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Nanocomposites based on bacterial cellulose in combination with osteogenic growth peptide for bone repair: cytotoxic, genotoxic and mutagenic evaluations

Raquel Mantuaneli Scarel-Caminaga [a,] , Sybele Saska [b], Leonardo Pereira Franchi [c], Raquel A. Santos [e], Ana Maria Minarelli Gaspar [a], Ticiana S.O. Capote [a*], Sidney José Lima Ribeiro [b], Younés Messaddeq [b], Reinaldo Marchetto [b], Catarina S. Takahashi [c,d]

[a] School of Dentistry at Araraquara, São Paulo State University, UNESP, Department of Morphology. Rua Humaitá, 1680, Araraquara- SP, Brazil. [b]Institute of Chemistry, São Paulo State University, UNESP. Rua Francisco Degni- Araraquara- SP, Brazil. [c] Faculty of Medicine of Ribeirão Preto, São Paulo University, USP, Department of Genetic. Bloco G. Av. Bandeirantes, 3900, Ribeirão Preto, SP, Brazil. [d] Faculty of Phylosophy, Sciences and Lyrics, São Paulo University, USP, Department of Biology. Av. Bandeirantes, 3900, Ribeirão Preto, SP, Brazil. [e] University of Franca, UNIFRAN. Avenida Dr. Armando Salles Oliveira- Franca-SP, Brazil.

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ABSTRACT

Bacterial cellulose membranes functionalized with hydroxyapatite or collagen with addition or not of osteogenic growth peptides (OGP) or its C-terminal pentapeptide OGP[10-14] were developed for improving bone repair. The aim of this study was to evaluate the potential cytotoxic, genotoxic and mutagenic effects of those nanocomposites in order to know whether they would be safe for biomedical applications. All nanocomposites (BC, BC-HA, BC-Col, BC-HA OGP, BC-Col-OGP, BC-HA OGP[10-14] and BC-Col-OGP[10-14]) were prepared as discs (5 mm in diameter) and submitted to *in vitro* tests in 24-well plates seeded with CHO-K1 cells. Cell viability was evaluated by the XTT assay and reproductive cell death was detected by the clonogenic assay. Genotoxicity was assessed by the comet assay and the cytokinesis-blocked micronucleus (CBMN) assay was used to detect mutagenicity. Only BC-HA OGP[10-14] showed a slight mutagenic effect, all other nanocomposites materials demonstrated no cytotoxic, genotoxic or mutagenic effects. In conclusion, the BC-HA OGP[10-14] promoted a slight mutagenic effect and future studies must be investigated for better understanding this result. The utilization of the investigated materials is promising for biomedical applications, such as bone repair and tissue engineering.

1. INTRODUCTION

Many nanomaterials present distinct physicochemical properties which could lead to variable toxicity in comparison with the bulk material of a similar chemical nature. Considering the growing industrial use of nanomaterials, there is an urgent need for information about their potential health effects [1-4]. Bacterial cellulose (BC) is characterized as a gelatinous membrane with a 3-D structure consisting of an ultrafine network of cellulose nanofibres [5]. It is obtained from cultures of the Gram-negative bacterium *Gluconacetobacter xylinus*, which produces highly hydrated membranes (up to 99% water), free of lignin and hemicelluloses, with a large nano-porous surface area and a higher molecular weight and degree of crystallinity than plant cellulose. Also, BC membranes show great elasticity, high wet strength and conformability [6]. The unique properties provided by the nanometric fibers structure have led to a number of commercial products and medical applications such as wound dressings and skin substitutes[7]. BC membranes exhibit important properties such as biocompatibility, bioinertness and selective permeability. Furthermore, they also serve as a barrier against microorganisms in wounds and burns, thus accelerating the healing process, providing pain relief and reducing scar formation[8-10]. Composite materials based on hydroxyapatite (HA) have been developed by different synthetic routes and techniques in order to improve both the bioactivity and mechanical properties of various orthopedic prosthetic and dental implants [11]. Many of them have shown excellent biocompatibility and ideal bioactivity both *in vitro* and *in vivo* [12-14]. Recently, we demonstrated that BC-hydroxyapatite (BC-HA) nanocomposites induce no inflammatory reaction in non-critical bone defects in rat tibiae one week after of surgery. Also, after four weeks, the defects were completely filled by bone tissue, demonstrating that the BC-HA membranes were

* *Corresponding Author*
Ticiana Sidorenko de Oliveira Capote
e-mail: ticiana@foar.unesp.br, Fax: 55 16 3301-6488.
Phone: 55 16 981118170

effective for bone repair [15]. Moreover, another material widely studied in the literature is collagen (Col), which is employed in development materials for hard and soft tissue repair with several clinical applications. Thus, these suitable results have encouraged researchers to develop nanocomposites based on BC and collagen for tissue repair [16-18], whose nanocomposites BC-collagen showed potential for application in tissue engineering.

Osteogenic growth peptide (OGP) is an endogenous tetradecapeptide physiologically present in the blood circulation at micromolar concentrations. It can promote the proliferation and differentiation of osteogenic cells in the marrow and bone repair callus and thus stimulates the osteogenic activity of osteoblasts and enhances fracture healing *in vivo* when administered systemically [19-23]. The regulatory role of OGP and its derivative, the C-terminal pentapeptide OGP[10-14], is to stimulate osteogenesis and hematopoiesis in the differentiation and/or proliferation of osteoprogenitor cells and hematopoietic progenitor cells [19, 24, 25]. Therefore, these peptides (OGP and OGP[10-14]) were chosen for synthesis and incorporation into nanocomposites based on BC. Recently, BC, BC-OGP and BC-OGP[10-14] nanocomposite membranes were characterized, showing osteoinductive properties without cytotoxic, genotoxic or mutagenic effects [26].

Several materials based on BC have been recognized as non-genotoxic and non-cytotoxic [27-29]. However, once a chemical modification is performed on such a material, the toxic hazards need to be investigated. Safety assessments of medical materials are conducted by the toxicological guidelines recommended by the International Organization of Standardization (ISO 10993-1/EN 30993-1). Depending on the type and extent of contact with the patient's material, a standardized battery of biological safety tests is suggested by the ISO. Considering that the materials presented here are intended to be used as bone implant devices that are required to be in contact with the patient's body for periods longer than 30 days, some assessments demanded by the ISO guidance are cytotoxicity and genotoxicity.

To further confirm the biocompatibility of nanocomposites based on BC with hydroxyapatite or collagen and functionalized with/without the peptide modulators of growth factors (OGP and OGP[10-14]), the aim of this study was to evaluate whether they exhibit any cytotoxic, genotoxic and mutagenic effects.

2. MATERIALS AND METHODS

2.1 Preparation of composites

The preparation of the BC-HA and BC-Col nanocomposites was performed following the methodology proposed by Saska *et al.*,(2011) [15] and Saska *et al.*,(2012) [17], respectively. HA was formed in highly hydrated BC membranes by alternating incubation cycles in 0.05 mol.L^{-1} $CaCl_2$ and 0.1 mol.L^{-1} Na_2HPO_4 solutions at room temperature (RT) for 24 h each cycle. The incorporation of collagen (COL) (type I collagen from rat tail tendon – Sigma®, Saint Louis/EUA) into the BC hydrogel was initiated by the exchange of water in the highly hydrated BC by dimethylformamide (DMF). The BC surface was then initially modified by Fmoc-glycine (Fmoc-Gly) esterification to the free hydroxyl groups of BC, through a solid-phase synthesis strategy employing Fmoc-based chemistry (9-fluorenylmethyl oxycarbonyl-Fmoc). For this reaction, a solution was prepared containing 0.7 mol.L^{-1} Fmoc-Gly, 0.1 mol.L^{-1} 1,10-cabonyldiimidazole, 0.02 mol.L^{-1} N-methylimidazole in DMF. The esterification reaction was carried out under shaking at RT for 2 h. After the samples were washed with DMF for the removal of excess reagents. Deprotection of BC-Gly-Fmoc was carried out using piperidine in DMF (20%) at RT for 2 h, followed by successive washes with DMF to remove the excess piperidine solution. The exchange of DMF for deionized was carried out incorporation of collagen. Then, each BC-Gly sample in water was soaked in 4 mmol.L^{-1} aqueous collagen solution containing 5 mmol.L^{-1} 1-ethyl-3-(3dimethylaminopropyl) carbodiimide at 4 °C for 24 h (pH 6). The samples were washed with deionized water under vacuum to remove excess reagents.

The peptides OGP (H_2N-ALKRQGRTLYGFGG-OH) and OGP[10-14] (H_2N-YGFGG-OH) were synthesized manually by the solid-phase method, according to the standard Fmoc/tert-butyl (tBut) protocol [30] by the Synthesis, Structure and Applications of Peptides and Proteins Laboratory (Instituto de Química de Araraquara, UNESP, Brazil). The peptides were purified by preparative HPLC (≥ 97% purity) and the identity of the peptides was confirmed by positive electrospray mass spectrometry (ESI-MS) (ESI-MS, $m/z\ (M+2H)^{+2}$ = 763 (OGP) and $(M + H)^+$ = 500 (OGP [10-14]), and M_T = 1523.8 and 499.5 for OGP and OGP [10-14], respectively).

Incorporation of the peptides was carried out by adsorption. This process promotes the interaction between the peptide and OH groups in cellulose by hydrogen bonding, and drug delivery occurs by increasing the ionic strength of the solution (biological conditions). The concentration used for both peptides was 10^{-9} mol.L^{-1}; this concentration was pre-established in a previous study [26]. Thus, BC-HA and BC-Col nanocomposites 5 mm in diameter were immersed in 5 mL of the respective peptide solution, OGP or OGP[10-14] for 72 h at 10ºC. All nanocomposites materials, BC only, BC-HA, BC-Col, BC-HA OGP, BC-Col-OGP, BC-HA OGP[10-14] and BC-Col-OGP[10-14] were dried into molds at 37ºC and sterilized by gamma radiation (20 kGy). The dimensions of all nanocomposites materials were 5 mm in diameter and 150 µm for BC-HA and 60 µm for BC-COL, including or not peptides.

2.2 Cell culture experiments

The CHO-K1 cells were cultured in 1:1 Ham-F10 + D-MEM medium (Sigma®, St. Louis, MO) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil) and antibiotics [(0.06 g.L^{-1} penicillin (Sigma®), 0.10 g.L^{-1} streptomycin (Sigma®), 1% kanamycin (Gibco, Carlsbad, CA) and 1% ciprofloxacin (Hifloxan®, Halexistar)] in 25 cm^2 culture flasks at 37°C, 5% CO_2. Cells were used between the third and eighth passages.

2.3 Cell viability

To perform these experiments CHO-K1 cells were used and the Cell Proliferation Kit II (Roche Applied Science). The technique principle is based on the cleavage of the yellow tetrazolium salt XTT by metabolically active cells, forming an orange formazan dye. Thus, this conversion occurs only in viable cells. After 24 h of seeding, cells (2×10^4 cells seeded) were exposed for 24 h to the BC, BC-HA, BC-Col, BC-HA OGP, BC-Col-OGP, BC-HA OGP[10-14] and BC-Col-OGP [10-14] membranes (in duplicate) in 24-well plates. Negative controls (NC) were wells without any BC membrane (untreated controls), while positive controls (PC) were treated with doxorubicin (3 $\mu g.mL^{-1}$) for 24 h (all experiments were carried out in duplicate). After exposure, the cultures were washed with Hank's solution and fresh medium was added. After 24 h of incubation, the cultures were washed with Hank's solution and immediately 500 µL of DMEM without phenol red were added, followed by the addition of 60 µL of the XTT/electron solution (50:1); this was incubated for 30 min. Next, the supernatant was transferred to a cuvette and a colorimetric reading was taken in a spectrophotometer (Ultrospec™ 2100 pro UV/Visible Spectrophotometer). The result of the absorbance measured at 492 and 690 nm is directly proportional to the number of viable cells in each treatment after 24 h of exposure. Three independent experiments were conducted.

2.4 Clonogenic assay

The clonogenic assay or clonogenic survival test is an *in vitro* assay based on the capacity of a single cell to grow into a colony. Only mitotically viable cells are able to produce progenitor cells; therefore, the number of colonies formed following or during the treatment is an excellent indicator of cell viability and proliferation. A colony is defined as being composed of at least 50 cells. We used CHO-K1 cells plated after treatment [31].

After 24 h of seeding, cells (5×10^4 cells seeded) were exposed for 24 h to BC, BC-HA, BC-Col, BC-HA OGP, BC-Col-OGP, BC-HA OGP[10-14] and BC-Col-OGP [10-14] membranes in 24-well plates; negative controls (NC) were wells without any BC membrane, while positive controls (PC) were treated with doxorubicin (0.3 $\mu g.mL^{-1}$) for 4 h (all experiments were carried out in duplicate). After exposure, the cultures were washed with Hank's solution and fresh medium was added. Exponentially growing cells were seeded at a number of 150 cells per 25 cm^2 flasks, in duplicate for each treatment. The flasks were incubated at 37ºC, 5% CO_2, for 7 days without media change. The colonies that formed were fixed with methanol:acetic acid:water (1:1:8 v/v/v) and stained with 5% Giemsa. The colonies were counted, and the cell surviving fraction was calculated as percent colonies in treated dishes relative to untreated controls (NC). Three independent experiments were conducted.

2.5 Comet assay

Prior to the comet assay, CHO-K1 cells were assayed for viability using trypan blue dye exclusion [32]; those cultures that were considered suitable for the comet assay showed cell viability above 70%. The comet assay was performed as described previously by [33]. After 24 h of seeding, cells (5×10^4 cells seeded) were exposed for 24 h to BC, BC-HA, BC-Col, BC-HA OGP, BC-Col-OGP, BC-HA OGP[10-14] and BC-Col-OGP [10-14] membranes in 24-well plates; negative controls (NC) were wells without any BC membrane, while positive controls (PC) were treated with doxorubicin (0.3 $\mu g.mL^{-1}$) for 4 h (all experiments were carried out in duplicate). After exposure, the cultures were washed with Hank's solution and harvested with trypsin.

Five hundred microliters of cells in suspension were obtained and kept on ice and protected from light. This cell suspension was centrifuged at 500 rpm for 5 min at 4°C. The pellet was resuspended in 160 µL of 0.5% (w/v) low melting point agarose and the mixture was 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 were immersed in cold (4°C) 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 and subsequently electrophoresis was performed at 25 V, 300 mA for 20 min. Afterwards, the slides were gently immersed in neutralization buffer (0.4 $mol.L^{-1}$ Tris–HCl, pH 7.5) for 15 min and then fixed with ethanol. The slides were then stained with 50 µL of SYBR Green 2X (Invitrogen, Eugene, Oregon). DNA damage was determined in 100 nucleoids (50 per slide) in a blind test using a fluorescent microscope (Zeiss Axiovision, Germany) equipped with an excitation filter at 515–560 nm and a barrier filter at 590 nm (40x objective). All the steps of comet assay were conducted under subdued light. Three independent experiments were conducted.

In order to quantify the extent of DNA damage, each nucleoid was classified visually according to the migration of the fragments as: class 0 (no damage, or < 5% migrated DNA); class 1 (little damage with a short tail length smaller than the diameter of the nucleus, or 5-20% migrated DNA); class 2 (medium damage with a tail length one or two times the diameter of the nucleus, or 20-40% migrated DNA); class 3 (significant damage with a tail length between two and a half to three times the diameter of the nucleus, or 40-95% migrated DNA); class 4 (significant damage with a long tail greater than three times the diameter of the nucleus, or < 95% migrated DNA) [34, 35]. To facilitate management of the data, an average of DNA migration (DNA damage index) was calculated as follows: [(number of cells with score 1) × 1 + (number of cells with score 2) × 2 + (number of cells with score 3) × 3 + (number of cells with score 4) × 4]/100 [36].

2.6 Cytokinesis-blocked micronucleus (CBMN) assay

The CBMN assay was performed according to published procedures [37] with minor modifications. CHO-K1 cells were seeded in 24-well plates at a density 5×10^4 cells/well. After 24 h of seeding, similar to the clonogenic assay, cells were exposed for 24 h to the BC, BC-HA, BC-Col, BC-HA OGP, BC-Col-OGP, BC-HA OGP[10-14] and BC-Col-OGP [10-14] membranes; negative controls (NC) were wells without any BC membranes and positive controls (PC) were treated with doxorubicin (0.3 $\mu g.mL^{-1}$) for 4 h (all experiments were carried out in duplicate). Cytochalasin-B (CytB) was added to the CHO-K1 cultures at a final concentration of 5 $\mu g.mL^{-1}$ and left for 20 h.

After the treatments, the cultures were washed with Hank's solution and trypsinized. The two wells containing cells submitted to each treatment, as well as those belonging to the negative or positive controls, were pooled and centrifuged for 7 min at 1500 rpm.

The pellet was then resuspended in cold hypotonic solution (0.3% KCl w/v) for 3 min. The cells were fixed twice with methanol:glacial acetic acid (3:1,v/v) and with three drops of formaldehyde and homogenized carefully with a Pasteur pipette. The cell suspensions were dripped on a slide with a film of distilled water at 4°C. The 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. One thousand (1,000) cells were scored to evaluate the percentage of mono-, bi-, tri- and tetra-nucleated cells. The nuclear division index (NDI) was calculated according to 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 scored cells. Micronuclei (MN) were scored in 1,000 binucleated cells. MN is a biomarker of DNA damage and instability. The criteria for identifying MN were based on a previous study [37].

2.7 Statistical analysis

We conducted at least three independent experiments for each parameter analyzed. The experimental results are expressed as mean and standard error. The Shapiro-Wilk test was utilized to assess the normality of the data and for homogeneity the Levene test was utilized. In view of the results, parametric tests were utilized for Cell viability (XTT), Clonogenic assay, Comet assay and NDI.

One-way analysis of variance (ANOVA) followed by the Tukey test was applied to these data while ANOVA on Ranks followed by Student-Newman-Keuls test was applied to the frequency of micronucleated binucleated cells (MNBCF). Data from treated groups were compared to the negative control of its experiments. We used the BioEstat statistical package v.5 (UFPA, Belém, Brazil) and SigmaStat v.3.5 (USA) to perform the tests. Differences were considered statistically significant when $p<0.05$.

3. RESULTS AND DISCUSSION

BC, BC-HA, BC-Col, BC-HA OGP, BC-Col-OGP, BC-HA OGP[10-14] and BC-Col-OGP [10-14] nanocomposites did not exhibit cytotoxicity in CHO-K1 cells, since exposure to these membranes did not alter the degree of cell viability (Fig. 1) and cell surviving fraction (Fig. 2) when compared to the NC.

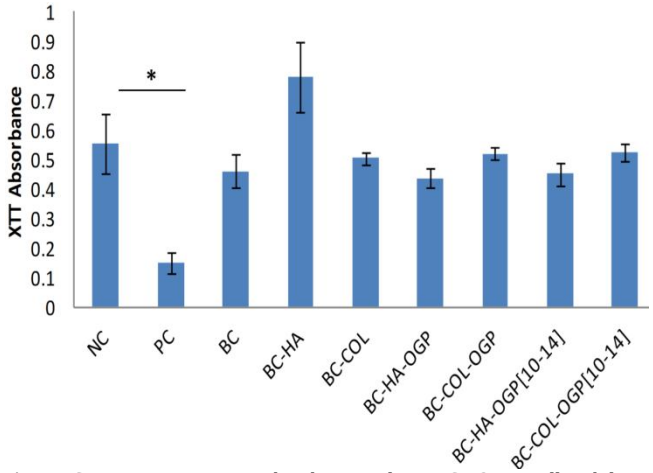

Fig. 1: Cytotoxic assay. XTT absorbance indicates CHO-K1 cell viability after treatment with different nanocomposites. ANOVA followed by the Tukey test. * = $p<0.01$; columns = mean; bars = standard error.

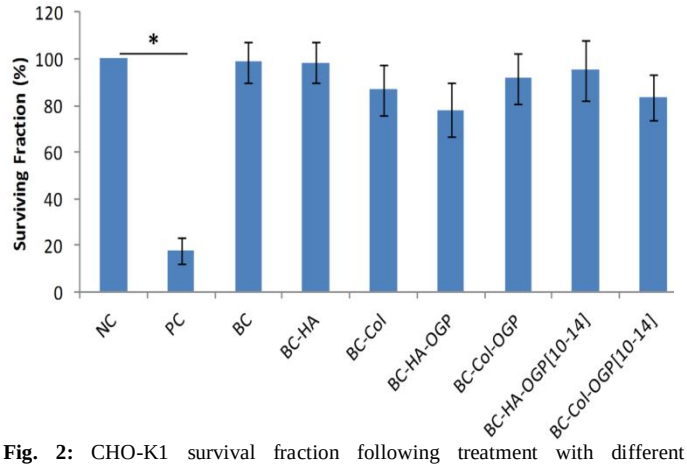

Fig. 2: CHO-K1 survival fraction following treatment with different nanocomposites. ANOVA followed by the Tukey test. * = $p<0.01$; columns = mean; bars = standard error.

Genotoxicity was evaluated by the comet assay and the results of the DNA damage index (DDI) of CHO-K1 cells treated with BC nanocomposites are shown in Table 1. The DDI of the NC and the PC were statistically different ($p<0.05$), but no statistical differences were obtained for any nanocomposites in comparison with the NC. Mutagenicity was assessed by the CBMN test. Results from both the nuclear division index (NDI) and frequency of binucleated cells with micronuclei (MNBCF) tests are presented in Table 1. The NDI was similar among all groups ($p=0.9181$), indicating that the exposure of CHO-K1 to different membranes did not induce nuclear division arrest, thus the nanocomposites did not influence cell division. However, the BC-HA OGP[10-14] induced a statistical increase of the MNBCF

(18.3) corresponding to ~ 3-fold induction when compared to NC (p<0.05, Table 1). All others BC nanocomposites materials did not increase statistically the MNBCF in comparison with the NC. We also observed a significant difference between the NC and PC (p<0.05, Table 1).

Table. 1: DNA damage after exposure to different BC membranes evaluated by the micronuclei and comet assays in the CHO-K1 cell line.

Treatment	NDI	MNBCF	DDI
	Mean ± SE	Mean ± SE	Mean ± SE
NC	1.81 ± 0.02	6.3 ± 0.3	0.82 ± 0.21
PC	1.83 ± 0.03	350.3 [a] ± 42.0	1.81 [a] ± 0.17
BC	1.79 ± 0.01	12.0 ± 1.2	1.08 ± 0.20
BC-HA	1.81 ± 0.07	14.0 ± 2.7	1.04 ± 0.08
BC-Col	1.84 ± 0.06	14.3 ± 0.6	0.61 ± 0.11
BC-HA-OGP	1.76 ± 0.09	15.3 ± 1.8	1.12 ± 0.08
BC-Col-OGP	1.81 ± 0.09	11.7 ± 0.8	1.01 ± 0.17
BC-HA-OGP[10-14]	1.78 ± 0.11	18.3 [a] ± 0.9	1.12 ± 0.09
BC-Col-OGP[10-14]	1.84 ± 0.08	12.0 ± 1.5	1.04 ± 0.01
	p = 0.9181		

NDI = nuclear division index; MNBCF = frequency of micronucleated binucleated cells; DDI = DNA damage index; SE = Standard Error. Different letters in a column indicate a statistically significant difference between groups (ANOVA, Tukey test – Comet Assay; ANOVA on Ranks, Student-Newman-Keuls test – MNBCF; p<0.05).

In the present study, BC-HA and BC-Col nanocomposites with/without the peptide modulators of growth factors (OGP and OGP[10-14]) demonstrated no cytotoxic or genotoxic effects. However, when mutagenicity was evaluated by CBMN assay the nanocomposite BC-HA OGP[10-14] showed a weak mutagenic effect, considering the MNBCF result.

The respective nanocomposites were investigated with the following dimensions: 5 mm in diameter and 150 µm (BC-HA) and 60 µm (BC-Col) in thickness, according to ISO 10993-3(2003), which states that whenever possible, the medical device shall be tested in a form representative of its "ready-to-use" state. In addition, we followed the ISO 10993-5(2009), which states, for direct-contact tests, materials that have various shapes, sizes or physical states (i.e. liquid, gels, solids, etc.) may be tested without modification in the cytotoxicity assays. The preferred test sample of a solid material should have at least one flat surface. Similarly to our study, Giavaresi *et al.*,(2006) [38]also investigated cytotoxicity using polyester films of about 0.5 mm in thickness, while Bäckdahl *et al.*,(2008) [39] utilized pieces of BC for cytotoxicity tests, and Ren *et al.*,(2008) [40] utilized discs of Poly (D,L-lactide)/nano-hydroxyapatite (PDLLA/NHA) composites.

The cytotoxic effect of HA has been previously investigated. Two different brands of HA extracts were tested regarding cytotoxicity by the MTT assay on the murine fibroblast L929 cell line, demonstrating negative results [41]. The cytotoxicity of HA, fluorhydroxyapatite (FHA) and fluorapatite (FA) discs [42] and five-day eluates [43] were comparatively evaluated on murine fibroblast NIH-3T3 cells. A direct contact study on NIH-3T3 cells demonstrated very slight cytotoxicity of biomaterials discs, in the range of 3.1-25.9% [42]. The eluate assay on NIH-3T3 cells showed that biomaterials induced different antiproliferative effects increasing in the order HA < FHA < FA; these effects were time-and concentration-dependent [43].

Cytotoxic effects on V79 Chinese hamster lung fibroblasts treated with HA different concentration eluates were investigated by the cell colony-formation assay, showing that HA was not cytotoxic at concentrations of 1%, 10% and 50% [11]. Clonogenic surveillance, also known as the colony formation test (CFA), measures clonogenic potential, i.e. the proliferative ability of single cells to form a clone, resulting in a colony [44]. Besides colony formation, this assay measures cell survival, and it is routinely used as a sensitive model for assessing long-term cytotoxicity. Tests measuring metabolic death are short-term assays for measuring cell growth within two to three days of drug exposure, but clonogenic assays are necessary for an assessment of the long-term cytotoxicity of drugs which cause reversible growth inhibition [44, 45]. Taken together, the data obtained from the viability tests indicate that BC nanocomposites are not cytotoxic to CHO-K1 cells. The Chinese Hamster Ovary (CHO) cell line has been widely used for studies measuring the cytotoxicity and genotoxicity of drugs [46]. The only previous study which investigated 3T3 fibroblast and CHO cell proliferation in the presence of BC nanofibres demonstrated that BC had no cytotoxic effect [28]. According to our previous study, BC membranes supplemented with OGP and OGP[10-14] do not exhibit cytotoxicity [26]. Furthermore, the addition of HA to BC functionalized with OGP or OGP[10-14] membranes does not alter the surviving fraction of CHO-K1 cells, demonstrating the complete absence of cytotoxic effects.

In the present study, the addition of collagen to BC membranes did not cause cytotoxicity. Also, slightly higher proliferation was observed for the BC-Col (5.5%), BC-Col-OGP (8.4%) and BC-Col-OGP[10-14] (9.4%) membranes, similar to what has been previously observed for the BC-OGP[10-14] membrane [26]. Research with BC-collagen composites have revealed slight increase in cell adhesion and proliferation in terms of 3T3 fibroblast cell culture [16] and BC-Col nanocomposites have showed osteoblastic differentiation with higher levels of ALP activity for primary osteogenic cell cultures of calvarial bone from newborn [17]. Moreover , type I collagen added to recombinant human platelet-derived growth factor-BB (rhPDGF-BB) and to beta-tricalcium phosphate, also showed no cytotoxicity [47].

In regard to the OGP, human osteoblast cells (hOB) treated with OGP[10-14] at different concentrations (10^{-13}-10^{-8} mol.L^{-1}) demonstrated an enhanced proliferative response. In comparison with hOB cells grown without the addition of OGP[10-14], the proliferation rate of hOB cells stimulated with OGP [10-14] at 10^{-12} mol.L^{-1} increased by 20% (p<0.05) after 24 h and 35% (p<0.05) after 72 h [48]. Very low doses of exogenous OGP or OGP[10–14] stimulate endogenous OGP, which is a precursor of bioactive OGP[10–14] [19,48,49]. OGP released into the culture medium binds to proteins, forming OGP-OGPBP complexes; upon dissociation, the inactive OGP undergoes proteolytic activation, generating the bioactive OGP[10–14] [17, 19]. The cellular response to this peptide is probably triggered by the stimulation of ERK phosphorylation through the G protein-MAP kinase signaling cascade, as previously demonstrated [50].

Phosphorylation of ERKs has been demonstrated to enhance cell survival, and these families of protein kinases are activated by several anti-apoptotic agents like PTH and bisphosphonates [51, 52]. Consequently, OGP[10–14] seems to have an antiapoptotic effect [48].

Moreover, nanomaterials may also affect cells mechanically, especially nanomaterials composed of long fibrous materials may physically interfere with cellular functions, e.g. the mitotic apparatus and chromosome segregation [1]. Also, nanomaterials may cause DNA damage indirectly, by promoting oxidative stress and inflammatory responses [53, 54]. Given the considerable uncertainty about the safety of engineered nanomaterials, it is imperative that we understand and thereafter minimize any potential toxicological hazards associated with them, not only to protect human health and the environment, but also to avoid damaging the nanotechnology industry in the longer term [53, 55]. In the present study, no genotoxic effects were found for the investigated materials based on BC membranes. The non-genotoxic effect of BC was described previously [29], as was the non-genotoxic effect of BC nanofibres [28] and BC membranes supplemented with OGP or OGP[10-14] [26]. In the present study, the addition of HA or collagen did not cause genotoxicity detected by comet assay. This is in agreement with a previous study that evaluated two different brands HA of extract by the comet assay, showing no significant extension of DNA damage in L929 cells [41]. The comet assay was also used to assess DNA damage in FHA, FA and HA five-day eluates on V79, showing no genotoxic effect for HA at 1-5%; although from 10 to 100% concentration a weak genotoxic effects were detected for HA. Similar results were observed to FHA and FA with this weak genotoxic effect classified as HA<FHA<FA [11]. Moreover, eluates of 1% to 50% of HA after 24 h used to treat NIH-3T3 increased the levels of DNA strand breaks proportionally to the concentration of HA (in the range of 6.77-49.25%) [43]. It is worth mentioning that the comet assay is not used to detect mutations, but to detect genomic lesions that could render a mutation [56]. *Hprt* and bacterial mutagenicity tests showed that FHA, FA and HA used to treat V79 cells had no mutagenic effects [11]. Considering bacterial assays, non-mutagenic effects have been observed for another material that was also supplemented with type I collagen, i.e. rhPDGF-BB with the β-tricalcium phosphate [47]. Recently, extracts from HA-bioglass and HA-ethyl vinyl acetate composite materials did not induce any significant damage to the chromosomes of human lymphocytes[57]. Here, we used the CBMN assay to detect chromosomal damage, drastic lesions that cannot longer be repaired by the cell DNA repair machinery [58]. BC-Col nanocomposites with/without the peptides, OGP or OGP[10-14] and BC-HA with/without the peptide OGP, did not have mutagenic effects on CHO-K1 cells, similar to what was observed in our previous study of BC-OGP and BC-OGP[10-14] membranes [26]. In contrast to these results, a positive response was detected to the composite BC-HA OGP[10-14]. The increase detected can be classified as a slight mutagenic effect, while doxorubicin (0.3 $\mu g.mL^{-1}$) induced a 55-fold increase in the frequency of micronuclei in binucleated CHO-K1 cells, BC-HA OGP[10-14] just induced a 3-fold increase in this frequency (Table 1). As mentioned before we do not observe increase in mutagenicity by BC-OGP[10-14] [26] or BC, BC-HA membranes alone, thus suggesting an additive or synergistic effect. As observed by other studies, modifications of hydroxyapatite can modify their toxic response [11, 43]. Thus, these results reinforce and corroborate the need to perform a genotoxicity evaluation when chemical modifications are held on such a material.

4. CONCLUSION

The BC-HA OGP[10-14] promoted a slight mutagenic effect and future studies with other cell lines must be investigated for better understanding this result. The studied nanocomposite membranes demonstrated no cytotoxic, genotoxic or mutagenic effects, indicating that these materials are promising for utilization in biomedical applications, such as bone repair and tissue engineering.

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