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Toxicity of therapeutic contact lenses based on bacterial cellulose with coatings to provide transparency

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ABSTRACT

Therapeutic contact lenses were developed from bacterial cellulose (BC) by the Institute of Chemistry at Brazil's São Paulo State University (UNESP). In a previous study, cyclodextrins (CD) and medications such as ciprofloxacin (CP) and diclofenac sodium (DS) were incorporated into the lenses to provide therapeutic properties and control drug release. However, significant opacity was seen in the material inherent to cellulose. In order to achieve full material transparency, the lenses were coated with an organic-inorganic hybrid compound containing aluminum alkoxide and glycidoxypropyltrimethoxysilane (GPTS)(H), or chitosan (Q) nanoparticles. This study evaluated the toxicity of these contact lenses to ensure the safety of these materials for future availability to the medical device industry. Lenses composed of BC and coated with either GPTS (H) or chitosan (Q), incorporating ciclodextrin (CD) to release diclofenac sodium (DS) or ciprofloxacin (CP), were submitted to cytotoxicity assays (XTT and Clonogenic Survival), genotoxicity (Comet Assay) and mutagenicity (Cytokinesis-blocked micronucleus assay) directly in cell culture. Statistical analyses were performed using the Tukey and Dunnett or Kruskal-Wallis and Dunn tests. All of the nanoparticles used in the lense coatings did not show cytotoxic effects by the XTT test ($p > 0.05$; Dunnett). Only materials associated with diclofenac sodium (BC-H-CD-DS and BC-Q-CD-DS) presented significantly different survival fractions compared to negative control ($p < 0.001$; Dunnett). Genotoxicity evaluation revealed a genotoxic effect in BC-H-CD-DS ($p < 0.05$; Dunn). All tested lenses did not present any mutagenic effect. These results indicate that improvements in DS incorporation are needed to eliminate toxicity. We demonstrated promising results in the safety of employing BC lenses functionalized with a drug delivery system permitting the bioavailability of ophthalmic drugs. Further studies utilizing other specific tests, such as corneal lineage are required before safe and efficient ophthalmologic use.

Abbreviations: BC, bacterial cellulose; CD, scyclodextrins; CP, ciprofloxacin; DS, diclofenac sodium; GPTMS, glycidoxypropyltrimethoxysilane; CS, clonogenic Survival; CA, comet Assay; CBMN, cytokinesis-blocked micronucleus assay; TCL, therapeutic contact lenses; HP-γ-CD, hydroxypropyl-γ-cyclodextrin; Boe-GPTS, boehmite-3-glycidyloxy-propyl trimethoxysilane system; PC, positive Control; NC, negative Control; CytB, cytochalasin-B; NDI, nuclear Division Index; FBMN, frequency of binucleate cells with micronucleus; FMN, frequency of micronucleus; NSAID, snonsteroidal anti-inflammatory drugs

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1. Introduction

The therapeutic contact lenses (TCL's) based on bacterial cellulose incorporating drug-cyclodextrin complexes [1] were developed to solve problems caused by the use of traditional contact lenses such as discomfort, irritation and oxygen deprivation. TCL's are widely used to reduce corneal nerve ending exposure and consequent pain, protect the cornea, assist epithelial healing, restore the anterior chamber, in corneal perforating wounds, and release medications on the ocular surface [2]. Moreover, TCL's have been used in treating a number of ocular surface diseases such as bullous keratopathy, recurrent erosion of the corneal epithelium, dry eye, and postoperative epithelial defects [2]. According to Boone et al. [2], choosing the type of TCL and its constituent materials is dependent on the target disease, the time of use, and the physiological needs of the diseased eye.

Cavicchioli et al. [3] developed a TCL made with bacterial cellulose and associated to cyclodextrin and ciprofloxacin. Based on their results, our research group has been working to improve some properties of those TCL's.

Bacterial cellulose (BC) is considered one of the most abundant biopolymers in the world; most of this polymer is produced in plant cell walls, where the cellulose is in the form of semicrystalline microfibers [4]. BC can be obtained from the bacterium *Gluconacetobacter xylinus* (*Acetobacter xylinum*), which produces a bacterial cellulose membrane free of lignin and hemicellulose, and in a 3-D network is composed of much more nanometric sized microfibrillary beams [5].

BC has excellent liquid retention capability which is an important property for medical and tissue engineering applications [6], such as dressing second or third-degree burns, artificial microvessels, and cartilage and bone tissue engineering [7,8]. Ophthalmological antibiotics or anti-inflammatories should be administered locally using appropriate devices [9]. Thus, the nanofibrillary structure of BC must represent a appropriate macromolecular support for the inclusion of active compounds and a specific controlled release system for drugs (antibiotics, analgesics, anti-inflammatories, hormones and anticancer) [10]. According to Ullah et al. [11], BC is a potential candidate for contact lens manufacture because of its transparency, light transmission, and permeability to liquids and gases.

Cyclodextrins (CDs) are complex carbohydrates composed of glucose (α-D-glucopyranose) units linked by α-1,4-type bonds, similar in structure to a cone trunk [12]. CDs act mainly as solubilizing vehicles in water and release systems, because the apolar substances are inside the cone, and their interaction with water occurs with the polar part, which is outside the trunk [13].

Hybrid organic-inorganic materials are prepared by combining organic and inorganic components and are an alternative way of producing new multifunctional materials with a wide range of applications. The boehmite-siloxane hybrid is one of the molecular scale composites that present macroscopic properties resulting from the synergy between two nanometric component phases. For example, the varying the phase proportions allows control of refractive index, transparency, and homogeneity. These materials can be used as transparent coatings with high abrasion resistance obtained by curing at low temperatures [14].

Chitosan is a polysaccharide-type biopolymer obtained from the deacetylation of chitin, extracted from the exoskeleton of crustaceans such as the crab [15]. Chitosan has been used in cosmetics, medicines and food additives, and in the development of several types of biomaterial. For example, dissolving chitosan in 0.1 M acetic acid and then evaporating the solvent, it can be made into a fully transparent film. It can therefore be added to the pulp network to increase transparency [16].

Benefiting from its biological properties, BC based contact lenses were associated to cyclodextrin and ciprofloxacin (CP) inclusion [3]. In order to obtain full lens transparency, while maintaining its protective and regenerative properties, bacterial cellulose overlayment was tested with aluminum-organic-inorganic hybrid complex of GPTS (Boehmita-GPTS) (H) or chitosan (Q).

The development of a material to be considered as a compatible biomaterial requires that it should not cause toxic, allergic or carcinogenic reactions [17]. This is essential for a compound to be classified as a biomaterial, since it must be biocompatible and chemically inert or stable.

Therefore, the aim of our study was to demonstrate the development of a TCL consisting of BC incorporating ciclodextrin (CD) to release diclofenac sodium (DS) or ciprofloxacin (CP) and coated with Boehmita-GPTS (H) or chitosan (Q), including the basic initial toxicity evaluation results of those lenses.

Importantly, according to Aberdam et al. [18], the ocular toxicity testing is required to evaluate the risks of drug and cosmetic products before their application to human patients, avoiding irritation or damage to the eyes. Due to the global ban on animal use, many human-derived alternatives have been proposed, such as the ex-vivo enucleated post-death cornea, culture of primary corneal cells, and immortalized corneal epithelial cell lines, all of which share limitations for routine use. Considering these aspects, our study evaluates in-vitro cytotoxicity, genotoxicity and mutagenicity as a first step in investigating these promising materials in TCL's. Further, these biomaterials should be submitted to more specific tests for ophthalmic utilization.

2. Materials and methods

Therapeutic contact lenses were developed from bacterial cellulose (BC) by the Institute of Chemistry of São Paulo State University (UNESP), whose patent has already been lodged (Messaddeq et al., PI 0603704-6 A, publication date 08/04/2008).

2.1. Bacterial cellulose

BC membranes were synthesized by the bacterium *Gluconacetobacter xylinus* (*Acetobacter xylinum*). Cultures were incubated for 96 h at 28 °C in 13 cm diameter petri dishes containing 50 g/l glucose compound, 4 g/l yeast extracts, 2 g/l anhydrous disodium phosphate, 0.8 g/l magnesium heptahydrate sulfate, and 20 g/l ethanol. After 96 h, hydrated BC membranes (3 mm thick) containing up to 99% water and 1% cellulose were obtained. The membranes were washed several times with water, followed by 1% by weight of aqueous NaOH at 80 °C to remove the bacteria, and water until reaching a neutral pH. Finally, the membranes were washed with NaClO.

2.2. Inclusion of drugs and CDs in BC

The wet BC membranes measuring 66 cm^2 were weighed and 50% of their water mass was removed by pressure. The drained BC membranes were immersed in 3 ml of an aqueous solution of CP and/or CDs at the concentrations described in Table 1.

Concentrations were based on commercial eyedrop formulations which were converted from mg/mL to mg/cm^2, the unit used in

Table 1
Compound concentrations used in the bacterial cellulose (BC) lenses.

Lenses	HP-γ-CD (Hydroxypropyl-γ-cyclodextrin) (%)[a]	Diclofenac sodium (%)[a]	Ciprofloxacin hydrochloride (%)[a]
BC + H + CD	0.5538		
BC + Q + CD	0.5538		
BC + H + CD + DS	0.1648	0.0332	
BC + Q + CD + DS	0.1648	0.0332	
BC + H + CD + CP	0.5538		0.1161
BC + Q + CD + CP	0.5538		0.1161

[a] Mass/volume (mg/mL) percentage. H = Hybrid-Boehmita-GPTS; Q = chitosan; CD = cyclodextrin; DS = diclofenac sodium; CP = ciprofloxacin.

pharmaceutical formulations for contact and dressing lenses. The ciprofloxacin concentration was based on that used by Alcon Laboratories Limited (U.K.) for ciloxan. However, the value was divided by 2 to avoid solid precipitation on the surface of the BC membrane.

The membranes were shaken at 100 rpm and 30 °C for 1 h to allow complete absorption of the drug solutions. After absorbing the whole solution, the BC membranes were placed on a plastic Petri dish and dried at 40 °C in a ventilated oven for 16 h. Aqueous solution concentrations in Table 1 were calculated in order to obtain a concentration of 1.75 mg/cm^2 of CP on a dry BC membrane. CD concentrations were calculated considering a 1:1 M ratio. Solutions and membranes with CP were prepared in the dark to avoid decomposition.

2.3. Preparation and application of organic-inorganic hybrid aluminum alkoxide and GPTS (Bohemita-GPTS)

The Boehmite-3-glycidyloxy-propyl trimethoxysilane system (Boe-GPTS) was synthesized according to Caiut et al. [19]. The synthesis proceeded as follows: 25 g (0.10 mol) of aluminum tri-sec-butoxide (Acros Organic) was added to 200 ml of water at 80 °C under vigorous stirring. After one hour of stirring, 0.440 ml of HNO_3 (Synth, 65%) in a ratio of 0.07 mol of HNO_3 to 1 mol of Al^{3+} were added. The temperature was raised to 90 °C, releasing butanol as a by product of the evaporation reaction. After 2 h, the resulting salt was then cooled to room temperature. Then GPTS (Acros Organic) was added to 5 ml of boehmite sol ($[Al^{3+}] = 0.5$ mol/L) under stirring at a Boehmite:GPTS (Al:Si) molar ratio of 1:1. The reaction medium was under continuous stirring for 12 h.

The resulting Boe-GPTS (H) system was used in the preparation of optically clear organic-inorganic bacterial cellulose hybrids. This hybrid was then applied to one side of the membrane using a small brush. Membranes were left in a ventilated oven at 50 °C (instead of 37 °C by the previous method) for complete drying.

2.4. Preparation and application of chitosan

Three grams of chitosan (Q) (Aldrich) were dissolved in 300 ml of 0.1 M acetic acid under stirring and heating at 60 °C for 12 h. The resulting solution was filtered and used for preparing films on BC membrane.

2.5. Functionalized BC prepared in a suitable format for conducting the tests

A mechanical press developed by the Institute of Chemistry (UNESP) was used to shape the BC membranes to the outer curvature of the human eye and create a contact lens (Fig. 3). However, flat samples were more suitable for cutting and processing in the tests performed in this study. After drying, the membranes were cut into 7 mm diameter spherical shapes by a manual cutter. They were sterilized by 15 kGy gamma radiation (Embrarad - Brazilian Company of Radiations Ltda, São Paulo, Brazil).

2.6. Cytotoxicity, genotoxicity and mutagenicity evaluation

2.6.1. Materials

Nine types of lenses with different combinations (Table 2) were tested to evaluate the effect of the separate and associated compounds.

Table 2
Nine types of BC lenses evaluated in cytotoxicity, genotoxicity and mutagenicity assays.

Lenses	Description
BC	Bacterial Cellulose
BC-H	Bacterial Cellulose + Hybrid
BC-Q	Bacterial Cellulose + Chitosan
BC-H-CD	Bacterial Cellulose + Hybrid + Cyclodextrin
BC-Q-CD	Bacterial Cellulose + Chitosan + Cyclodextrin
BC-H-CD-CP	Bacterial Cellulose + Hybrid + Cyclodextrin + Ciprofloxacin
BC-Q-CD-CP	Bacterial Cellulose + Chitosan + Cyclodextrin + Ciprofloxacin
BC-H-CD-DS	Bacterial Cellulose + Hybrid + Cyclodextrin + Diclofenac sodium
BC-Q-CD-DS	Bacterial Cellulose + Chitosan + Cyclodextrin + Diclofenac sodium

2.6.2. Cell culture experiments

CHO-K1, a subclone of the parental CHO cell line, which was derived from the ovary of an adult Chinese hamster, is often used in biological and medical research. CHO cell line has been widely used for studies that assess cytotoxicity and genotoxicity (Yalkinoglu et al. [20] and is recommended by the Organization for Economic Cooperation and Development (2014) (OECD) [21] for genotoxicity screening.

CHO-K1 cells were cultured in HAM-F10: D-MEM (1:1) (Sigma®, St. Louis, MO) culture medium, supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, Brazil) and 1% kanamycin (Gibco, Carlsbad, CA) in 25 cm^2 culture flasks at 37 °C in a 5% CO_2 atmosphere. Each assay was performed in triplicate.

2.6.3. Cell viability (XTT assay)

The XTT assay was performd with CHO-K1 cells and the Cell Proliferation Kit II (Roche Applied Science). This test is based on the cleavage of tetrazolium salt (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) to form an orange formazan dye. However, this conversion occurs only in viable cells due to mitochondrial dehydrogenase activity. Cells were seeded (2×10^4) in 24-well plates in a volume of 1 ml of HAM-F10: D-MEM medium (1:1) supplemented with 10% FBS, and incubated at 37 °C in a 5% CO_2 for 24 h. Nine types of lenses were tested (Table 1). They were placed on the culture medium surface of the wells, in contact with the cells for 24 h. Negative control (NC) consisted of wells containing only cells and culture medium without any material. Positive control (PC) used doxorubicin hydrochloride (3.0 µg/ml for 24 h). All treatments, and controls were performed in duplicate.

At the end of incubation time materials were removed from each well, cultures were washed with PBS, then 500 µl of DMEM without phenol red was 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 colorimetric spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, CA) at 492 and 690 nm. Absorbance is directly proportional to the number of metabolically active cells (viable cells). Cell viability was calculated from absorbance.

2.6.4. Clonogenic survival assay

The clonogenic survival test is an in-vitro assay based on the ability of a single cell to grow, multiply and form a colony, which is defined as being composed of at least 50 cells. Only mitotically viable cells are capable of producing progenitor cells; the number of colonies formed after treatment is an indicator of cell viability and proliferation [22]. After 24 h of seeding, CHO-K1 cells (4×10^4) were exposed to the nine lens types for 24 h. NC consisted of wells containing only cells and culture medium, and for PC cells were treated with doxorubicin (0.3 µg/ml) for 4 h. After exposure, cultures were washed with PBS solution and fresh medium was added. Exponentially growing cells were seeded after treatment, at 150 cells per 25 cm^2 culture flask in duplicate for each treatment. Flasks were incubated at 37 °C, 5% CO_2 for 7 days without changing culture medium. The colonies were fixed with methanol: acetic acid: water (1:1:8 v/v/v) for 30 min and stained with 5% Giemsa. The number of colonies counted in NC was considered 100%. Survival fractions (FS) calculations were performed: FS = number of colonies counted in each treatment × 100 / Number of colonies in NC.

2.6.5. Comet assay

The alkaline version of the Comet Assay was used according to the methodology described by Singh et al. [23]. After 24 h of seeding, CHO-K1 cells (40×10^3) were exposed to the nine types of lenses for 24 h. NC wells containing only cells and culture medium, and PC cells were treated with hydrogen peroxide (80 µmol/L for 10 min). The cells were released by trypsinization, and 500 µL of the cell suspension was transferred to a microtube. After centrifugation, the cell pellet was homogenized with 200 µL 0.5% low melting point agarose. The cell solution was dripped on pregelatinized histological slides (Knittel, Germany) with 1.5% normal melting point agarose (Gibco). Slides were then covered with coverslips and left in the refrigerator for 10 min when, after careful removal of the cover slip, they were immersed in a lysis solution (1% Triton X-100, 10% DMSO, 2.5 mmol/L NaCl, 100 mmol/L Na_2EDTA, 100 mmol/L Tris, pH 10) for 24 h protected from light in the refrigerator. Subsequently, the slides were immersed in alkaline electrophoresis (1 mmol/L Na_2EDTA, 300 mmol/L NaOH, pH > 13) for 20 min for DNA denaturation. Electrophoresis was performed at 43 V and 308 mA for 25 min. Slides were immersed in a neutralization buffer (0.4 mol/L Tris – HCl, pH 7.5) for 15 min and then fixed with ethanol for 3 min.

Slides were stained with ethidium bromide (0.02 mg/ml) and photographed under a fluorescence microscope (ZEISS®, Jena, Thuringia, DEU) equipped with an excitation filter of 515–560 nm, a barrier filter of 590 nm and a 40× objective. DNA damage was evaluated by an image analysis system (TriTek CometScore®1.5, 2006, Sumerduck, VA, USA) to obtain the percentage of tail DNA and Tail Moment for each treatment, with 100 nucleotides analyzed for each repetition. Tail Moment analysis allows us to relate the amount of fragmented DNA to the distance the fragments migrated during electrophoresis [24].

2.6.6. Cytokinesis-blocked micronucleus (CBMN) assay

The Fenech [25] protocol with some modifications was used. CHO-K1 cells were seeded in 25 cm^2 flasks (37×10^4 cells) containing 5 ml of HAM-F10: D-MEM medium (1:1), one culture flask for each treatment. After 24 h seeding, cells were exposed to the nine types of lenses for 24 h. NC wells containing only cells and culture medium, and PC cells were treated with doxorubicin hydrochloride (0.15 µg/mL) for 4 h. Cytochalasin-B (CytB) (1 mg/ml) was added to the CHO-K1 cultures for 24 h to stop cytokinesis. The cells were washed, trypsinized, and centrifuged for 7 min at 1000 rpm. The cell pellet was resuspended in a cold hypotonic solution (0.3% KCl) for 5 min. This cell suspension was centrifuged again and resuspended in 3 ml of methanol:acetic acid (3:1) with four drops of 1% formaldehyde. After further centrifugation, the supernatant was dripped on 2 previously cleaned slides. The slides were stained with 3% Giemsa solution diluted in phosphate buffer (0.06 M Na_2HPO_4 and 0.06 M KH_2PO_4 - pH 6.8) for 7 min, washed and dried at room temperature.

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, according to Eastmond and Tucker [26]. The frequency of binucleate cells with micronucleus (FBMN) and the total frequency of micronucleus (FMN) were analyzed in 1000 binucleated cells for each treatment. The criteria used to identify micronucleus were based on Fenech [25].

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 software. Parametric analysis was by one-way ANOVA followed by Tukey's and Dunnett's tests. Non-parametric analysis was by Kruskall-Wallis followed by Dunn's test. The level of significance was 5%.

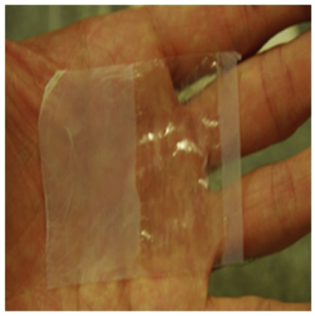

Fig. 1. Bacterial cellulose membrane impregnated with organic-inorganic hybrid.

3. Results

3.1. Morphology

Figs. 1 and 2 show membranes of pure BC and with organic-inorganic hybrid and chitosan, respectively. Visually, it may be noted that the presence of the coatings provided transparency to the BC.

The characterization of the bacterial cellulose membrane and the bacterial celulose coated with the hybrid material were described before by our research group in two manuscripts. These studies include the optical transmission spectra for both membranes [27,28].

3.2. Cytotoxicity

3.2.1. Cell viability (XTT assay)

All tested lenses showed high cellular viability with values larger or similar to NC (Fig. 4). Cell viability was lower in BC-Q-CD-CP and BC-Q-CD-DS compared to NC (88% for BC-Q-CD-CP and 83% for BC-Q-CD-DS). However, this decrease was not statistically significant ($p > 0.05$; Dunnet's)

3.2.2. Clonogenic survival assay

BC-H-CD-DS and BC-Q-CD-DS presented significantly different survival fractions compared to NC ($p < 0.001$; Dunnett's) (Fig. 5), indicating a compromise in cell proliferative capacity. BC, BC-H, BC-H-CD and BC-H-CD-CP did not present a statistically different survival fraction compared to NC ($p > 0.05$; Dunnett's).

Both cytotoxicity tests (XTT and Clonogenic Survival) present similar results showing that BC-H and BC-H-CD were not cytotoxic. They displayed higher cell viability in the XTT test and did not affect cell proliferative capacity in the Clonogenic Survival test. In contrast, BC-H-CD-DS and BC-Q-CD-DS despite not presenting a significant decrease in cell viability (XTT), significantly compromised cell proliferative capacity (Clonogenic Survival).

3.3. Genotoxicity - comet assay

Results obtained from the percentage of DNA in tail and Tail Moment were non-parametric; we therefore used the Kruskal-Wallis test followed by Dunn's test.

Analyzing percentage of DNA in the tail (Fig. 6) showed that BC-H-CD and BC-H-CD-DS presented a genotoxic effect ($p < 0.05$; Dunn's). The other lenses did not show a statistically significant difference compared to NC ($p > 0.05$; Dunn's).

Analysis of genotoxicity by Tail Moment (Fig. 7) showed BC-H-CD-DS was genotoxic ($p < 0.05$; Dunn's). The other materials did not present a genotoxic potential ($p > 0.05$; Dunn's).

3.4. Mutagenicity - cytokinesis-block micronucleus assay

A mutagenicity test was performed to complement genotoxicity

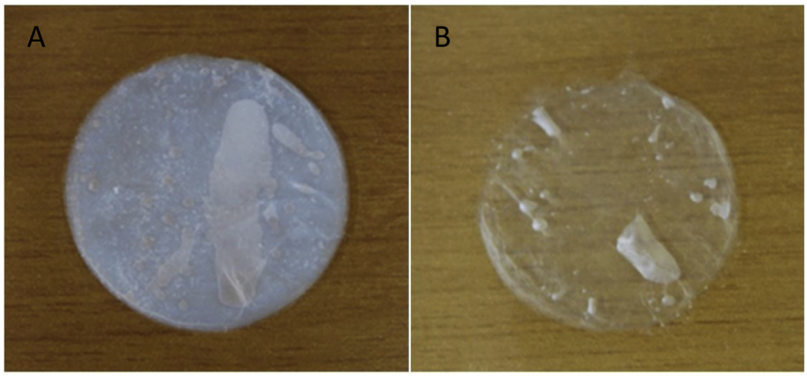

Fig. 2. A. Bacterial cellulose membrane without chitosan. **B.** Bacterial cellulose membrane impregnated with chitosan.

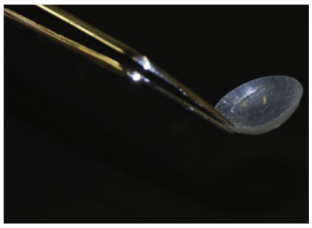

Fig. 3. BC combined with γ-CD in the shape of a contact lens. Reproduced from Cavicchioli et al. [15].

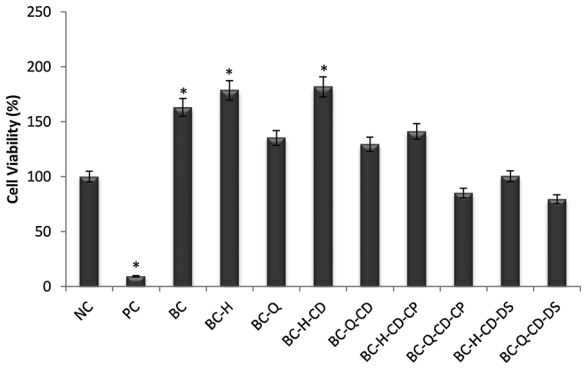

Fig. 4. Cytotoxicity (cell viability) assessed by the XTT assay. Columns indicate cell viability. Bars indicate standard error. * statistically significant compared to NC ($p < 0.05$; Dunnett's test).

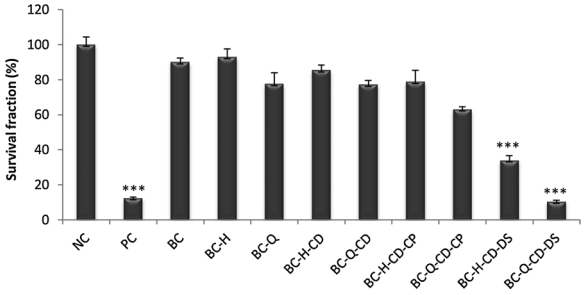

Fig. 5. Cytotoxicity assessed by Clonogenic Survival assay. Columns indicate the mean value of the Survival Fraction (%). NC represents 100% of survival fraction. Bars indicate the standard error. *** statistically significant compared to NC ($p < 0.001$). Dunnett's Test.

analysis. Cytokinesis-block micronucleus assay (CBMN) results from Nuclear Division Index (NDI), Frequency of Binucleate cells with Micronucleus (FBMN) and Frequency of Micronucleus (FMN) are shown in Table 3.

In NDI, statistically significant differences were seen between NC

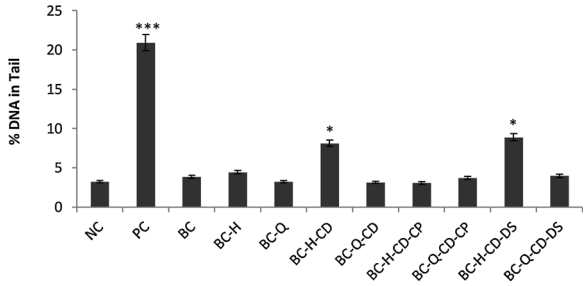

Fig. 6. Genotoxicity evaluated by the Comet Assay. Columns indicate the mean value of the percentage of DNA found in the nucleoid tail. Bars indicate standard error. * = $p < 0.05$; *** = $p < 0.001$. Dunn's Test.

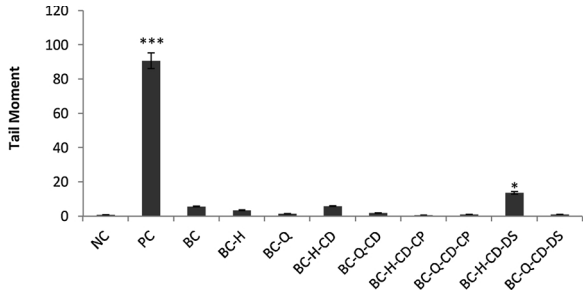

Fig. 7. Genotoxicity assessed by Comet Assay. Columns indicate mean Tail Moment. Bars indicate standard error. * = ($p < 0.05$); *** = ($p < 0.001$). Dunn's Test.

Table 3
Mean and standard error (SE) values for Nuclear Division Index (NDI), Frequency of Binucleate cells with Micronucleus (FBMN) and Frequency of Micronucleus (FMN) from each treatment.

Treatment	NDI Mean ± SE	FBMN Mean ± SE	FMN Mean ± SE
NC	1.83 ± 0.02	9.00 ± 1.73	9.33 ± 2.03
PC	1.71 ± 0.05[a]	65.00 ± 15.18[b]	103.70 ± 17.46[b]
BC	1.78 ± 0.05	11.33 ± 3.38	12.00 ± 4.04
BC-H	1.84 ± 0.03	11.00 ± 1.53	12.00 ± 1.00
BC-Q	1.85 ± 0.101	7.70 ± 1.04	7.70 ± 1.04
BC-H-CD	1.72 ± 0.05[a]	10.00 ± 1.00	10.67 ± 1.33
BC-Q-CD	1.84 ± 0.062	9.20 ± 3.55	9.20 ± 3.55
BC-H-CD-CP	1.79 ± 0.02	9.67 ± 0.88	11.00 ± 1.45
BC-Q-CD-CP	1.78 ± 0.125	14.80 ± 1.15	15.00 ± 1.32
BC-H-CD-DS	1.68 ± 0.04[a]	11.67 ± 0.33	12.33 ± 0.58
BC-Q-CD-DS	1.93 ± 0.047	12.00 ± 1.32	12.00 ± 1.32

[a] $p < 0.001$; Tukey's Test.
[b] $p < 0.001$; Dunnett's Test (compared to NC).

and PC, BC-H-CD and BC-H-CD-DS, showing that the cellular division process seemed to suffer some interference of those treatments.

In FBMN evaluation, no statistically significant differences were seen between tested lenses and NC. High micronucleus (FMN) number was only observed in PC as expected. Therefore, all tested lenses did not present a mutagenic effect.

4. Discussion

Biotechnological advances have been made in the development of biomaterials for tissue regeneration and in their use as medical devices due to the beneficial physicochemical characteristics they offer. However, relevant improvements in TCLs have been on demand. According to Dumbleton [29], silicone hydrogel contact lenses have overcome many of the hypoxic problems. However, when these lenses are worn on a continuous basis they may cause adverse events such as peripheral ulcers, acute red eye, infiltrative keratitis and papillary conjunctivitis. Although these conditions do not threaten sight, they can be painful and certainly inconvenient.

The creation of new bacterial cellulose-based contact lenses will provide a new strategy in minimizing or avoiding adverse events caused by silicone hydrogel contact lenses as they use another type of polymer; it could also lead to a reduction in the cost of contact lense therapies.

It is essential that development of or improvements in biomaterials should be accompanied by appropriate toxicity evaluations. For example, adverse effects such as genotoxicity or mutagenicity are likely to occur during the cell division process due to the possibility of free particles from such a biomaterial penetrating a cell in contact with the material and interacting with its DNA. According to Singh et al. [30], indirect DNA damage such as the promotion of oxidative stress and inflammatory response may also occur because of the presence of biomaterials. It is therefore necessary to ensure the safety of developed materials by minimizing any associated potential toxicological risks in order to protect human health and the environment. The first step in verifying the safety of a new biomaterial is evaluating its toxicity. In our study we present the cytotoxic, genotoxic and mutagenic results of these new therapeutic contact lenses.

Cytotoxicity is the ability of a material to cause damage that causes cell death or reduces its ability to proliferate. The XTT assay is considered a good method for evaluating cytotoxicity as it quantifies the ability of the dehydrogenase enzyme present in the mitochondria to convert tetrazolium salt XTT (yellow color) to formazan compounds (orange color) by spectrophotometry [31]. The Clonogenic Survival Test is another method of evaluating the ability of cells to form colonies and generate daughter cells after a treatment that induces death associated with cell division [22].

Genotoxicity is defined as the potential that a substance has to react with the DNA of the cell. Such contact may result in either a genotoxic effect if the endogenous DNA repair enzymes can repair single and/or double strands of DNA; a mutagenic effect happens when changes in cell DNA cannot repair and are stably transmitted to daughter cells in the cell division process [32]. Saska et al. [33] and Moreira et al. [34] demonstrated the non-cytotoxic nature of BC, which agree with our results in this study where XTT and Clonogenic Survival assays confirmed the non-cytotoxic nature of BC. BC biocompatibility was demonstrated *in-vivo* by the absence of inflammation after 12 weeks and 12 months application of the material in animal models [35,36].

The non-genotoxic effect of BC has previously been described [37], as has the non-genotoxic effect of BC nanofibers [34]. In agreement with Saska et al. [33], our study demonstrated no cytotoxicity, genotoxicity and mutagenicity of BC. It is worth noting that the Comet Test is not used to detect mutations, but to detect genomic lesions that can become mutations [32]. The Micronucleus Assay detects chromosomal breaks and aneuploidies, drastic lesions that cannot be repaired by the cellular DNA repair machinery [38].

In the lenses containing cyclodextrin and hybrid only (BC + H + CD), a genotoxic effect could be verified (Fig. 6). However, cytotoxic and mutagenic potentials were not observed. The genotoxic effect found in this material is in disagreement with Cavicchioli et al. [3], as no cyclodextrin related DNA damage was observed. Usually, cyclodextrin does not present cytotoxic or mutagenic effects. Further studies should investigate whether possible adjustments in cyclodextrin concentration could override this genotoxic potential. It can therefore be verified that the addition of CD to BC did not alter the non-cytotoxic behavior of BC and increased cell viability (XTT analysis) except when DS was present. Interestingly, the presence of HP-γ-CD has been verified in concentrations of up to 10 mM for a period of 1 h incubation in endothelial cells *in-vitro* with no cytotoxic effect [39], our study also did not demonstrate this effect.

The addition of organic-inorganic hybrid complex and chitosan to the contact lenses increased lens transparency and did not cause any toxic effects impairing cell viability, or leading to genotoxicity or mutagenicity. This suggests that the inorganic-organic hybrid complex and chitosan have a good potential for use in conjunction with BC in developing a good TCL.

The cytotoxicity of ciprofloxacin in BC-H-CP was not observed by XTT and Clonogenic Survival assays; this disagrees with Kloskowski et al. [40].

Our results showed that lenses with diclofenac sodium showed late cytotoxicity and genotoxic potential only when associated with organic-inorganic hybrid complex, but were not mutagenic. Cavicchioli et al. [3], who also studied BC based contact lenses with the same drugs, verified that the materials with added diclofenac sodium were cytotoxic, genotoxic and mutagenic.

Our study demonstrated cytotoxicity in materials with DS in TLC composition (BC-H-CD-DS and BC-Q-CD-DS). We did not evaluated different lense DS concentrations, but Takahashi et al. [41] verified a dose dependent cytotoxic effect with diclofenac sodium by observing cell proliferation inhibition. Lauer et al. [42] evaluated the cytotoxic effects of diclofenac sodium by measuring cellular ATP content as an indicator of cellular vitality. Different diclofenac doses have been used to treat primary rat and human hepatocytes in monolayer cultures in order to analyze species differences in drug-induced cytotoxicity. Both hepato-toxicants clearly showed a strong dose response effect on cellular ATP content as a measure of cell viability. The use of 10 μM diclofenac sodium showed 100% cell viability, reducing to 80% with 100μM, and 40% with 500 μM. The authors reported that diclofenac sodium dose was related to cytotoxicity effects.

The Clonogenic Survival or Colony Formation Assay is important for investigating whether a drug may have a long-term cytotoxic effect leading to cell lethality that could have a dose-dependent effect [43]. This test is performed to complement the investigation of possible cytotoxic action of tested materials and to evaluate possible damage in the ability of CHO-K1 cells to mitotically replicate. There is the possibility that, under the action of a substance, a cell does not undergo immediate cytotoxic action. The XTT assay showed cell viability, however some damage could have occurred compromising cell proliferative capacity. In the case of our study lenses, results from evaluating immediate (XTT) and late cytotoxicity (Clonogenic Survival) were in agreement, except for BC-H-CD-DS and BC-Q-CD-DS. Significant cytotoxicity (XTT) has been shown to significantly compromise cell proliferative capacity (Clonogenic Survival).

Comet assay did not find genotoxicity with diclofenac sodium (0.1 mM) and some other nonsteroidal antiinflammatory drugs (NSAIDs) including naproxen (1 mM), ketoprofen (1 mM), and ibuprofen (1 mM) [44]. This differs from our Comet assay results where genotoxicity was found in BC-H-CD-DS. Future studies are therefore needed to investigate whether cytotoxic and genotoxic effects could be absent at different diclofenac sodium concentrations in the TCL's developed by our group,.

NDI is a cell proliferation marker in cultures where it is considered a measure of general cytotoxicity. We observed decreased NDI in BC-H-

CD, BC-H-CD-DS, and PC. The hypothesis is that cells effected by mutagenic agents undergo DNA damage and cannot survive the cell division cycle and enter into a process of necrosis or apoptosis before the end of first division. Mitotic delay may also be induced, which by not allowing the repair of genotoxic lesions, will modify the number of cells entering mitosis, modifying the proportion of mono, bi, tri and tetranucleate cells. Thus, NDI is lower due to the smaller number of dividing cells. Moreover, there is a hypothesis of a clastogenic effect of mutagenic agents with aneugenic action, inducing some degree of cell cycle blockade. Therefore, more cells will not divide and NDI will remain low [45].

Analysis of the micronucleus test *in-vitro* in a human lymphocyte line verified that the quantity of micronuclei in binucleate cells increased according to the increase in DS dose, although the result was not statistically significant when compared to the negative control [46]. Ibrulj et al. [46] demonstrated that increased DS dose is related to increased mutagenic potential. Therefore, further studies should be performed to adjust DS dose or use another anti-inflammatory drug to replace DS in the TCL.

To eliminate the effect of BC-H-CD-DS and BC-Q-CD-DS on impairing the proliferative capacity of CHO-K1 cells, future studies should test different concentrations of DS as well as potentiate the action of cyclodextrin (CD) so that DS can be released more slowly.

Wilson et. al [47] conducted an overview of current ocular toxicity testing techniques. They stated that in recent years, legislation in many developed countries has been introduced to promote alternative techniques and attempt to reduce animal testing, such as the Draize test, which determines ocular toxicity level by applying substances to a live rabbit eye and evaluating biological response. New techniques include ex-vivo testing in dead animal tissue, computational models that use algorithms to apply existing data to new chemicals and in-vitro assays based on two-dimensional (2D) and three-dimensional (3D) cell culture models. From the reviewed tests, the authors stated that in-vitro toxicity testing using cultured cells are advantageous over in- and ex-vivo tests, because they are relatively inexpensive, simple and quick to manufacture.

Although not retinal like ARPE-19, the CHO-K1 lineage that we used in our initial toxicity tests is recommended by OECD [21]. Our study presents very important results demonstrating that the majority of the developed TCL's tested were safe for future use as medical devices. Obviously, this is an initial and extremely important step in the evaluation of biomaterials developed for safe and efficient use in ophthalmology. After evaluating the toxicity of these lenses by the aforementioned assays, further studies will be conducted to ensure their effectiveness as therapeutic contact lenses. In addition, no significant difference has been found between ARPE-19 and HEK 293 T (non-retinal cell lineage) in evaluating the cytotoxicity of chitosan nanoparticles for ocular administration [48]

5. Conclusions

We have demonstrated promising results in the safety of employing BC lenses functionalized with a drug delivery system permitting the bioavailability of ophthalmic drugs. Only BC-H-CD-DS presented cytotoxic and genotoxic effects and BC-Q-CD-DS showed a cytotoxic effect. These results were probably related to the influence of DS. Further studies utilizing other specific tests, such as employing corneal lineage are required before their safe and efficient ophthalmologic use.

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Conflict of interest

The authors have declared that there are no conflicts of interest.

Disclaimer

This document represents the consensus of the participants' and the views expressed by the individual scientists and does not necessarily represent the policies and procedures of their respective institutions.

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