BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902018000217438

Article

BONEFILL® block as alternative for bone substitute: a toxicological evaluation

Karine Melchior[1], Sybele Saska[2], Fernanda Coelho[2], Raquel Mantuaneli Scarel-Caminaga[2], Ticiana Sidorenko de Oliveira Capote[2*]

[1]São Paulo State University (UNESP), School of Pharmaceutical Sciences, Araraquara, São Paulo, Brazil, [2]São Paulo State University (UNESP), School of Dentistry, Araraquara, São Paulo, Brazil

Bone substitutes based on hydroxyapatite (HA) and Bonefill® (BO - inorganic bovine bone) associated with poly(lactic-co-glycolic acid) (PLGA) (HA/PLGA and BO/PLGA) were evaluated concerning cytotoxicity, genotoxicity and mutagenicity as potential candidates for bone repair. The materials were developed and provided by Bionnovation Biomedical Products Ltda. Eluates from these bone substitutes were prepared for toxicity evaluations using eukaryotic cell cultures. HA/PLGA was used as a comparison for Bonefill®. Cell viability was evaluated by XTT assay and surviving fraction was calculated for clonogenic survival. Additionally, tail moment was used to assess genotoxicity (comet assay). The frequencies of binucleated cells with micronucleus (FBMN), micronucleus (FMN), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs) were analysed by cytokinesis-block micronucleus assay (CBMN assay). Results showed no statistical difference in cell viability compared with negative control (NC) The eluates did not promote delayed cytotoxicity whereas the surviving fraction rate for cultured cells was similar to NC. Furthermore, no genotoxicity or mutagenicity effects were observed for cultured cells with the Bonefill/PLGA and HA/PLGA eluates. In conclusion, the negative cytotoxicity, genotoxicity and mutagenicity results indicate that these bone substitutes presented interesting preliminary results as potential biomaterials for bone repair.

Keywords: BONEFILL®/toxicity. Biomaterials/evaluation. Bone regeneration. Hydroxyapatite.

INTRODUCTION

Over the years, therapeutic approaches and alloplastic materials have been developed which improve bone repair/regeneration mainly in tissue engineering. However, bone grafts are still used as an alternative to support bone repair in traumatic or non-traumatic injuries (Suchanek, Yoshimura, 1998; Kaveh *et al.*, 2010; Dimitriou *et al.*, 2011). The gold standard for bone reconstruction is autograft, due to the low rejection factor and its osteogenic properties. However, the disadvantages of this graft include additional surgery and limited donor quantities (Costantino *et al.*, 1991; Suchanek, Yoshimura, 1998; Kannana *et al.*, 2014). In addition, allografts and xenografts have been used as alternatives for bone repair, although they may promote rejection, and diseases can be transmitted when not properly chemically processed (Suchanek, Yoshimura, 1998). For these reasons, alternative bone substitutes auto, allo and xenografts to replace auto, allo, and xeno bone grafts (Saska *et al.*, 2015) which contain materials with similar characteristics to bone tissue, such as osteoconductive, osteoinductive and osteogenic properties for decreasing immunogenic and compatibility problems (Saska *et al.*, 2015).

From the materials used for bone repair/regeneration, hydroxyapatite (HA) and derived apatite have shown good biocompatibility, bioactivity and osteocompatibility (Suchanek, Yoshimura, 1998; Shi *et al.*, 2015), as well as not stimulating toxic or foreign body giant cell reactions (Costantino *et al.*, 1991; Kannana *et al.*, 2014). Bioceramics have therefore become an interesting material in bone repair/regeneration, in spite of the weak mechanical

***Correspondence:** T. S. O. Capote. Departamento de Morfologia, Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista, UNESP. Rua Humaitá, 1680. CEP: 14801-903. Araraquara – SP. Brazil. E-mail: ticiana@foar.unesp.br

properties of pure HA ceramics (Suchanek, Yoshimura, 1998). Due to their bioactivity and reabsorption features, they promote direct binding to living tissue and their own slow and gradual degradation which is replaced by the tissues in which they are implanted (Wang *et al.*, 2005). However, metallic, ceramic and polymer materials have been combined with HA to improve its mechanical and biological properties (Suchanek, Yoshimura, 1998; Shi *et al.*, 2015).

Other alloplastic materials, such as synthetic polymers, have been used to improve bone replacement. One of those, poly(lactic-co-glycolic acid) (PLGA) is a biodegradable aliphatic polyester polymer obtained from hydroxyl acids (De Lima *et al.*, 2011); it is used in several biomedical applications, such as tissue and genetic engineering and drug delivery (Cieślik *et al.*, 2009). This polymer has the capacity to modulate the mechanical properties of others compounds and provide versatility in their structure (Saska *et al.*, 2015), and it can be used as supporting or stabilizing elements by creating composites based on them (Cieślik *et al.*, 2009). The association with other compounds provides biocompatibility, bioactivity, satisfactory mechanical properties, and osteoconduction, which makes them potential materials in regenerative medicine therapies (Saska *et al.*, 2015).

Although xenografts may induce some immunogenic and inflammatory reactions depending on the host, bovine grafts have been a good option for bone regeneration in humans. As their inorganic phases are similar to human bone, and if bovine grafts are correctly processed, they are a reliable and high available source (Galia *et al.*, 2008; Galia *et al.*, 2009). Moreover, xenografts have good biocompatibility without unfavourable immunologic responses (Rios *et al.*, 1996; Araújo *et al.*, 2009).

Engineered nanomaterials have become prevalent in our everyday life, raising awareness of nanotoxicology to accelerate our understanding of the ill effects that different nanomaterials can bring to biological systems (Setyawati *et al.*, 2013). In this context, this study aimed to evaluate toxicological effects of alloplastic materials based on hydroxyapatite and PLGA (HA/PLGA) and inorganic bovine bone and PLGA, named Bonefill® (Bionnovation Biomedical Products Ltda, Brazil). We therefore evaluated the cytotoxicity, genotoxicity and mutagenicity of the bone substitutes in block, HA/PLGA and Bonefill®, as the biologic safety of medical devices form part of the risk management and analysis process, and this information also plays an important role in the safe use of the biomaterial.

MATERIALS AND METHODS

Materials

The HA/PLGA and Bonefill® block materials were provided by Bionnovation Biomedical Products Ltda (Brazil), and sterilized by gamma irradiation at dose of 25 kGy.

Cell culture experiments

Chinese hamster ovary cells (CHO-K1) were grown in 1:1 Ham-F10+D-MEM (Sigma®, St. Louis, MO) culture medium supplemented with 10 % (v/v) fetal bovine serum (FBS) (fetal bovine serum-Cultilab, Campinas, Brazil) and kanamycin (1%) (Gibco, Carlsbad, CA) at 37 ºC in a 5% CO_2 atmosphere. Cells were used between the 3rd and 8th passages. CHO cell line has been widely used for studies that assess cytotoxicity and genotoxicity (Yalkinoglu, Schlehofer, Hausen, 1990) and are recommended by The Organization for Economic Cooperation and Development (2004) (OECD, 2014) for genotoxicity screening.

Eluates from each HA/PLGA and Bonefill® (Bonefill/PLGA) block were prepared considering weight (0.2 g mL). The materials were immersed in 1:1 Ham-F10+D-MEM medium (Sigma®) without fetal bovine serum (FBS) at 37 ºC for 72 h (Iso, 2008), shaking at 180 ×*g* in an incubator (New Brunswick Scientific – Excella E24 Incubator Shaker Series).

Cytotoxicity tests

XTT assay. These experiments used CHO-K1 cells, a Cell Proliferation Kit II (Roche Applied Science), and 24 h of seeding. CHO-K1 cells (2×10^4 cells seeded) were treated with Bonefill® or HA/PLGA eluates at 100% concentration for 24 h in 24-well plates. Each well containing the respective eluate was supplemented with 10% FBS. Negative controls (NC) were wells containing culture medium supplemented with 10% FBS without any eluate (untreated controls), while positive controls (PC) were wells containing CHO-K1 cells, treated with doxorubicin (3 $\mu g.mL^{-1}$) for 24 h (all treatments performed in triplicate). After treatment, cultures were washed with PBS and fresh medium was added. Subsequently, cultures were washed with PBS and 500 µL DMEM without phenol red was immediately added, followed by 60 µL of XTT/electron solution (50:1) (Cell Proliferation Kit II – Roche Applied Science). After 3 h reaction, the supernatant was transferred to a 96-well culture plate, and absorbance

measured by a Microplate Reader (VersaMax, Molecular Devices, Sunnyvale,CA) at 492 and 690 nm. Absorbance is directly proportional to the number of metabolically active cells (viable cells) in each treatment after 24 h of exposure. Cell viability was calculated from the absorbance. Three independent experiments were conducted.

Clonogenic assay. Clonogenic assay or colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined as consisting of at least 50 cells (Franken *et al.*, 2006). After 24 h of seeding, CHO-K1 cells (5×10^4 cells seeded) were exposed to Bonefill® or HA/PLGA eluates at 100% concentration for 24 h in 24-well plates. Each well containing the respective eluate was supplemented with 10% FBS. NC were wells containing culture medium supplemented with 10% FBS without any eluate, and PC were wells containing CHO-K1 cells, treated with hydrogen peroxide (80 µmol.L^{-1}) for 10 min. After exposure, the cultures were washed with PBS and fresh medium added. Exponentially growing cells were seeded after treatment at 150 cells per 25 cm^2 flasks in duplicate for each treatment. The flasks were incubated at 37 ºC, 5% CO_2, for 7 days without medium exchange. Colonies were fixed with methanol:acetic acid:water (1:1:8, v/v/v) and stained with 5% Giemsa. The number of colonies counted in the negative control group was considered 100%. From this, survival fraction (FS) calculations were performed: FS = number of colonies counted in each treatment × 100/Number of colonies observed in the negative control group. Three independent experiments were conducted.

Genotoxicity and mutagenicity assays

Comet assay. The alkaline version of the comet assay was used according to a previously described method (Singh *et al.*, 1988). CHO-K1 cells were seeded (5×10^4 cells seeded) and after 24 h exposed to Bonefill® or HA/PLGA eluates at 100% concentration for 24 h in 24-well plates. Each well containing an eluate was supplemented with 10% FBS. NC were wells with culture medium supplemented with 10% FBS without any eluate and PC were wells containing CHO-K1 cells treated with hydrogen peroxide (80 µmol.L^{-1}) for 10 min (all treatments were carried out in duplicate). After exposure, cultures were washed with PBS and harvested with trypsin. Five hundred microliters of cells in suspension were obtained, kept on ice, and protected from light. After centrifugation, the pellet was re-suspended in 200 µL of 0.5% (w/v) low melting point agarose and the mixture spread onto two microscope slides (Knittel, Germany) pre-coated with 1.5% (w/v) normal melting point agarose (Gibco).

Coverslips were placed over the gel. When the gels had solidified, the coverslips were gently removed and the slides immersed in cold (4 ºC) lysis solution (1% Triton X-100, 10% DMSO, 2.5 mmol.L^{-1} NaCl, 100 mmol.L^{-1} Na_2EDTA, 100 mmol.L^{-1} Tris, pH 10) for 24 h. Immediately after this step, slides were placed in a horizontal electrophoresis unit containing freshly prepared electrophoresis buffer (1 mmol.L^{-1} Na_2EDTA, 300 mmol L^{-1} NaOH, pH > 13). The DNA was allowed to unwind for 20 min; electrophoresis was then performed at 43V, 308 mA for 25 min. The slides were then gently immersed in neutralization buffer (0.4 mol.L^{-1} Tris– HCl, pH 7.5) for 15 min and then fixed with ethanol. All steps of the comet assay were conducted under subdued light. Three independent experiments were conducted. DNA damage was determined blinded regarding treatment in 100 nucleoids per slide. Slides were prepared in triplicate, stained with ethidium bromide, and screened with a fluorescent microscope (ZEISS®, Jena,Thuringia, DEU) equipped with a 515–560 nm excitation filter, a 590 nm barrier filter, and a 40X objective. The level of DNA damage was assessed by an image analysis system (TriTek CometScore®1.5, 2006, Sumerduck, VA, USA), and the percentage of DNA in the tail and Tail Moment were obtained for each treatment.

Cytokinesis-blocked micronucleus assay (CBMN). CBMN assay for mutagenicity evaluation was performed according to a reliable study (Fenech, 2000) with minor modifications. CHO-K1 cells (37×10^4 cells/culture flask) were seeded in 25 cm^2 culture flasks at 37 ºC, 5% CO_2. After 24 h of seeding, cells were exposed for 24 h to Bonefill® or HA/PLGA eluates at 100% concentration. Each culture flask containing an eluate was supplemented with 10% FBS. NC were culture flasks containing culture medium supplemented with 10% FBS without any eluate (untreated controls), and PC were culture flasks containing CHO-K1 cells treated with doxorubicin (0.3 µg.mL^{-1}) for 4 h. Cytochalasin-B (CytB) was added to the CHO-K1 cultures at a final concentration of 5 µg.mL^{-1} and left for 24 h. After treatments, the cultures were washed with PBS, trypsinized and centrifuged for 5 min at 406×*g*. The pellet was then resuspended in cold hypotonic solution (0.3% KCl, w/v) for 3 min. Cells were fixed twice with methanol:glacial acetic acid (3:1, v/v) and with four drops of formaldehyde, and then carefully homogenized with a Pasteur pipette. The cell suspensions were dripped on to a slide with a film of distilled water at 4 ºC. Slides were stained with 5% Giemsa solution diluted in phosphate buffer (Na_2HPO^4 0.06 mol.L^{-1}, KH_2PO^4 0.06 mol.L^{-1} – pH 6.8) for 7 min, washed with distilled water, air dried, and examined by light microscopy (400× magnification). Three independent experiments were conducted.

Five hundred (500) viable cells were scored to determine the frequency of cells with 1, 2, 3, or 4 nuclei. The nuclear division index (NDI) was calculated using the formula: [NDI = M1 + 2(M2) + 3(M3) + 4 (M4)/N], where M1–M4 represents the number of cells with 1–4 nuclei, respectively, and N is the total number of viable cells scored (Eastmond, Tucker, 1989; Fenech, 2000). The frequency of binucleated cells with micronuclei (MNBCF), total frequency of micronuclei (MF), frequency of nucleoplasmic bridges (FNPBs) and the frequency of nuclear buds (NBUDs) were scored in 1000 binucleated cells for each treatment. The criteria used for identifying micronuclei were based on Fenech (2000).

Statistical analysis

At least 3 experiments were conducted for each analysed parameter. The experimental results were expressed as mean and standard error (SE). The Shapiro-Wilk test was used to assess data normality and Levene's test for data homogeneity. In view of the results, parametric tests were utilized. For XTT, Clonogenic Survival, Comet, and CBMN assays, one-way ANOVA test followed by Tukey's test were applied to the data. In addition, data from treated groups were compared with negative controls (Dunnett's test). The non-parametric Kruskal-Wallis test was applied for NDI (CBMN), followed by Dunn's test. Graphpad Prism 5.01 was used to perform the statistical tests. Differences were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Although there is some published research on the toxicity of PLGA, HA and xenograft from bovine bone, inasmuch as a chemical modification takes place, toxicity potential regarding these materials requires investigation. Hence, toxicity data obtained by these studies may determine whether the material is safe for medical implants (Galia *et al.*, 2008). Safety assessments of medical materials can be conducted by toxicological guidelines recommended by the International Organization of Standardization (ISO 10993-1/EN 30993-1). Depending on the type and extent of contact of a material with the patient, a standardized battery of biological safety tests are suggested by the ISO (Scarel-Caminaga *et al.*, 2014).

Considering that the materials investigated in this study are potential candidates for bone substitute as alloplastic block grafts requiring long-term contact with host fluids and tissues, some cytotoxicity and genotoxicity assessments are required under ISO guidance (Scarel-Caminaga *et al.*, 2014).

Cytotoxicity results are shown in Figure 1 (XTT cell viability assay). Cell viability is related to absorbance. Negative control corresponded to 100% cell viability. The results obtained for NC and HA/PLGA and Bonefill® eluates did not significantly differ ($p>0.05$, Dunnett's test), indicating that the materials did not affect cell viability.

Literature describes several assays that can be used to determine toxicity in polymeric nanostructured systems, including assays involving cell viability analyses

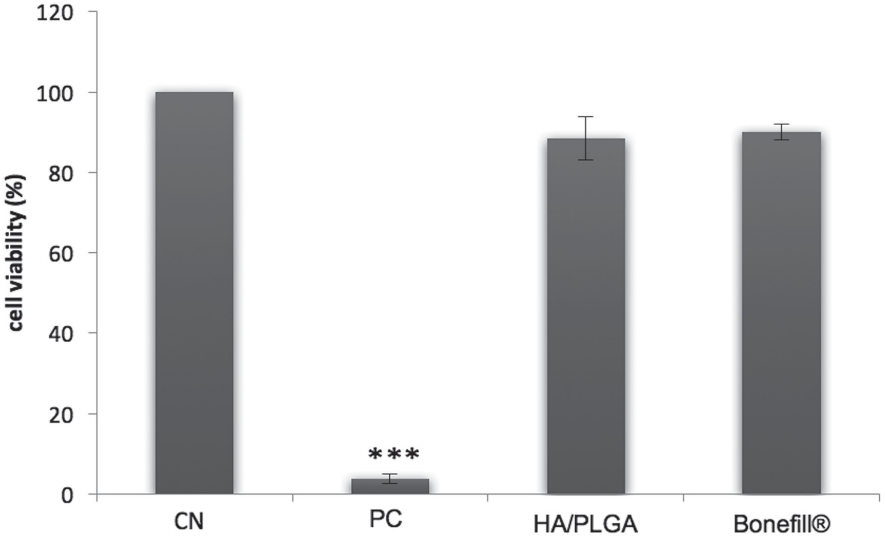

FIGURE 1 - XTT assay in CHO-K1 (Cell viability). Cells treated with 100% concentration HA/PLGA and Bonefill® eluates. NC: negative control; PC: positive control. NC represents 100% cell viability. Columns = mean of cell viability (%); bars = Standard error. *** = $p<0.0001$ compared to NC; Dunnett's test.

of mammalian cells, such as the tetrazolium salt reduction assay (XTT), MTT assay and genotoxicity assays (Cieślik *et al.*, 2009). Therefore, the XTT assay was used to evaluate cell viability after exposure to eluates from HA/PLGA and Bonefill® materials. This assay is based on cell metabolic activity where the reduction of yellow tetrazolium salt to orange formazan dye only occurs in viable cells by mitochondrial dehydrogenases; this can be measured by absorbance. It is important to bear in mind that the XTT assay aims to demonstrate immediate cytotoxic effect on cultured cells, whereas the clonogenic survival assay shows whether other damage has occurred to cells that interfere with or stop their proliferative capacity at a later time (Sumantran *et al.*, 2007). The results of this study showed the absence of cytotoxic effects from HA/PLGA and Bonefill® eluates, which are in accordance with literature demonstrating HA, bovine bone, and PLGA as biocompatible materials (Galia *et al.*, 2008; Cieślik *et al.*, 2009; Trif *et al.*, 2015). PLGA nanoparticles associated with polyethylene glycol (PEG) and poly L-lysine (PLL) (PEG-PLL-PLGA) have demonstrated low cytotoxicity by MTT assay (Guo *et al.*, 2015).

Furthermore, different concentrations of PLGA nanoparticles have shown no cytotoxicity to fibroblasts (3T3) by MTT assay (De Lima *et al.*, 2011). In contrast, high concentrations (5,000 $\mu g.mL^{-1}$) of PLGA nanoparticles induced moderate cytotoxicity in Madin–Darby bovine kidney (MDBK) cells (Trif *et al.*, 2015). However, the same concentration did not induce a cytotoxic effect in human colorectal adenocarcinoma (Colo 205) cells. Also, no cytotoxicity was seen from PLGA and HA/PLGA composites by the lactate dehydrogenase (LDH) test (Cieślik *et al.*, 2009). Additionally, lyophilized bovine bone prepared on a semi-industrial scale showed no cytotoxicity potential by the agar diffusion test (Galia *et al.*, 2008). Studies have verified that even with a high particle surface area, particles concentrations between 5.4 and 540 µg/mL are incapable of inducing cell toxicity (Semete *et al.*, 2010; De Lima *et al.*, 2011), agreeing with previously presented explanations.

The surviving fraction obtained by clonogenic survival assay revealed no statistical difference with NC ($p>0.05$, Dunnett's test; Figure 2).

The clonogenic assay evaluated whether the material interferes in mitotic replication of CHO-K1 cells, since only mitotically viable cells can produce progenitor cells. We verified that HA/PLGA and Bonefill® eluate did not interfere in cell survival or the proliferation capacity of a single cell. Increasing eluate HA concentration was shown to increase cytotoxic effects by inhibiting cell-colony formation (Jantová *et al.*, 2008).

The general cytotoxicity results revealed that HA/PLGA and Bonefill® were not cytotoxic, showing no disturbance in mitotic cell replication.

Tail moment evaluation verified that neither HA/PLGA or Bonefill® promoted genotoxic effects ($p>0.05$, Dunnett's test; Figure 3).

The alkaline version of Comet assay can detect DNA double-strand breaks, alkali-labile sites, and single-strand breaks associated with incomplete excision repair sites (Tice *et al.*, 2000; Araújo *et al.*, 2009). Tail moment evaluation verified that HA/PLGA and Bonefill® bone substitutes did not promote genotoxic effects. The concept of tail moment (calculated as the product of tail length and total DNA fraction in the tail) is a measurement of DNA migration. This method incorporates relative

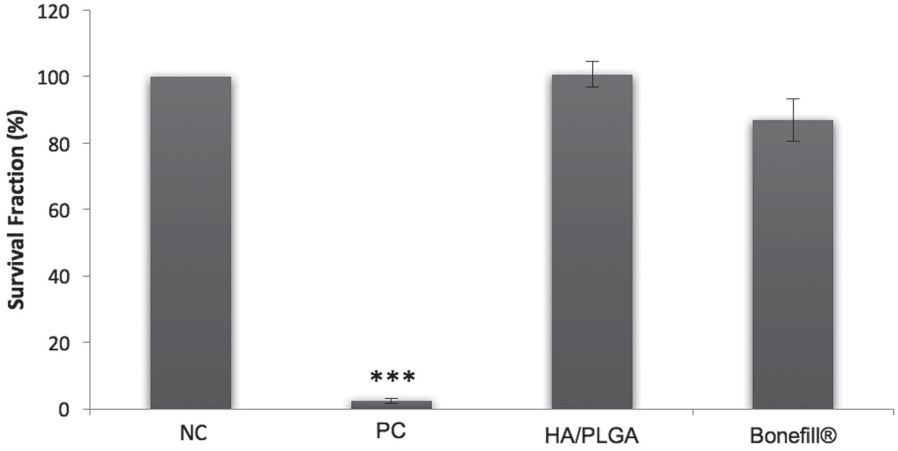

FIGURE 2 - Clonogenic survival assay in CHO-K1. Cells treated with 100% concentration HA/PLGA and Bonefill® eluates. NC: negative control; PC: positive control. NC represents 100% survival fraction. Columns = mean of survival fraction (%); bars = Standard error. *** = $p<0.0001$ compared to NC; Dunnett's test.

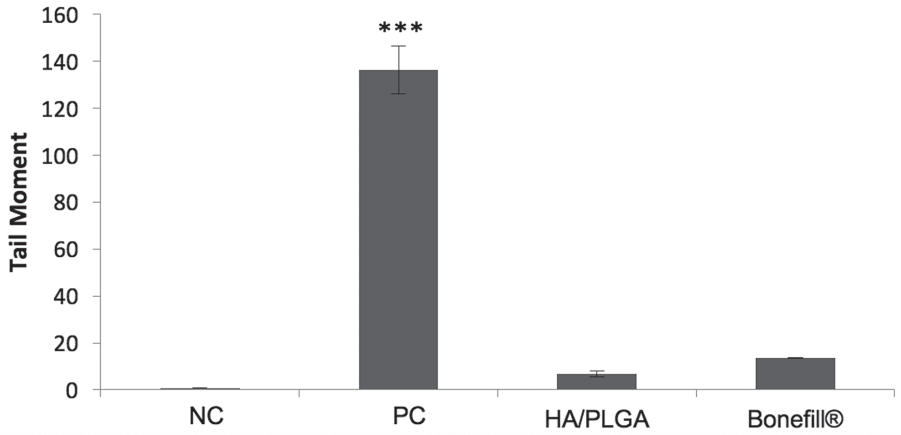

FIGURE 3 - Comet assay in CHO-K1. Cells treat with 100% concentration HA/PLGA and Bonefill® eluates. NC: negative control; PC: positive control. Columns = mean of tail moment; bars = Standard error. *** = $p<0.0001$ compared to NC; Dunnett's test.

measurements of both the smallest detectable size of migrating DNA (reflected by comet tail length) and the number of broken pieces of DNA (represented by the staining intensity of DNA in the tail) (Liao, Mcnutt, Zhu, 2009). The study of DNA damage at the chromosome level is an essential part of genetic toxicology whereas chromosomal mutation is an important event in carcinogenesis. Studies investigating HA toxicity have shown that it does not induce genotoxic effects (Kannana *et al.*, 2014), however comet assay showed increasing HA concentrations (10% to 100%; v/v) induced DNA damage (between 13.1 and 14.2%), which was dose-dependent (Jantová *et al.*, 2008).

Table I shows mutagenicity assay results of: nuclear division index (NDI), the frequency of binucleated cells with micronuclei (BCMN), and micronucleus (MN), nucleoplasmic bridge (NPB), nuclear bud (NBUD) frequencies.

NDI was similar between groups ($p>0.05$; Kruskal Wallis's), except for PC and Bonefill® ($p<0.05$; Kruskal Wallis's). Nuclear Division Index (NDI) is a marker of cell proliferation in cultures and is considered a measure of general cytotoxicity (Eastmond, Tucker, 1989; Fenech, 2000; Ionescu *et al.*, 2011). There may be an induction of mitotic delay which, by not allowing the repair of genotoxic lesions, will modify the number of cells entering mitosis and modify the proportion of mono-/bi-/tri- and tetranucleated cells (Ionescu *et al.*, 2011). Thus, lower NDI can signify fewer cell divisions. There is also the hypothesis of a clastogenic effect from mutagens with an aneugenic action, inducing some degree of cell cycle blockade. Therefore, more cells will not divide and NDI will again be low. There was no significant difference in NDI between NC and both Bonefill® or HA/PLGA materials, indicating that these materials did not induce CHO-K1 nuclear division arrest. Therefore, Bonefill® and HA/PLGA did not decreased cell division.

HA/PLGA and Bonefill® were not mutagenic, as only PC was statistically higher than NC for FBCMN, FMN, and FNBUD. In FNPB, only Bonefill® was statistically lower than NC emphasizing that this material did not induce nucleoplasmic bridges.

TABLE I - CBMN assay in CHO-K1. Mean and standard error of nuclear division index (NDI), frequency of binucleated cells with micronucleus (FBCMN), frequency of micronuclei (FMN), frequency of nucleoplasmic bridges (FNPBs) and frequency of nuclear buds (NBUDs), in 1000 binucleated cell for each treatment. Cells treated with 100% concentration of HA/PLGA and Boneffil® eluates. NC: negative control; PC: positive control; *$p<0.05$ compared to NC group (Dunnett's test); [a,b] = $p<0.05$ (Kruskal-Wallis's test).

TREATMENT	NDI Mean ± SE	FBCMN Mean ± SE	FMN Mean ± SE	FNPBs Mean ± SE	FNBUDs Mean ± SE
NC	1.914± 0.002	16.33 ±0.298	21.67±1.075	5.33±0.789	16.57±2.024
PC	1.754±0.005[a]	91.67±1.814*	143.67±5.873*	40.6±1.300*	101.29±7.928*
HA/PLGA	1.977±0.006	15.33±1.193	20.33 ± 1.578	4.33±0.298	11.86±2.436
Bonefill®	2.032±0.013[b]	11.67±1.660	12.67±1.193	2.33±0.298*	13.71±1.723

The micronucleus assay (or cytokinesis-blocked micronucleus) has emerged as one of the preferred methods for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be reliably measured (Fenech, 2000). Although using a different method, the *Salmonella* Typhimurium bacterial test, Jantová *et al*., (2008) also observed no mutagenic effect in 100% HA-concentration eluates. Therefore, HA was not mutagenic, even at high concentration, by two different reliable tests (CBMN and bacterial mutagenicity test).

Our study showed that 100% HA/PLGA eluate concentration (v/v) did not promote cytotoxic, genotoxic, or mutagenic effects. This is in agreement with Cieślik *et al*. (2009) who demonstrated that an HA/PLGA composite was fully biocompatible and the bone defects were fully repaired after 48 weeks. They concluded that this composite did not induce toxic effects on bone-forming cells.

Cyto- and genotoxicity effects on materials prepared with PLGA nanoparticles require further study, even though this polymer is one of the most widely used in the preparation of polymer nanoparticles, mainly for pharmaceutical and medical processes. Studies concerning the impact of these nanostructures on living organisms and the environment are therefore needed so that the safety of these nanosystems can be assessed before they become even more widely commercialized (De Lima *et al.*, 2011). For example, the aforementioned PEG-PLL-PLGA showed no blood toxicity and mutagenicity by MN (Guo *et al.*, 2015). Positively charged PLGA nanoparticles led to chromosomal aberrations without primary DNA damage in human bronchial epithelial cells (Platel *et al.*, 2016). Human peripheral blood mononuclear cell cultures treated with PLGA-PEO nanoparticles revealed no increase in the number of micronucleated binucleated cells (Tulinska *et al.*, 2015). On the other hand, PLGA-PEO lead to a weak but significant increase in the level of MN in TK6 human B-lymphoblastoid cells, which did not induce DNA strand-breaks (detected by comet assay), nor was it cytotoxic (measured by relative cell growth activity and cytokinesis-block proliferation index (CBPI)) (Kazimirova *et al.*, 2012).

Previous studies have shown that HA/PLGA composites not only promote adequate bone regeneration (Galia *et al.*, 2008), but also do not induce cytotoxicity (Galia *et al.*, 2008; Cieślik *et al.*, 2009; Kannana *et al.*, 2014), mutagenicity (Kazimirova *et al.*, 2012; Kannana *et al.*, 2014), or genotoxicity (Cieślik *et al.*, 2009; Kannana *et al.*, 2014), which corroborate with the results of this study. In this way, HA/PLGA results were used as a bench mark to evaluate whether Bonefill® could have the similar toxicity profile to that of HA/PLGA. Both materials demonstrated similar results - not inducing cytotoxic, genotoxic, or mutagenic effects in CHO-K1 cells.

CONCLUSION

Considering the negative cytotoxicity, genotoxicity and mutagenicity results found for HA/PLGA and Bonefill®, it was concluded that these bone substitutes presented interesting preliminary results as potential biomaterials for bone repair.

DECLARATION OF INTERESTS

The authors certify that they have no commercial or associate interests that represent a conflict of interest in connection with the manuscript.

ACKNOWLEDGMENTS

The authors are grateful to Bionnovation Biomedical Products Ltda. Brazil, for providing the materials (HA/PLGA and Bonefill® Block).

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Received for publication on 28th July 2017
Accepted for publication on 12th November 2017