixed with Bouin solution for 24 hours and embedded in paraplast (Sigma-Aldrich®). All paraplast-embedded histological sections were stained using Hematoxilyn and Eosin and histopathologically examined under a light microscope. The quantification of biochemical toxicological markers from kidneys (Creatinine), liver (TGO, TGP) and muscles (Total Creatine Kinase - Total CK) was performed using enzymatic kits (Interkit®).The normality of the data was attested by the kolmogorov smirnov test (KS test) and the results of the different groups were then compared using One-way ANOVA test with Tukey post hoc test.

Micronucleus test. Micronucleus induction is a key characteristic of genotoxic compounds and the analysis of micronuclei formation, resulting from DNA strand breakage (clastogens) or interference with chromosome segregation (aneugens), is an important component of toxicology screening of new biomaterials. For this assay, we collected bone marrow from rat's femur using a disposable syringe containing 1 mL of 0.9% saline at room temperature. The harvested bone marrow was transferred to a sterile tube, homogenized and centrifuged for 10 minutes at 900 rpm and part of its supernatant was discarded. The sediment was re-suspended in the remaining supernatant. One drop of this bone marrow suspension was placed on one extremity of a microscope slide. Using another glass slide a smear was made and left to dry at room temperature. The dry slides were fixed in 100% methanol for 10 minutes and dried at room temperature. Once fixed, the slides were stained with Giemsa diluted in Sorensen buffer (Na_2HPO_4 0,06 M e KH_2PO_4 0,06 M - pH 6,8), at the proportion of 1 mL of Giemsa for 20 mL of buffer solution for 10 minutes, washed with distilled water, and dried at room temperature. Entellan® was used to mount the histological slides for further analysis. For the calculation of the relative number of micronucleus 3000 erythrocytes were counted per animal in five animals per group (45S5, BGPN2.6 and SHAM). The means of micronucleus were compared to the SHAM group using One-way ANOVA test with Tukey post hoc test.

Computed Microtomography of rats' calvaria. Immediately after the euthanasia, the calvarias were dissected and fixed with 10% buffered formaldehyde for 24 hours and kept in 70% ethanol up to the analysis. Prior to the microcomputed tomography scanning specimens were left to dry at room temperature for at least 60 minutes. We scanned 5 calvarias per group. Scanning was performed using SkyScan 1278 with 84.6 µm of pixel size, 53kV, 0.5 mm of Al filter and 0.18° of rotation. For the image reconstruction a correction of 10% of Beam Hardening was applied together with a ring artefact correction of 5 and Gaussian smoothing of zero. The amount of bone formed within the defect was calculated as a percentage of the total volume of a pre-determined volume of interest and normalized by the mean values of the SHAM group (which represented 100%). The amount of bone found in the

control group served as a blank that was subtracted from the other values. The sample size used for this experiment is detailed on **table 1**.

Results

pH behaviour in culture media containing bioactive glasses. It is known that pH is a variable that significantly affects cell behaviour. For the cell culture experiments we decided to remove this variable in order to investigate the precise role of the ionic products derived from glass dissolution. For this, we studied the kinetics of media buffering in the cell incubator over time to determine how long it would take to neutralize media's pH.

We observed that all glass compositions caused an increase in pH which can be attributed to the release of Ca^{2+} and Na^{+} ions from the glass into the cell medium as previously described.[30] After 24 hours mixing the different glass compositions in media at a concentration of 10 mg·mL we measured their pH. The pH reached 10.27 in the medium conditioned with 45S5 and 9.46 in the group BGPN2.6 (**Figure 2**). No glass was added to the control group (growth medium) and osteogenic group (osteogenic medium) and their pHs were 7.77 and 8.22 respectively.

Cell culture medium containing living cells tends to become acidic over time due to normal cell metabolism reactions. Chemical reactions related to HCO_3^- present in the cell medium and the CO_2 present in the cell incubator are responsible for buffering the system maintaining medium's pH around 7.4-8.2 which is suitable for living mammal cells. We observed that it takes 4 hours inside the incubator for the media's pH to reach physiological levels (**Figure 2**). Thus, to exclude pH as a variable all media were left to buffer in cell incubator for 4 hours prior being used to treat cells.

Viability of bone-marrow-derived MSCs. The survival of Normal Human Osteoblasts (NHOsts) within the different media over 72 hours was assessed using MTT and Live/Dead assay (**Figure 3**). It can be seen that none of the glass-conditioned media were cytotoxic to the cells. It proves that the

addition of niobium species did not compromise the cytocompatibility of 45S5 and that niobium can replace phosphorous pentoxide (P_2O_5) without damaging living bone cells.

Systemic Biocompatibility: Biochemical Markers. In all groups the levels of TGO/AST, TGP/ALT were similar to those of the Control and SHAM groups (**Figure 4**, upper row). This result shows that the presence of the implants did not cause any kind of damage to the hepatic cells. Results of the levels of renal (Creatinine) and cardiac (Total CK) biochemical markers support the claim that these materials are biocompatible as none of them showed significant differences between the glass-treated rats and those from the control and SHAM groups (**Figure 4**, bottom row).

Histopathology. Histological sections of liver and kidneys were stained with Haematoxylin and Eosin and were qualitatively analysed. We looked for any sign of tissue damage or cellular disturbance. A thorough examination of the organs confirmed the results for the biochemical markers. After 56 postoperative days no organ showed any noticeable sign of damage (**Figure 5**). In the livers all hepatocytes presented normal morphology as did the hepatic parenchyma, showing no aggregates of connective tissue or the presence of any inflammatory cells which could represent a sign of toxicity (**Figure 5**, upper row, A-C). In the kidneys, their functional units, the glomerulus appeared normal as well as the proximal and distal convoluted tubules (**Figure 5**, bottom row, D-F). Just as in the liver, no sign of inflammation or tissue damage was observed in any group attesting the integrity of the kidneys and that none of the materials caused any harm to these organs.

Mutagenic Potential. The carcinogenic risk of Nb-containing glass was tested by means of the micronucleus test which is a method to assess chromosomal damage in cells exposed to genotoxic agents. We counted 3000 erythrocytes per animal and compared the mean of each group with the SHAM group. The SHAM group exhibited, on average, 1.87 micronucleus per thousand of erythrocytes. No significant difference was observed between the experimental groups and the SHAM group ($P=0.0863$) (**Figure 6**). This result reveals that none of the glass compositions are genetically toxic and suggest they display no mutagenic potential.

MicroCTs of rat calvaria. The regeneration of a 5 mm calvarial defect was assessed by means of microcomputed tomography (microCT). This analysis is a powerful tool for the evaluation of bone tissue because it provides clear visualization of the 3D microarchitecture of the bone. In the present work the qualitative and quantitative microCT analysis of the rat`s calvarias were performed *ex vivo*. MicroCT images showed that the calvarial defect maintained its size even 56 days after surgery in the control group, proving that it was in fact a critical-sized defect. The treatment with 45S5, on average, filled 65.63% of the bone defect whereas BGPN2.6 filled 91.66% of the defect, on average. In one rat BGPN2.6 completely filled the defect (**Figure 7**).

Discussion

We performed both *in vitro* and *in vivo* analysis in order to verify the biological properties of Nb-containing bioactive glass. The glass showed excellent cytocompatibility with normal Human Osteoblasts (NHOsts) as demonstrated in the *in vitro* assay. In the *in vivo* experiments it did not compromise high metabolic and excretory organs such as the liver and kidneys throughout the eight week period tested. Through microcomputed tomography we observed that Nb-containing glass was capable of stimulating the regeneration of a 5-mm calvarial defect in 56 days.

When implanted into the body a biomaterial for bone replacement must be compatible with its surrounding cells in order to enable efficient osteointegration. The biocompatibility of 45S5 Bioglass® dissolution products (Ca, Si, Na and P) has been described in various studies.[5, 6, 8] Elemental analysis using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) revealed that the ionic products of Niobium-containing glass are similar to those from 45S5, apart from trace amounts of Nb, thus, this material is not expected to be toxic to living cells.[23] Previous reports demonstrated the cytocompatibity of other niobium doped biomaterials with different types of cells.[13, 17, 31] Pure niobium discs were shown to be cytocompatible with mesenchymal stem cells derived from human bone marrow (HBMSCs) seeded and grown onto its surface up to 10 day.[13] Cells adhered well to the surface of pure-niobium discs presenting a spread morphology which is an evidence that they were active.[13]

Mesenchymal stem cells were also observed to attach and proliferate well onto niobium-doped fluorapatite glass-ceramics. The dissolution products of Nb-doped glass-ceramic were also shown to stimulate greater osteogenic differentiation of HBMSCs when mixed with osteogenic medium.[17] Pure metalic Nb also proved to be bioinert when implanted into rats for 4 weeks.[31]

In addition to the analysis of the cytocompatibility of the materials, which is a good sign of non-toxicity, it is also important to take into account the fact that the implant will progressively dissolve in the body fluid. Part of its dissolution products and even microscopic particles may get into the blood stream and end up in high metabolic and excretory organs such as liver and kidneys which may be of concern. This is the first study in which organs have been verified to test the systemic behavior of Nb-doped bioactive glass to ensure that no damage was caused by its dissolution products after eight weeks of implantation (**Figure 4** and **Figure 5**). The observed results are in accordance with an investigation in which Swiss male mice were treated with a single dose (1 mL) of 3 % niobium species diluted in phosphate-buffered saline (PBS), intraperitoneally. In this cited study rats hepatocytes showed some signs of degeneration between the third and seventh day after the intraperitoneal application. However, after 12 days all livers appeared to regenerate with cellular mitoses.[32] In our investigation we did not observe any sign of degeneration nor regeneration at any of the studied time points. We believe that in the aforesaid investigation they might have provoked a mild hepatic degeneration due to the direct intraperitoneal injection of a much higher dose of niobium species (~30µg per rat) compared to the present study (~3.4 µg per rat).

The assessment of the genotoxic potential of new biomaterials is imperative. In order to evaluate the genotoxicity of Nb-containing glass we performed micronuclei analysis. This is considered to be one of the gold-standard analyses for the recognition of the mutagenic potential of any treatment. In the present study 3,000 erythrocytes were taken into account per animal and the number of micronuclei found was compared to the SHAM group (group in which no fracture nor treatment was performed). Nb-glass showed no sign of genotoxicity demonstrating that, at least in this

particular dose, niobium is a safe material to be used in biomaterials (**Figure 6**). Other metallic ions have been shown to possess genotoxic effects. Vanadium, one of the components of the titanium alloy (Ti-6Al-4V), a well-recognized primary metallic biomaterial for orthopedic implants, can generate long-term health problems such as peripheral neuropathy, osteomalacia and Alzheimers disease.[33] Furthermore, lanthanum and nickel were shown to cause some degree of DNA damage.[34-35] Therefore, Nb-containing glass appears to be a safe biomaterial that causes no cellular or DNA damage and is therefore a very interesting candidate for use in biomedical devices for bone replacement.

The osteoestimulative capacity of Nb-containing glass was tested by treating critical-sized calvarial defect for up to eight weeks. A critical size defect is defined as "the smallest size tissue defect that will not completely heal over the natural lifetime of an animal".[36] For the rat calvarial defect, 8 mm is generally accepted to be of critical size.[37] Nevertheless, we chose to work with 5 mm size defect because it can be made in one rat's parietal bone without crossing the sagittal suture. The sutures are nearly immovable fibrous joints that connect two or more bones. In the case of the sagittal suture it connects the two parietal bones. It is important to take into account the fact that fibrous joints (such as the sagittal suture) show a very different pattern of regeneration from flat bones (such as parietal bones). In view of this, we believe the occurrence of these two different rates of regeneration at the fracture site might constitute an extra variable that could confuse the interpretation of the results as the bioactive glass may interact with these two tissues in different ways. Furthermore, even though our 5 mm size calvarial defect showed very low regeneration along eight weeks, in order to avoid any influence of such regeneration over the results, the mean value of the control group (empty defect) served as a blank that was subtracted from the means of all other groups during the quantification of bone formation using MicroCT images.

The analysis of MicroCT images showed that treating circular calvarial defect with Nb-doped glass promoted greater bone regeneration over eight postoperative weeks revealing the great osteostimulative capacity of this glass (**Figure 7**). Our study is the first one to report osteostimulative

effects of Nb-containing bioactive glass using critical-sized calvarial defect. Two other studies investigated the osteintegrative properties of other types of Nb-containing biomaterials.[13, 21] Wang at al. implanted Ti–Nb-Zr-Ta-Si alloy into rabbit's femur to assess its mineral apposition rate and bone-implant contact.[21] The authors concluded that Ti-Nb-Zr-Ta-Si alloy showed significantly higher mineral apposition rate compared to CpTi implants after 4 postoperative weeks and showed no difference in bone-implant contact (BIC). They suggested that Ti–Nb–Zr–Ta–Si alloy had favorable biocompatibility and had an effect on the promotion of osteogenesis.[21] Furthermore, Bartolomé and colleagues verified the biological tolerance of new zirconia/Nb biocermets implants in rabbit's tibias. Their results demonstrated the effectiveness of osseointegration after six postoperative months as new bone was observed around the implants at retrieval date.[13] Together these results indicate that the presence of niobium in the bulk of biomaterials seems to improve material's biocompatibility and increase bioactive properties such as osteoconduction and osteostimulation.

It is important to considerate that the calvarial defect serves as a model for intramembranous bone formation and thus may be less applicable to biomaterials or strategies for endochondral bone formation;[37] thus, this result may be applicable mainly for large fractures in flat bones such as those of the skull, ribs, pelvis and some parts of the vertebras.

Conclusion

In summary we conclude that the Nb-containing glass BGPN2.6 is not cytotoxic to Human Osteoblasts. Its biocompatibility was confirmed by the results of the *in vivo* experiments showing that the material does not cause any harm to high metabolic and excretory organs such as liver and kidneys. Moreover, the Nb-containing glass does not show any genetic toxicity, therefore can be used without risk of mutagenicity. These results attest this glass composition is biocompatible and, up to the used concentration, can be implanted into the body without any harm. Microcomputed tomography demonstrated that Nb-containing glasses stimulated the regeneration of a large calvarial defect and the

results showed a 40% increase in defect repair compared to 45S5 bioglass. Taken together these results support the claim that Nb-glass is a safe and efficient biomaterial to be used for bone replacement.

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Author Contributions

J. H. L. and L. P. L. S. produced the bioactive glasses. L. P. L. S conceived and performed the experiments and analyzed the results. J. H. L. and L. P. L. S. wrote the paper. All authors reviewed the paper.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

Data Availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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Captions for Figures

Figure 1. Development of a critical size calvarial defect in rat: (a) an incision was made starting at the nose bridge, and ending at the base of the skull, using a scalpel; (b) skin, subcutaneous tissue, temporal muscle, and the periosteum were pulled aside for complete exposure of the parietal bones; (c) 5 mm round full-thickness calvarial defect was created in the parietal bone with a 5 mm-diameter tissue punch (Richter®); (d) in the control group, the defect was left empty, in the other groups it was filled with different glass compositions (in powder form); (e) The periosteum was sutured using a 6-0 Nylon non-absorbent monofilament (ETHILON®). (f) The skin was sutured using a 4-0 Nylon non-absorbent monofilament (ETHILON®). In the SHAM group the periosteum and skin were sutured immediately after step "(b)", without making the bone defect.

Figure 2. pH variation of different culture media along 8 h of incubation at 37 ºC and 5% CO_2. It takes 4 hours inside the cell incubator for the media's pH to reach physiological levels (7.4 - 8.2).

Figure 3. Live/Dead assay photomicroscopies of normal human osteoblasts (NHOsts) treated with Control medium (A), 45S5 (B), BGPN2.6 (C), and ETOPOSIDE (D) for 72 hours (100x magnification). Living cells appear in bright green whereas the dead cells appear in red. None of the glass conditioned media was cytotoxic to the human osteoblasts (E). Quantification of MTT assay displaying the absorbance as % of the control group and standard deviation of the mean (SEM) (E). One-way ANOVA revealed no significant difference between the groups treated with glass-conditioned media and the control group (ns bars). All groups showed significantly higher viability than the negative control group (ETOPOSIDE) (*).

Figure 4. Blood serum concentration of biochemical markers of hepatic, renal and muscular damage after 56 postoperative days. Data are displayed as mean and SEM. One-way ANOVA and Tukey's post-test were performed to compare the experimental groups (45S5 and BGPN2.6) with the SHAM group. No significant difference was found between treatment groups and SHAM group. This result reveals that none of the glass compositions were toxic to these three organs.

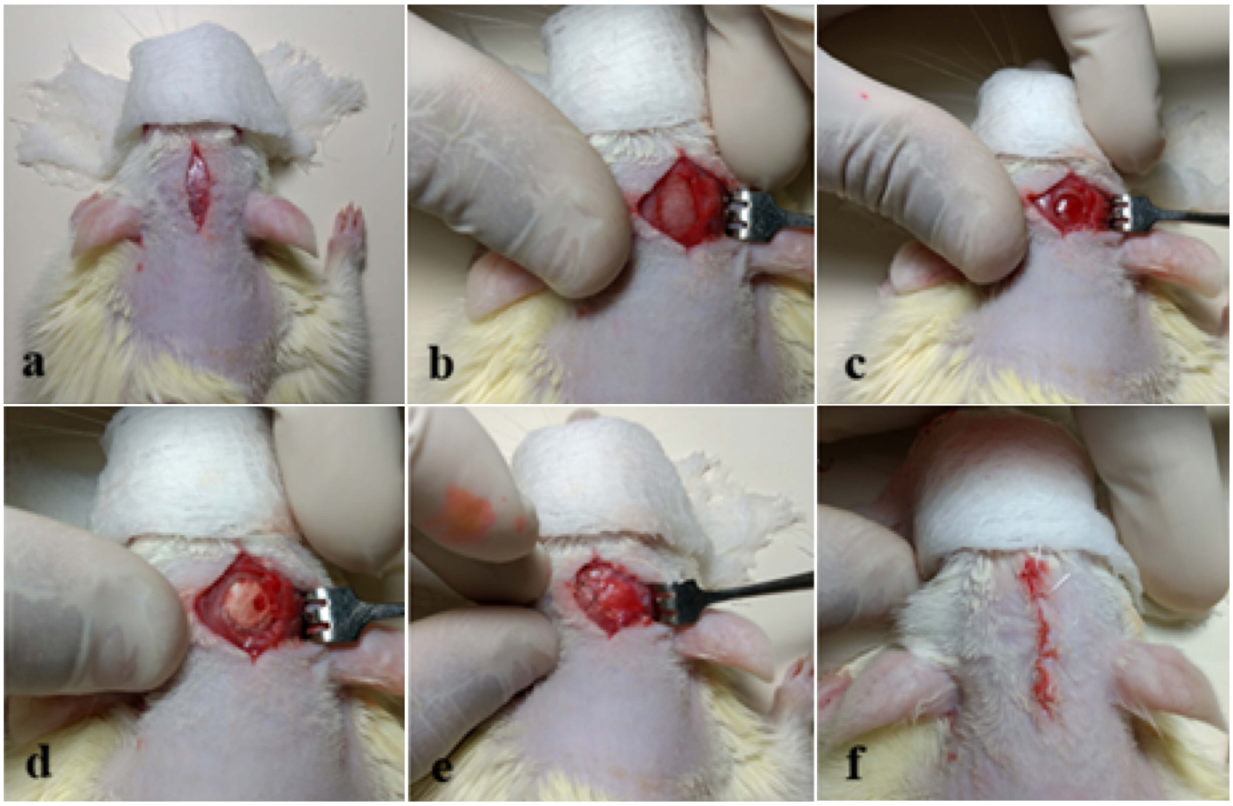
Figure 5. Photomicrographs of histological sections of liver (upper row) and kidneys (lower row) after 56 postoperative days. Control (A and D), 45S5 (B and E), and BGPN2.6 (C and F). Magnification 200x. Haematoxylin and Eosin staining. No sign of damage was observed in any of the analyzed organs. None of the glass compositions were toxic to these organs in the studied experimental time.

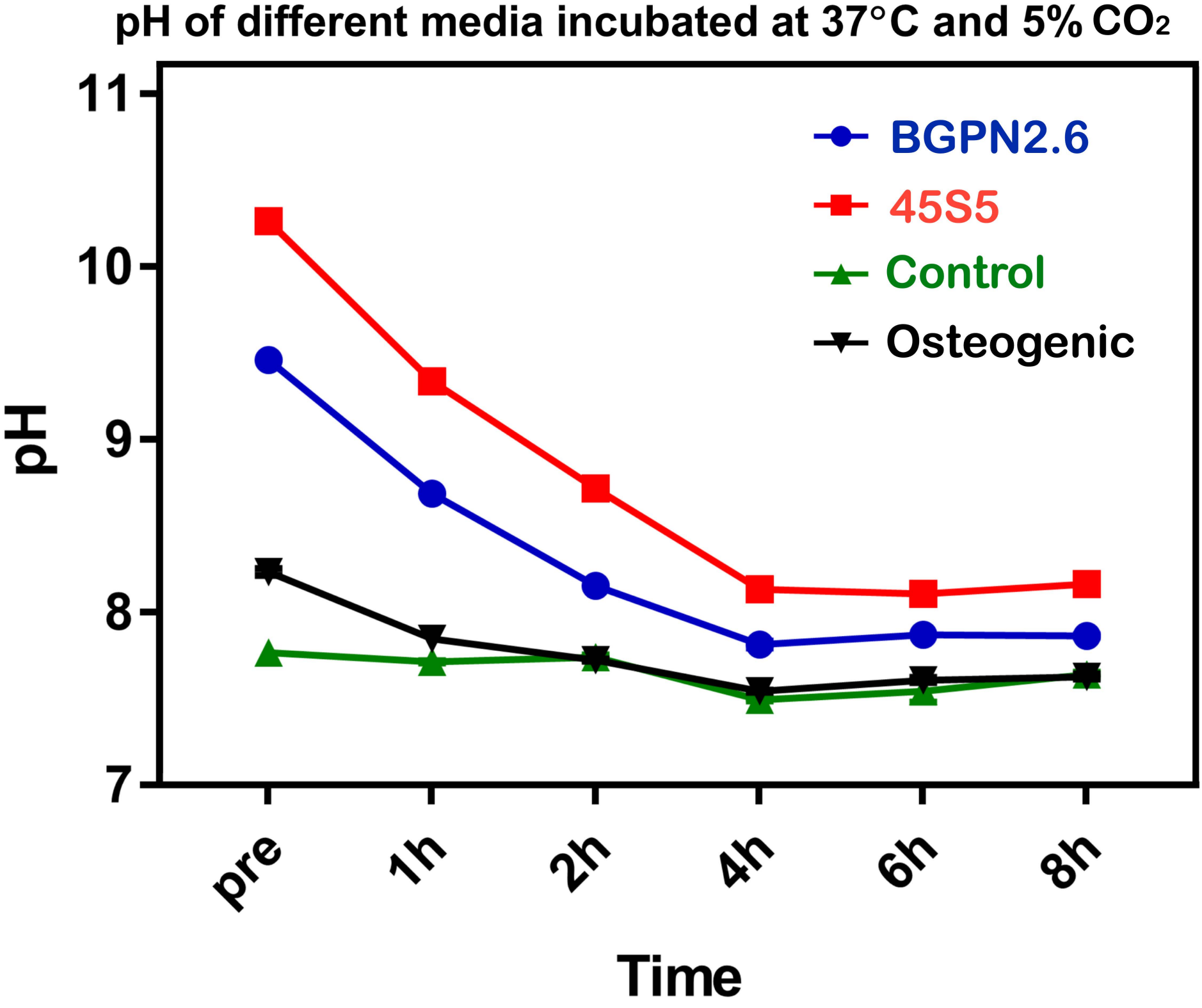
Figure 6. Number of micronuclei per 3000 erythrocytes. Data are displayed as mean and SEM. One-way ANOVA and Tukey's post-test were performed to compare the experimental groups (45S5 and BGPN2.6) with SHAM. No significant difference was found between the experimental groups and the SHAM group ($p=0.0863$). This result shows that none of the glass compositions possesses mutagenic potential. The red arrow points to one micronucleus.

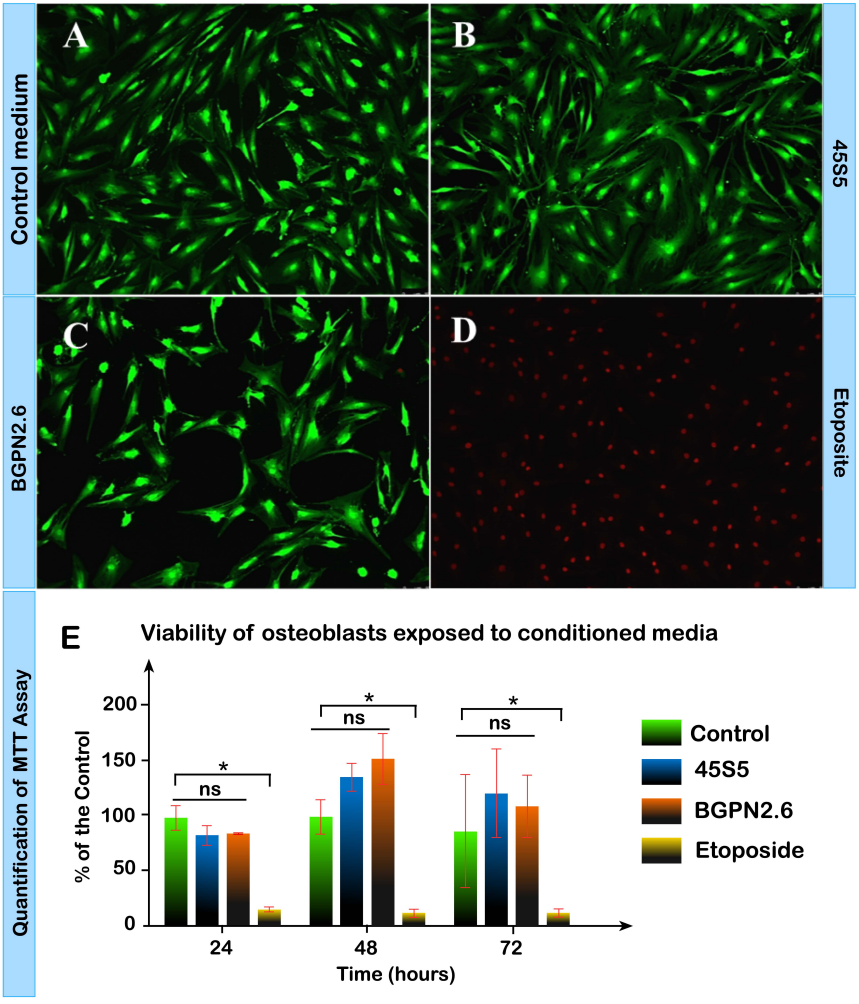
Figure 7. 3D reconstructions of microcomputed tomography images of rats' calvarias from the three experimental groups: Control (A-B), 45S5 (C-D), and BGPN2.6 (E-F). Images taken after 56 postoperative days using SkyScan 1278. Almost no bone formation can be observed in the control group (A) after 56 days which confirms the model of a critical-sized defect. The group treated with 45S5 (C) showed a good amount of bone within the defect yet not enough to completely fill it. The composition BGPN2.6 (C) was the only one that was able to completely fill the 5 mm calvarial defect after 56 days in one of the subjects.

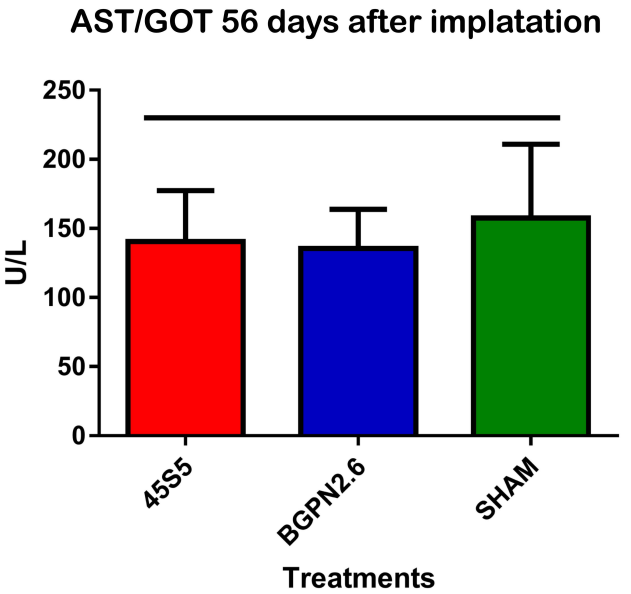
Table 1. Sample division into the different experimental groups

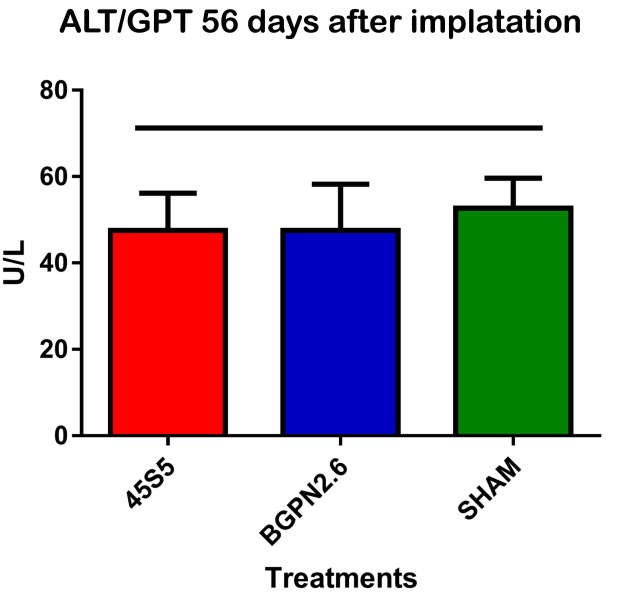
	Number of rats		
Material	14 days	28 days	56 days
Control	6	6	6
Sham	6	6	6
45S5	6	6	6
BGPN2.6	6	6	6
Total:			72 rats

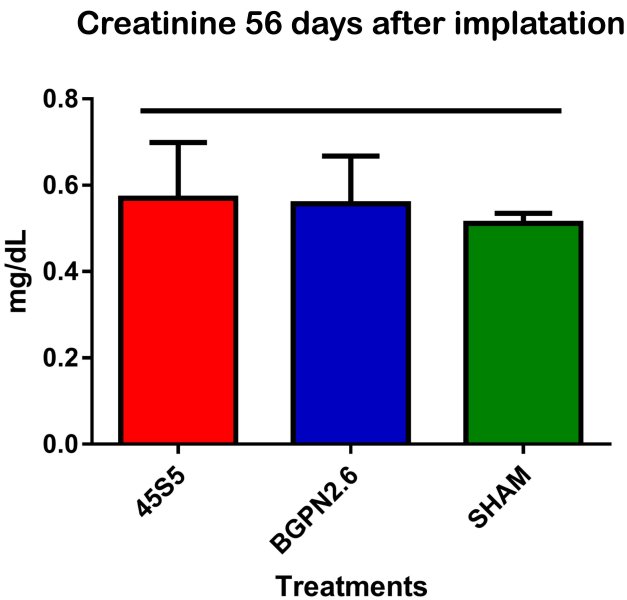

a
b
c
d
e
f

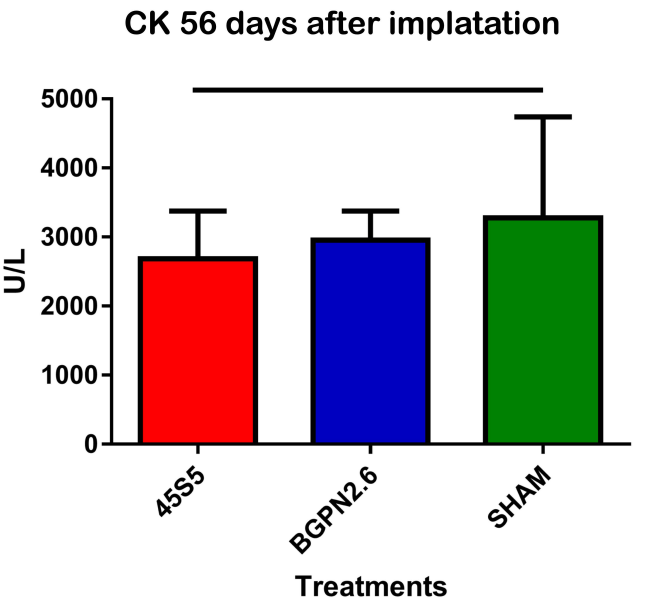

pH of different media incubated at 37°C and 5% CO2
11
10
9
8
7
pH
BGPN2.6
45S5
Control
Osteogenic
pre
1h
2h
4h
6h
8h
Time

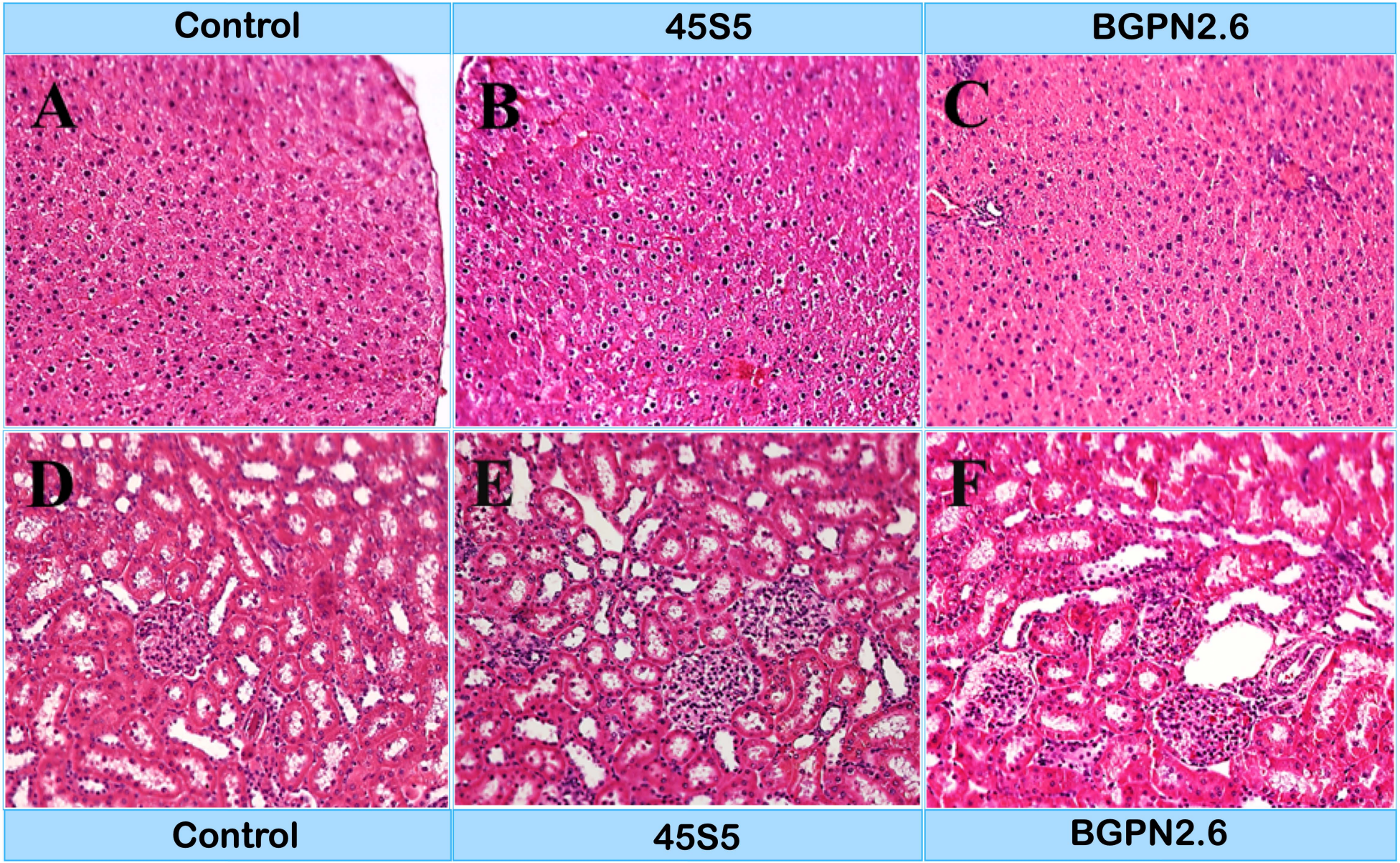

A
B
C
D
Control medium
45S5
BGPN2.6
Etoposite
Quantification of MTT Assay
E
Viability of osteoblasts exposed to conditioned media
% of the Control
200
150
100
50
0
*
ns
*
ns
*
ns
24
48
72
Time (hours)
Control
45S5
BGPN2.6
Etoposide


AST/GOT 56 days after implatation
250
200
150
100
50
0
U/L
45S5
BGPN2.6
SHAM
Treatments

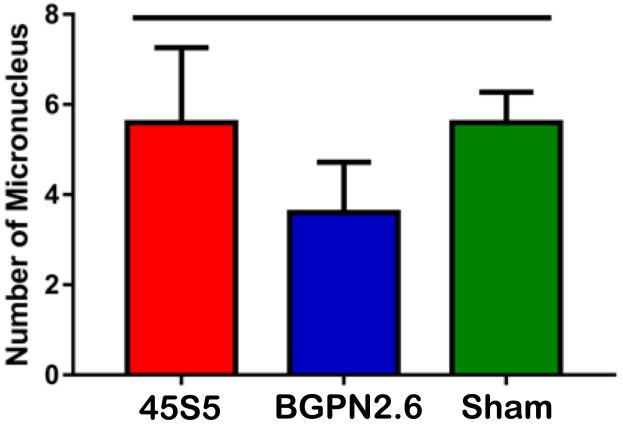

ALT/GPT 56 days after implatation
80
60
40
20
0
U/L
45S5
BGPN2.6
SHAM
Treatments

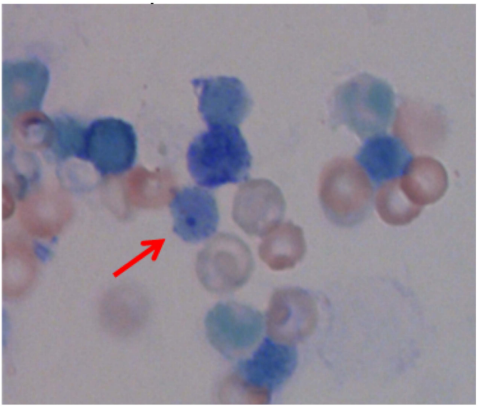

Creatinine 56 days after implatation
0.8
0.6
0.4
0.2
0.0
mg/dL
45S5
BGPN2.6
SHAM
Treatments

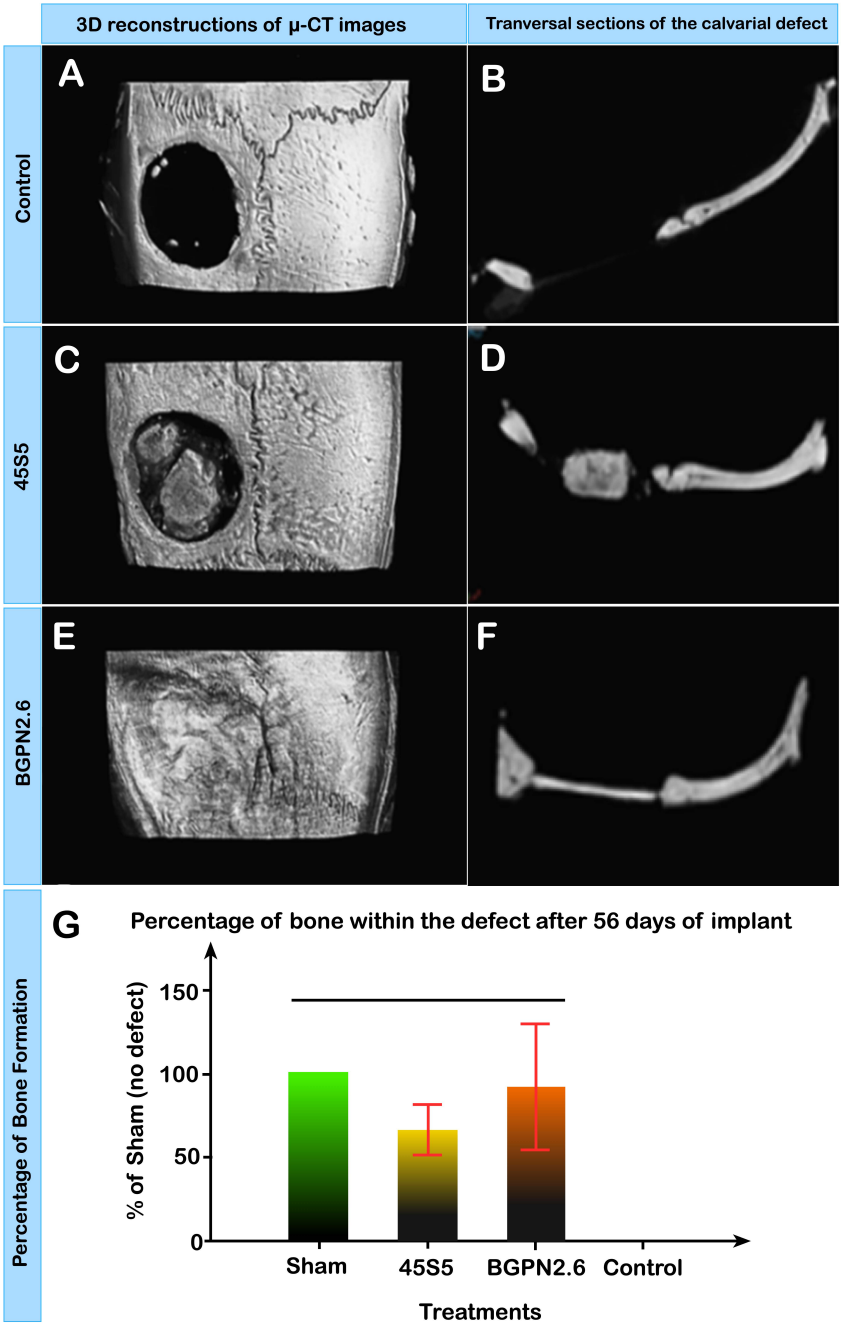

CK 56 days after implatation
5000
4000
3000
2000
1000
0
U/L
45S5
BGPN2.6
SHAM
Treatments


Control
45S5
BGPN2.6
A
B
C
D
E
F
Control
45S5
BGPN2.6

Number of Micronucleus per 3000 eritrocites after 56 days of implantion






3D reconstructions of µ-CT images
Tranversal sections of the calvarial defect
Control
45S5
BGPN2.6
A
B
C
D
E
F
Percentage of Bone Formation
G
Percentage of bone within the defect after 56 days of implant
% of Sham (no defect)
150
100
50
0
Sham
45S5
BGPN2.6
Control
Treatments