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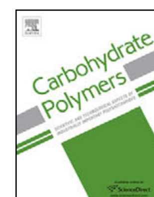

Acute toxicity, cytotoxicity, genotoxicity and antigenotoxic effects of a cellulosic exopolysaccharide obtained from sugarcane molasses

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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.

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