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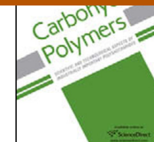

Acute toxicity, cytotoxicity, genotoxicity and antigenotoxic effects of a cellulosic exopolysaccharide obtained from sugarcane molasses

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ABSTRACT

The acute toxicity, cytotoxicity, genotoxicity and antigenotoxic effects of BC were studied. Cytotoxicity of BC was evaluated in cultured C3A hepatoma cells (HepG2/C3A) using a lactate dehydrogenase (LDH) activity assay. Acute toxicity was tested in adults Wistar rats treated with a single dose of BC. The genotoxicity of BC was evaluated *in vivo* by the micronucleus assay. BC (0.33–170 μg/mL) added to C3A cell culture medium caused no elevation in LDH release over the background level recorded in untreated cell wells. The treatment with the BC in a single oral dose (2000 mg/kg body weight) caused no deaths or signs of toxicity. BC attenuated CP-induced and inhibition the incidence of MNPCE (female: 46.94%; male: 22.7%) and increased the ratio of PCE/NCE (female: 46.10%; male: 35.25%). There was no alteration in the LDH release in the wells where C3A cells were treated with increasing concentrations of BC compared to the wells where the cells received the cell culture medium only (background of approximately 20% cell death), indicated that in the dose range tested BC was not cytotoxic. BC was not cytotoxic, genotoxic or acutely toxic. BC attenuated CP-induced genotoxic and myelotoxic effects.

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1. Introduction

Bacterial Cellulose (BC) is an exopolysaccharide obtained from sugar cane molasses by flotation in the form of a gelatinous matrix (Paterson-Beedle, Kennedy, Melo, Lloyd, & Medeiros, 2000). It is composed of stable polymerized sugars. Owing to its chemical composition and physical properties, BC is a promising biomaterial for many medical and biological uses (Coelho et al., 2002; Lee, Buldum, Mantalaris, & Bismarck, 2014; Martins, Lima, Araujo, Vilar, & Cavalcante, 2013; Silva, Aguiar, Marques, Coelho, & Rolim Filho, 2006; Teixeira, Pereira, Ferreira, Miranda, & Aguiar, 2014). It has been used in different areas of surgery, such as urethral reconstruction (Chagas, Aguiar, Vilar, & Lima, 2005), bio-sling for treatment of urinary incontinence (Gonçalves et al., 2006; Lucena et al., 2005), and as a bulking agent in orthopedics (Albuquerque, Santos, Aguiar, Pontes, & Melo, 2011), ophthalmology (Cordeiro-Barbosa, Aguiar, Lira, Pontes Filho, & Bernardino-Araújo, 2012) and urology (Lima et al., 2015).

Although offering no foreseeable risks to patients, an experimental assessment of the cytotoxic and genotoxic potential of BC remains necessary to ensure that it is safe for use in medical products.

The present study was undertaken to evaluate BC cytotoxicity on the human cell line (HepG2/C3A) and its *in vivo* genotoxic potential. A possible modulation of cyclophosphamide (CP)-caused genotoxic and myelotoxic effects by BC was also investigated using the mouse bone marrow micronucleus test.

2. Materials and methods

2.1. Test material: Bacterial Cellulose (BC)

BC was produced from sugar cane at the Carpina Experimental Station of the Federal Rural University of Pernambuco, Brazil (Paterson-Beedle et al., 2000). Sugar cane molasses is the only raw material used for the synthesis of BC. The molasses is adjusted to the ideal brix in order to facilitate the digestion process. Once synthesized, the biopolymer is submitted to a chemical procedure to reduce residual sugars. It is then converted to a gel by a fragmentation technique and by mechanical shock. At this preparation step, the product undergoes a water vacuum extraction process

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that produces the BC matrix. From the BC matrix, three products are obtained: a film, a hydrogel and lyophilized forms of the BC.

BC samples tested in this study were prepared from 0.8% hydrogel.

Preparations of BC acquire viscoelastic properties and remain stable at concentrations of 0.6% and 0.8% at the usual storage temperatures and in biological fluids (0–40 °C). These properties make it applicable *in vivo* (Pita et al., 2015). Owing to its chemical composition and physical properties, BC does not induce immune responses and thus it is regarded as a promising biomaterial with an extensive range of applications in biological and medical sciences (Lee et al., 2014).

2.2. Cytotoxicity assay

2.2.1. Cell culture conditions

C3A hepatoma cells [HepG2/C3A, derivative of HepG2 (ATCC HB-8065)] (ATCC® CRL-10741™) were maintained in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM) (Sigma D6046) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 100 U/mL penicillin/100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere (Küblbeck et al., 2011). The cells were subcultured once a week and cells used in this study were from passages 15 and 16. Cell viability was evaluated at each subculture using a 0.4% trypan blue solution.

2.2.2. Treatment of C3A cells

BC was dissolved in the culture medium and solutions were prepared to reach the following target concentrations in the wells (in replicates of 4 wells for each BC concentration tested): 170 μg/mL, 85 μg/mL, 42.5 μg/mL, 21.25 μg/mL, 10.625 μg/mL, 5.3 μg/mL, 2.65 μg/mL, 1.328 μg/mL, 0.66 μg/L and 0.33 μg/mL.

C3A cells were plated on 48-well culture plate wells (5.3×10^4 cells/cm^2). The cells were treated with BC solutions and incubated at 37 °C in a 5% CO_2 incubator for 20 h. Negative controls received an equivalent volume of culture medium in place of BC solution.

2.2.3. Measurement of lactate dehydrogenase (LDH) activity

At the end of the incubation period, BC cytotoxicity was evaluated by measuring lactate dehydrogenase (LDH) activity (CytoTox 96® Non-Radioactive Cytotoxicity Assay kit; Promega G1780) according to manufacturers' instructions. Briefly, 50 μL cell-culture medium from each well from the 48-well plate was collected and transferred to the wells in a 96-well plate. The 48-well plate was submitted to a freeze–thaw cycle and afterwards 50 μL of the cell culture medium was collected and transferred to wells in the 96-well plate. The reconstituted substrate mix (50 μL) was added to all wells and let stand, protected from light at room temperature for 30 min. 50 μL of the Stop solution was added to each well and the plate was taken to read the absorbance at 490 nm in a microplate spectrophotometer reader *Spectramax Plus – Molecular Devices*®, with 4.0 *SoftmaxPro software* for *Macintosh*® and *Windows*®. Background values (cell culture medium) were subtracted from the sample readings for phenol red correction. An LDH positive control (bovine heart LDH) supplied with the kit was used to validate the assay. Percentages (%) of cell death (percentage of LDH release) were determined using the formula: (experimental LDH release/maximum LDH release) × 100.

2.3. Acute toxicity test

2.3.1. Animals

Adult Wistar rats, 5 males and 5 females, approximately 85 days old, were used in the experiment. The animals were individually housed in rat standard plastic cages with stainless steel coverlids and wood shavings, under controlled environmental conditions (light–dark cycle of 12 h; temperature 22 ± 1 °C; relative humidity approximately, 70%). A standard rodent pellet diet (Nuvital, Nuvilab Ltd., Curitiba, PR, Brazil) and water were provided *ad libitum*.

2.3.2. Treatment

The animals received a single dose of BC (2.000 mg of BC to 0.8%/kg of body weight, bw) by gavage. The maximum (limit) dose was chosen based on OECD 423 protocol recommendations (OECD, 2001). Since this upper limit dose caused no deaths and any discernible signs of toxicity, lower doses were not tested.

The animals were weighed daily and BC was administered after a 12-h fasting period.

2.3.3. Signs of toxicity

Rats were examined for behavioral changes or any clinical sign of toxicity every 30 min during the first 4 h following the treatment and thereafter once a day for 14 consecutive days. The animals were euthanized with a lethal dose of sodium thiopental, administered intraperitoneally. After euthanasia, all animals were submitted to necropsy and organs were macroscopically inspected for any abnormality.

The procedures were performed at the Center for Experimental Surgery/UFPE, Recife, PE, Brazil.

2.4. In vivo *mouse bone marrow micronucleus assay*

2.4.1. Animals

Adult Swiss Webster (*Mus musculus*) mice (25 males and 25 females), approximately 55 day-old, from the Fiocruz Central Animal House breeding stock, were used in the experiments. The animals were housed individually in standard mouse plastic cages with stainless steel coverlids, and kept under controlled environmental conditions (light–dark cycle of 12 h; room temperature 22 ± 1 °C; relative humidity approximately, 70%). A standard rodent pellet diet (Nuvital, Nuvilab Ltd., Curitiba, PR, Brazil) and filtered water were provided *ad libitum*. This study was performed in accordance with International Agency recommendations (FDA, 2000; OECD, 2013).

2.4.2. Treatment

For this study, animals were divided into six groups, each group consisting of 5 male and 5 female mice. Groups were one vehicle (water) control group, one positive control group (cyclophosphamide, CP), two BC-treated groups and an additional group of mice that received BC orally for 3 days and a single dose of CP by the ip route on the third day (group 6).

Vehicle controls received water orally (po) at a dose of 10 mL/kg bw/d (group 1). BC-treated groups were administered with a dose as high as 200 mg BC 0.8%/kg bw/d for 3 consecutives days administered by gavage (group 2) or by intraperitoneal injection (group 3). Positive control mice (group 4) were treated intraperitoneally (ip) with a single dose of cyclophosphamide (CP, 25 mg/kg bw ip). To evaluate whether BC would alter CP-induced clastogenic effects, animals were treated (group 5) by gavage (po) with BC (200 mg BC to 0.8%/kg bw/day) for 3 consecutive days and with CP (25 mg/kg bw ip) 45 min after the third dose of BC.

Animals were observed once a day for clinical signs of toxicity. Mouse body weights were recorded on treatment days. Twenty-four hours after CP injection or the last dose of BC or the vehicle, all animals were euthanized. The bone marrow was flushed from both femur bones with an injection of fetal calf serum to obtain bone marrow cell suspensions.

2.4.3. Preparation of bone marrow cell slides, analysis and data interpretation

The air-dried slides of bone marrow cell suspensions were stained with May-Grunwald and Giemsa for evaluation (Krishna & Hayashi, 2000). Slides were scored under a light microscope (magnification 100× with an immersion objective) by an experienced evaluator kept unaware of mouse prior treatment. To determine the ratio of PCE to total erythrocytes (PCE + NCE), at least 200 erythrocytes (*i.e.*, polychromatic erythrocytes (PCE) plus normochromatic erythrocytes (NCE)) from each animal were scored PCE/NCE = [(PCE/(PCE + NCE) − PCE)]. The increase of the PCE (%) in the group 5 was calculated by the formula: [(PCE/NCE in group 5) − (PCE/NCE in group 4)]. To calculate the incidence of micronucleated polychromatic erythrocytes (MNPCE) a minimum of 2000 PCEs were scored per mouse. The percentage of reduction of CP-caused increase in MNPCE was calculated using the formula as follows (Waters, Brady, Stack, & Brockman, 1990): Reduction of CP-genotoxicity (%) = [(MNPCE in CP-treated) − (MNPCE in BC + CP)/(MNPCE in CP − MNPCE in vehicle control)] × 100. All experiments were performed at the Laboratory of Environmental Toxicology, National School of Public Health, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil.

2.5. Ethical clearance for animal experimentation

The study protocol was approved by the Ethics Committee for Animal Care of the Oswaldo Cruz Foundation, FIOCRUZ (CEUA-FIOCRUZ, Protocol No. P-19/14-4; License No. LW-3/15).

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) or, alternatively, by the Kruskal–Wallis test whenever the data did not fit a normal distribution. Differences between two groups were tested by two-tailed Student's *t*-test, Tukey test *post hoc* test, or Mann–Whitney *U*-test. Proportions were evaluated by the chi-square test or, alternatively, by the Fisher exact test. Statistical analysis was performed using the GraphPad Prism 5.0 program (GraphPad Software Inc., USA), and a difference was considered statistically significant at $p < 0.05$.

3. Results

3.1. Cytotoxicity – LDH release

Cytotoxicity was assessed by measuring LDH in the medium after 20 h cell treatment with different concentrations of BC. Results (LDH release expressed as % of maximum LDH obtained for total cell lysis) presented here are the mean of two independent experiments. Statistical analysis (ANOVA) showed that there was no alteration in the LDH release in the wells where C3A cells were treated with increasing concentrations of BC (0.33–170 μg/mL) compared to the wells where the cells received the cell culture medium only (background of approximately 20% cell death) (Fig. 1). These results indicated that in the BC dose range tested, the BC was not cytotoxic.

3.2. Acute toxicity test

No deaths or any other clinical signs of toxicity were noted in rats treated with BC. All BC-treated rats (2000 mg/kg bw po) increased their body weight during the 14 post-treatment days. The mean body wt before treatment was 346.4 ± 41.2 g and after treatment it was 370.4 ± 38.4 g. No gross pathological abnormality was observed in major organs at necropsy and so histology was considered unnecessary. Since no toxicity was noted at this rather high dose, lower doses were not tested.

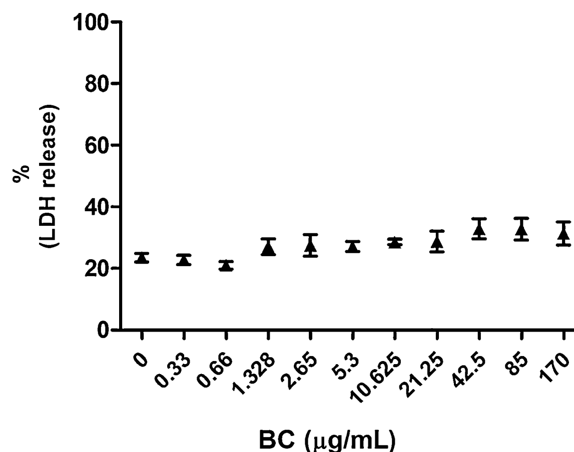

Fig. 1. LDH release (%) in culture medium after a 20 h treatment of C3A cells with BC (0, 0.33, 0.66, 1.328, 2.65, 5.3, 10.625, 21.25, 42.5 and 170 μg/mL).

3.3. In vivo *mouse bone marrow cell micronucleus assay*

BC caused no deaths or any clinical signs of toxicity in treated mice. Three-day treatment with BC (200 mg/kg bw/d × 3d, ip and po) caused no statistically significant changes in mouse mean body weights (females, $p = 0.2977$; males, $p = 0.1844$).

Results of the bone-marrow micronucleus assay are summarized in Table 1. The ratios of MNPCE to PCE in male and female mice treated with BC (200 mg/kg bw/d × 3d, po, ip) were comparable to those background ratios obtained for vehicle-control animals. Nonetheless, in the group of mice treated with BC the myelotoxic (ratio of PCE/NCE) and clastogenic (% of MNPCE) effects of CP were clearly attenuated.

4. Discussion

Bacterial Cellulose (BC), a by-product of the sugarcane production process, has a chemical structure that consists of polymerized sugars. This biopolymer is stable and is not digested by the surrounding tissues (Paterson-Beedle et al., 2000; Pita et al., 2015).

Although a product of natural origin, BC must undergo a rigorous safety assessment to meet national and international safety testing requirements, before it can be placed on the market in different countries. Product safety assessment involves a number of tests to disclose any potential health risk under normal conditions or reasonably foreseeable conditions of use (ANVISA, 2001; FDA, 1994).

In this article we presented and discussed results from a set of preliminary toxicity tests including an *in vitro* cytotoxicity test (LDH release in cultured HepG2 cells) (Küblbeck et al., 2011), an acute toxicity test (OECD, 2001) and an *in vivo* genotoxicity assay (mouse bone marrow micronucleus test) (FDA, 2000; OECD, 2013).

The LDH cytotoxicity assay is based on the release of lactate dehydrogenase into the culture medium whenever plasma cell membranes are broken (*e.g.*, cell lysis). In this study, an *in vitro* standardized assay was performed to investigate whether BC (0.8%) in tested in growing concentrations up to 170 μg/mL would damage seeded C3A cells. Negative results obtained in this assay are consistent with those of a previous study, in which *in vitro* cytotoxicity of Bacterial Cellulose was evaluated in rat alveolar macrophages using [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay, cells adhesion rate and nitric oxide production,

Table 1
Summary of bone marrow cell micronucleus test results after administration of BC (200 mg/kg bw/d × 3d) by oral (po, gavage) or intraperitoneal route (ip) to male and female Swiss Webster mice.

Groups	Females				Males			
	MNPCE (%)	Inhibition (%)	PCE/NCE	Increase (%)	MNPCE (%)	Inhibition (%)	PCE/NCE	Increase (%)
1. Vehicle-control (po)	0.19 ± 0.09[b,e]	–	1.09 ± 0.09[b]	–	0.10 ± 0.09[b,e]	–	1.12 ± 0.13[b,e]	–
2. BC (ip)	0.21 ± 0.04[b,e]	–	1.07 ± 0.16[b]	–	0.13 ± 0.04[b,e]	–	1.20 ± 0.26[b,e]	–
3. BC (po)	0.17 ± 0.04[b,e]	–	1.20 ± 0.16[b,e]	–	0.16 ± 0.06[b,e]	–	1.17 ± 0.07[b,e]	–
4. CP (25 mg/kg bw, ip)	1.66 ± 0.15	–	0.38 ± 0.03	–	1.33 ± 0.20	–	0.42 ± 0.12	–
5. BC (po) + CP (25 mg/kg bw, ip)	0.97 ± 0.07[a,b,c,d]	46.94	0.84 ± 0.07[b]	46.10	1.05 ± 0.15[a,b,c,d]	22.76	0.77 ± 0.09[a,b,c,d]	35.25

CP: cyclophosphamide (positive control, 2 mg/kg bw, ip); BC: Bacterial Cellulose; po: gavage; ip: intraperitoneal injection; MNPCE: micronucleated polychromatic erythrocytes; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes. Values are expressed as mean ± SD and percentages (%). Tukey's test, if $p < 0.05$.
[a] Vehicle control.
[b] CP.
[c] BC ip.
[d] BC po.
[e] BC po + CP ip.
Mice were euthanized 24-h after the last administration of BC, CP or the vehicle. Increase or inhibition % compared to group 4 (CP = 100%).

where results did not differ from the negative controls (Castro et al., 2004).

The *in vivo* tests (rat acute toxicity, and mouse bone marrow micronucleus test) conducted with BC are also recommended by international guidelines (ANVISA, 2001; FDA, 1994, 2000; OECD, 2001, 2013).

Acute toxicity tests are intended to evaluate the detrimental effects that may arise from a single or several doses administered over a period not exceeding 24 h. Since toxic effects may occur after a long latency period, treated animals should be observed for at least one or two weeks after treatment (ANVISA, 2001; Lucena et al., 2015; Naik, Rozman, & Bhat, 2013). Dose selection is one of the most critical issues when designing the study. Choosing an adequate dose and avoiding killing unnecessarily a large number of animals should be a priority goal. In this study we selected 2000 mg/kg because it is a reasonably high dose for this test. As no sign of toxicity was found at this very large dose, we decided against testing lower doses.

The mouse bone marrow micronucleus (MN) assay is one of the most used in *in vivo* tests for the evaluation of the genotoxic potential of chemicals. Rodent micronucleus assay is considered to be a reliable, quick and sensitive screening method for genotoxic carcinogens (carcinogens which act by causing genetic damage). The frequency of micronuclei in treated cells is a biomarker for chromosomal damage and chromosomal losses (clastogenic) (OECD, 2013). In the MN assay, reductions in polychromatic erythrocyte (PCE)/normochromatic erythrocyte (NCE) ratio compared to vehicle controls indicate unspecific toxicity to bone marrow cells or myelotoxic, thus providing an index of mitotic activity (Naik et al., 2013). In this study, BC caused no alteration of the PCE/NCE ratio, while the positive control agent CP dose (25 mg/kg bw ip) produced a marked reduction of the ratio (in male and female mice) compared to the vehicle control group ratio. BC also did not alter the incidence of MNPCE (in males and females), indicating that this biopolymer was not genotoxic/clastogenic to Swiss Webster mice.

Moreover, BC clearly antagonized the myelotoxic and genotoxic effects of CP in the assay. The mode of action (MOA) by which BC protects against CP-genotoxicity and myelotoxic, however, remains unclear. Further research should clarify whether the BC-protective effect is dose-dependent and whether it is observed with direct clastogens and also with other indirect clastogens (*i.e.*, genotoxic compounds requiring metabolic activation). Possible BC MOAs might involve a blockade of CP-activation by CYPs and/or antioxidant effects, and/or other actions.

Data provided by experiments reported in this paper add to existing data suggesting that BC is a promising material for a number of medical uses. Previous studies have shown that mesenchymal stem cells (MSC) adhere to a biopolymer film and suggest that BC films could be used as cell culture substrates as a good platform for cell adhesion (Fragoso et al., 2014). It is known that BC is biocompatible and a material able to induce tissue remodeling and to integrate physiologically with tissue (Cordeiro-Barbosa et al., 2012; Fragoso et al., 2014; Lima et al., 2015; Lucena et al., 2015).

A recent study using biopolymer gel as implant in eviscerated rabbit eyes indicated that BC is similar to polytetrafluoroethylene (PTFE) and polypropylene (Prolene®) in biocompatibility, and integrates adequately with the surrounding tissues (Cordeiro-Barbosa et al., 2012).

Likewise, when implanted in the bladder wall, BC is uniformly integrated and preserves its function as a bulking agent. In the bladder wall, BC induces a remodeling process at the site of injection, fully replacing the normal bladder tissue, and inducing formation of new tissue and extracellular matrix with new vessels. The incorporation of BC into tissue has been clearly demonstrated by neovascularization, starting at the periphery and moving toward the center of the implant (Lima et al., 2015).

Therefore, BC seems to be a promising biopolymer that competes favorably with other materials due to its low toxicity, low production costs, biocompatibility and capability of integration with different living tissues (Fragoso et al., 2014; Lucena et al., 2015).

5. Conclusions

The results of our work suggest that BC is safe when administered orally in rats at 2000 mg/kg body weight in a single dose. Data provided by the lactate dehydrogenase (LDH) activity assay indicate that BC is not cytotoxic. Our data also suggest that BC exerts a protective effect against CP-induced myelotoxicity and genotoxicity.

Further *in vivo* studies should also be undertaken to evaluate the effects of repeated doses, considering biochemical and hematological parameters and to confirm the antimutagenic and antigenotoxic action observed in this study. Nevertheless this may be premature until completion of the studies concerning the antimutagenic effects of BC as well as its indication or contraindication as quimioprotector agent.

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Conflict of interest

None.

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