Non-clinical safety study of a sugarcane bacterial cellulose hydrogel

Estudo não clínico de segurança de um hidrogel de celulose bacteriana de cana-de-açúcar

Estudio no clínico de seguridad de un hidrogel de celulosa bacteriana de caña de azúcar

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Abstract

A hydrogel of bacterial cellulose of 0.8%, biopolymer produced from sugarcane molasses and synthesized from a bacteria called Zoogloea sp., was analyzed for its composition and tested by two routes of administration, subcutaneous and intraperitoneal, to clarify the local and systemic adverse effects after skin implantation in Wistar rats (24 males, 24 females, 55 days old). Analysis of two samples of BC films indicates carbon (42.94%; 43.43%), hydrogen (6.73%; 6.76%), nitrogen (0.28%, 0.23%) and oxygen (50.05%, 49.58%). No abnormal behavior, clinical signs of chronic toxicity or inflammation were observed. There was no change in liver injury biomarker levels (ALT, AST and ALP), as well as in renal histology, where it was evaluated to signs of tubular cell injury, glomerular or vascular damage, and renal morphometry, in which it was quantified the number of renal corpuscles, the number of cells per glomerulus and the capsular space area. Thus, the absence of signs and symptoms of toxicity suggests that subcutaneous or intraperitoneal injections of these polymers may be used in clinical situations.

Keywords: Bacterial cellulose; Skin; Toxicity; Biopolymer.

Resumo

Um hidrogel de celulose bacteriana a 0,8%, biopolímero produzido a partir do melaço de cana-de-açúcar e sintetizado a partir de bactéria *Zoogloea* sp., foi analisado quanto à sua composição e testado por duas vias de administração, subcutânea e intraperitoneal, para esclarecer os efeitos adversos locais e sistêmicos após implante na pele de ratos Wistar (24 machos, 24 fêmeas, 55 dias de idade). A análise de duas amostras de filmes CB indica carbono (42,94%; 43,43%), hidrogênio (6,73%; 6,76%), nitrogênio (0,28%, 0,23%) e oxigênio (50,05%, 49,58%). Nenhum comportamento anormal, sinais clínicos de toxicidade crônica ou inflamação foram observados. Não houve alteração nos níveis de biomarcadores de lesão hepática (ALT, AST e ALP), na histologia renal, onde se fez avaliação a procura de sinais de lesão celular tubular, dano glomerular ou vascular, nem na morfometria renal, onde foram quantificados os corpúsculos renais e a área do espaço capsular. Assim, a ausência de sinais e sintomas de toxicidade sugere que injeções subcutâneas ou intraperitoneais desses polímeros possam ser utilizadas em situações clínicas.

Palavras-chave: Celulose bacteriana; Pele; Toxicidade; Biopolímero.

Resumen

Se analizó un hidrogel de celulosa bacteriana del 0,8%, biopolímero producido a partir de melaza de caña de azúcar y sintetizado a partir de las bacterias Zoogloea sp., para su composición y probado por dos vías de administración, subcutánea e intraperitoneal, para aclarar los efectos adversos locales y sistémicos después de la implantación en la piel de ratas Wistar (24 machos, 24 hembras, 55 días de edad). El análisis de dos muestras de película cb indica carbono (42,94%; 43,43%), hidrógeno (6,73%; 6,76%), nitrógeno (0,28%, 0,23%) y oxígeno (50,05%, 49,58%). No se observaron comportamientos anormales, signos clínicos de toxicidad crónica o inflamación. No hubo cambios en los niveles de biomarcadores de lesión hepática (ALT, AST y ALP), en histología renal, donde se evaluó el análisis de signos de lesión de células tubulares, daño glomerular o vascular, o morfometría renal, donde se evaluaron los corpus renales y el área del espacial capsular. Por lo tanto, la ausencia de signos y síntomas de toxicidad sugiere que las inyecciones subcutáneas o intraperitoneales de estos polímeros pueden utilizarse en situaciones clínicas.

Palabras clave: Celulosa bacteriana; Piel; Toxicidad; Biopolímero.

1. Introduction

Bacteria-synthesized polysaccharides have suitable properties for biomedical applications (high degree of purity, biodegradability, elasticity, and flexibility) (Fragoso et al., 2014; Silveira et al., 2016; Gonçalves-Pimentel et al., 2018). The biocompatibility and lack of cytotoxicity of this biopolymer in preliminary tests, allied to its relatively low production costs, prompted research on the possible medical applications of this new and promising biomaterial (Lucena et al., 2015; Pinto et al., 2016; Donini et al., 2018).

Experimental and clinical studies suggested that sugarcane bacterial cellulose hydrogel (BC) is a biomaterial useful for a variety of surgical procedures, such as correction of hypospadias and other uses in urologic surgery (Martins et al., 2013; Lima et al., 2015; Lucena et al., 2015; Lima et al., 2017), treatment of fecal incontinence after anal trauma (Cavalcante et al., 2018), treatment of eviscerated eyes (Cordeiro-Barbosa et al., 2012), repair of tympanic membrane perforation (Silveira et al., 2016), vascular surgery procedures (Barros-Marques et al., 2012), pressure injury (Oliveira et al., 2019), orthopedic surgery (Albuquerque et al., 2011), and neurosurgery (Lima et al., 2017).

Although preliminary studies indicated that BC presents a rather low acute toxicity and has no genotoxic and cytotoxic effects (Castro et al., 2004, Pinto et al., 2016), additional

non-clinical safety studies are necessary before making a decision to start clinical trials, and to apply for BC products marketing approval.

Thus, the aim of this study was to evaluate the cellulose exopolysaccharide produced from sugarcane by a non-clinical safety study to shed light on the local and systemic adverse effects of the bacterial cellulose 0.8% hydrogel after implantation under the skin (subcutaneous administration) or injection into the peritoneal cavity of Wistar rats.

2. Materials and Methods

2.1 The Bacterial Cellulose

The Bacterial Cellulose 0.8% Hydrogel (BC) was produced by a bacterial fermentation of sugarcane molasses. BC was manufactured by POLISA Biopolymers for Health located at the Sugarcane Experimental Station of the Federal Rural University of Pernambuco (EECC/UFRPE). Disposable hypodermic syringes containing the BC 0.8% hydrogel ready for use were supplied as single units wrapped and sealed with surgical paper tape that were sterilized by gamma rays (25kGy) at the Laboratory of Metrology of the Department of Nuclear Energy of the Federal University of Pernambuco (UFPE).

2.2 Animals

Wistar rats (24 males, 24 females, 55 days old) from the Oswaldo Cruz Foundation (FIOCRUZ-RJ) breeding stock were used in the experiment. Upon arrival at the laboratory animal facilities, rats were housed in standard plastic cages with stainless coverlids and white pinewood shavings as bedding, and kept under controlled environmental conditions (22±0.5 ºC, 12 h photoperiod with lights on from 8:00 a.m. to 8:00 p.m.; 55-70% relative air humidity and controlled room air changes). A commercial pelleted diet for laboratory rodents (Labina®, Purina® Co) and filtered tap water were available ad libitum throughout the experiment.

The rats were handled in compliance with the Brazilian Animal Welfare and Protection legislation, and international (Guide for Care and Use of Laboratory Animals, 8th edition, 2011, of the US National Institutes of Health) and national guidelines for care and use of laboratory animals for scientific research. The research project was approved (License LW-3/15) by the Ethics Committee on the Use of Animals of the Oswaldo Cruz Foundation

(CEUA-FIOCRUZ).

2.3 Study design

Rats were allocated at random to three experimental groups shown in Figure 1. On experiment day 1 (D1), one group of rats (9♀ and 9♂) received a single subcutaneous injection of BC (200 mg/kg bw, 4 mL/kg bw) while a second group (9♀ and 9♂ rats) was administered with a single dose of BC (200 mg/kg bw, 4 mL/kg bw) by the intraperitoneal route. A third group, control, (6♀ and 6♂ rats) was treated with a single intraperitoneal injection of 0.9% NaCl solution (4 mL/kg bw).

Figure 1 – Diagram illustrating the design of the study.
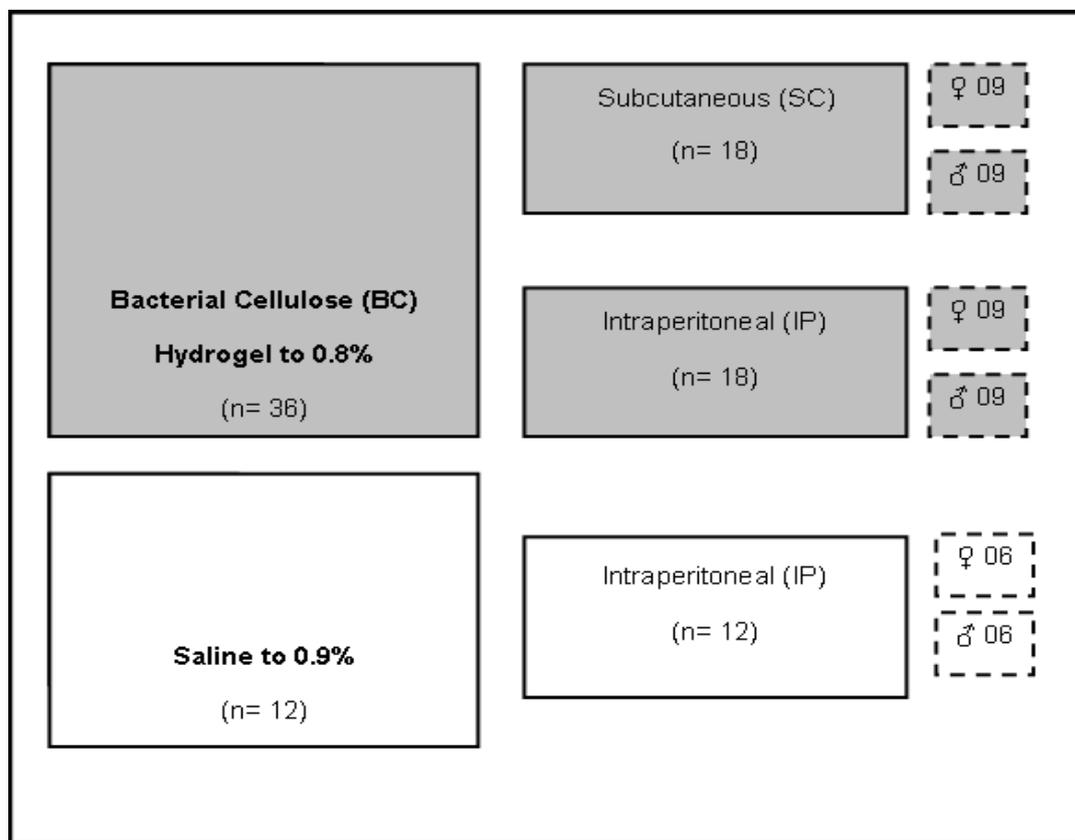
Source: Authors' research data (2020).

From the second (D2) through to the 20th day after the treatment (D20) all rats were daily weighed and examined for clinical signs and symptoms of toxicity. On the 20th day (D20), after the clinical examination and weighing procedure, rats were anesthetized by CO_2 inhalation, and a sample of blood (500µL) was taken by heart puncture immediately before

the euthanasia by decapitation. After blood clotting, the serum was separated by centrifugation, transferred to Eppendorf vials, frozen and stored at -20º C until further use.

The rats undertook laparotomy and the peritoneal cavity, and abdominal and thoracic organs were examined for gross pathology findings. Liver, spleen and kidneys were removed, weighed and fixed in a 10% buffered formalin solution. A piece of the skin and subcutaneous tissue of the site where BC was implanted was also excised and fixed in 10% buffered formalin solution for further histopathology evaluation.

2.4 Toxicological evaluation

To evaluate the acute/subacute toxicity of the cellulose exopolysaccharide, a non-clinical study was conducted on the local and systemic adverse effects of the 0.8% bacterial cellulose hydrogel after implantation under the skin (subcutaneous administration) or injection into the peritoneal cavity of Wistar rats.

Subcutaneous and intraperitoneal routes have been used in experimental studies to assess acute/subacute toxicity and carcinogenicity, according to the OECD Series on Testing and Assessment, No. 19 (OECD, 2000). This document indicates that the subcutaneous route should be used when the human exposure route is intended to be intravenous, and for pharmaceutical products. The subcutaneous or intraperitoneal injection reflects the expected route of administration in humans. The tests were performed based on the Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints (OECD Series on Testing and Assessment, No. 19).

The procedures performed are also in accordance with the specifications of the International Organization for Standardization (ISO), considering the Systemic Toxicity tests (ISO 10993-13), the Subacute Systemic Toxicity test (ISO 10993-11), and the implantation test (ISO 10993-6). This ISO specifies requirements and provides guidance on procedures to be followed in assessing the potential of medical device materials to cause systemic adverse reactions. Specifies test methods to assess local effects after implantation of biomaterials intended for use in medical devices (ISO 109936:2016; ISO 10993-11:2017; ISO 10993-13:2010).

2.4.1 Signs of toxicity

All rat cages were inspected once a day for any behavioral abnormality and clinical

signs of toxicity such as hypo- or hyperactivity, piloerection, vocalization, chromodacryorrhea, hair loss, lacrimation, lethargy, epistaxis, diarrhea and others. The rat body weight was also determined on a daily basis from D2 to D20.

2.4.2 Determination of biomarkers of liver injury in the blood serum

Alanine (ALT) and aspartate (AST) aminotransferase, and alkaline phosphatase (AP) activities were determined by a colorimetric method using a commercially available kit (Laborclin®, Pinhais, PR, Brazil) as reported elsewhere (Reitman & Frankel, 1957). Absorbance was measured at 505 nm in a microplate spectrophotometer reader (Biochrom EZRead 2000) and results were expressed as IU/L.

2.4.3 Histopathology

After fixation in 10% buffered formalin solution, tissue samples were processed according histotechnical procedures routinely used by the Laboratory of Immunopathology Keizo Asami (LIKA) of the Federal University of Pernambuco (UFPE). Tissue samples were embedded in paraffin blocks, sectioned (4 µm thick sections), mounted on slides and stained with hematoxylin-eosin (HE) and picrosirius red. The slides were examined under an optical microscope by pathologists who were kept unaware of the treatment received by the animal (blinded evaluation) and the diagnosis was confirmed by a second evaluator also blinded to the treatment.

The histological analysis of the skin excised from the site of injection evaluated the location and continuity (homogeneity) of BC subcutaneous implant, the inflammatory response, cellular infiltrate (polymorphonuclear cells, lymphocytes and giant multinuclear, GMN, cells), the presence of fibroblasts and the occurrence of neovascularization. Based on the percentage of cells and novel vessels within the BC implant, slides of the skin were scored as follows: 0 or Absence (0-5%), I or slight (5-25%), II or mild (25-50%) and III for the most intense findings (>50%).

Morphometry of renal tissue slides was performed under a Zeiss® light microscope using an ImageJ software® (Image Processing and Analysis in Java – 1:446 ImageJ, National Institutes of Health, US). The number of renal corpuscles, and the capsular space area (or Bowman's space) were quantified. The renal capsular space area was the Bowman's capsule area minus the glomerulus area.

2.5 Chemical Analysis of BC

Bacterial cellulose hydrogel samples were analyzed by 1H NMR spectroscopy while BC films were submitted to elementary analysis. 1H NMR spectra were performed using Varian Unity Plus® spectrometer, operating at 300 MHz, using D2O as solvent. RF Pulse of 45°, h acquisition time equal to 2.55s, presaturation time equal to 2.00s and 128 repetitions were used. Elementary analysis was performed in duplicate using a Perkin Elmer 2400 CHNS/O Series II System®. The mass percentage of carbon, hydrogen, nitrogen and sulfur was determined and the oxygen content was the difference. It was not feasible to make a reliable elementary analysis of the hydrogel because its weight (and water content) was not constant during the analysis.

2.6 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Bonferroni post hoc test. Proportions were compared by the Fisher exact test. In any case a difference was considered to be significant when $p \leq 0.05$. Statistical calculations were performed using GraphPad Prism® version 3.0 software.

**3. Results**

3.1 Chemical Analysis

The elementary analysis of two samples (S1 and S2) of BC films indicated that this biomaterial has carbon (S1, 42.94%; S2, 43.43%), hydrogen (S1, 6.73%; S2, 6.76%), nitrogen (S1, 0.28%, S2, 0.23%) and oxygen (S1, 50.05%, S2, 49.58%). This proportional elementary composition, except for a small content of nitrogen, corresponds to that expected for carbohydrates, the minimal formula of which is $(CH_2O)_n$, where *n* is the number of units of carbon. ^{1}H NMR spectra of BC hydrogel detected a single intense signal at δ 4.72 ppm that can be attributed to the presence of water and hydroxyl groups.

3.2 Systemic effects of subcutaneous and intraperitoneal injections of BC

On the 20th post-treatment day, the body weight of male and female rats that received a single (200 mg/kg bw) subcutaneous or intraperitoneal injection of BC did not differ from that

of males and females of the saline-treated control group (Table 1). The body weight gains from treatment day 1 to 20 (D20- D1) did not differ between the BC-injected groups and the control group either, yet a non-significant greater increase in body weight was noted among males treated by the subcutaneous route (Table 1). No abnormal behavior and no clinical signs of toxicity were noted among rats injected with BC by either one of the two parenteral routes in any of the days of the 20-day observation period. Moreover, the subcutaneous or intraperitoneal injections of BC did not alter liver, kidney and spleen weights (Table 1), nor did these injections increase the levels of biomarkers of liver injury such as activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) or alkaline phosphatase (ALP) in the blood serum on D20 (Figure 2).

Figure 2 - Absence of effects of a single subcutaneous (n = 9M, 9F) or intraperitoneal (n = 9M, 9F) injection of BC hydrogel (200 mg/kg bw) on serum biomarkers of liver toxicity measured 20 days after treatment. Histogram bar heights represent fold-changes (mean± SD) of alanine-aminotransferase (ALT), aspartate aminotransferase and alkaline phosphatase (AP) activities in the blood serum. Control rats (n = 6M, 6F) received an IP injection of 0.9% NaCl solution (4 mL/kg ip) Control group mean activity = 1. Differences were not significant (ANOVA p>0.05). M= male; F= female rats.
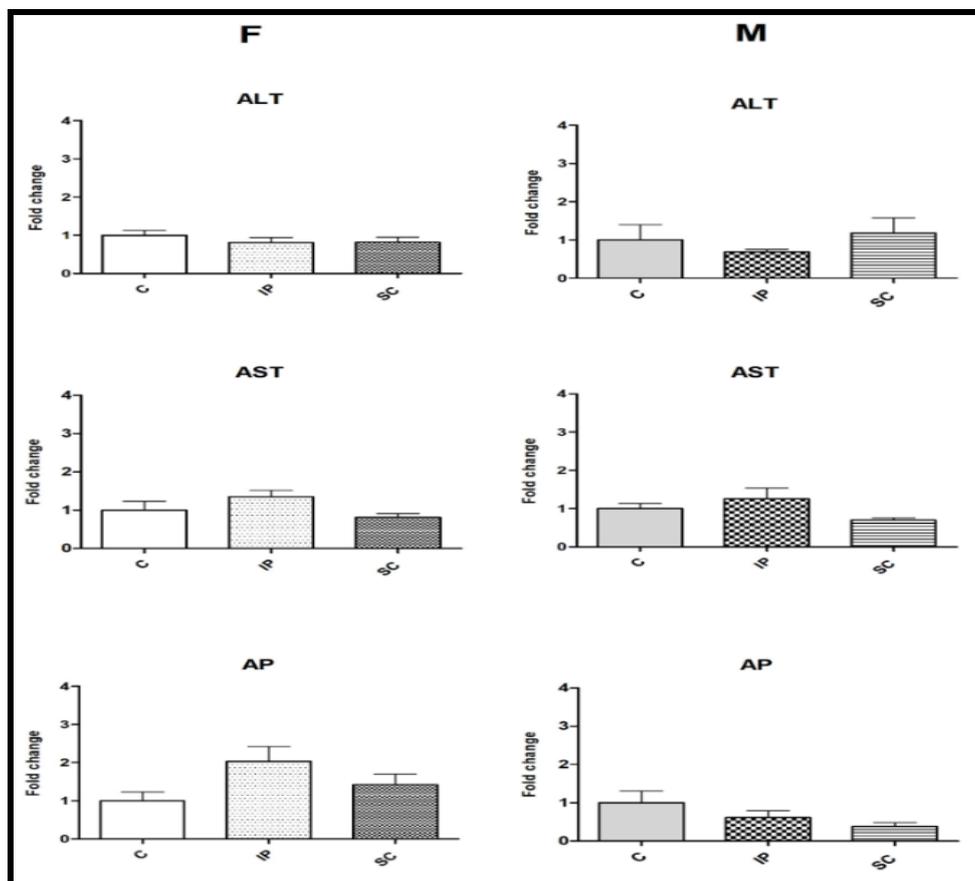
Source: Authors' research data (2020).

Table 1. Effects of a single injection of BC hydrogel (200 mg/kg bw) under the skin (BC-SC) or into the peritoneal cavity (BC-IP) on the body weight gain, and on the organ (liver, kidneys, spleen) weights on post-treatment day 20. Control group rats received an intraperitoneal injection of 0.9% NaCl solution (Saline IP, 4 mL/kg bw) on treatment day 1.

| Groups	Sex	N	Body weight on the 20th day (g)	Post-treatment body weight gain (day 20 - day 1) (g)	Liver weight (g)	Kidneys weight (g)		Spleen weight (g)
						Right	Left	
BC-SC	F	9	194.8±16.0	46.9±7.4	8.1±1.1	0.8±0.1	0.8±0.1	0.6±0.1
	M	9	310.0±24.3	86.6±10.5	11.8±1.0	1.2±0.1	1.2±0.1	0.9±0.2
BC-IP	F	9	183.8±14.2	40.8±6.9	7.3±1.0	0.8±0.1	0.8±0.0	0.6±0.1
	M	9	292.4±18.3	73.8±8.6	10.5±1.5	1.2±0.1	1.2±0.1	0.8±0.2
Saline IP	F	6	241.8±11.7	38.4±8.7	7.1±0.8	0.8±0.1	0.8±0.0	0.7±0.1
	M	6	294.7±21.6	75.8±8.9	9.8±2.1	1.1±0.1	1.3±0.4	0.8±0.1

Data are means ± SD. BC-SC, Bacterial Cellulose-Subcutaneous; BC-IP, Bacterial Cellulose-intraperitoneal. Body weight and weight of organs (liver, left kidney, right kidney or spleen): for comparisons within the same sex (M, male or F, female), statistical analysis (ANOVA and Bonferroni's post hoc test) did not find differences (P>0.05) between treatment groups (BC-SC, BC-IP or Saline IP).

3.3 Histopathological Analysis

3.3.1 Examination of the kidneys

Examination of renal tissue slides revealed no sign of tubular cell injury, glomerular or vascular damage, or any other abnormalities associated to treatment with BC (Figure 3).

Figure 3 - Photomicrographs of sections of the renal tissue (post treatment day 20) of rats treated with a single subcutaneous or intraperitoneal injection of BC (200 mg/kg bw) on D1. **a** and **b** - No toxicologically significant histological change was noted. HE staining.
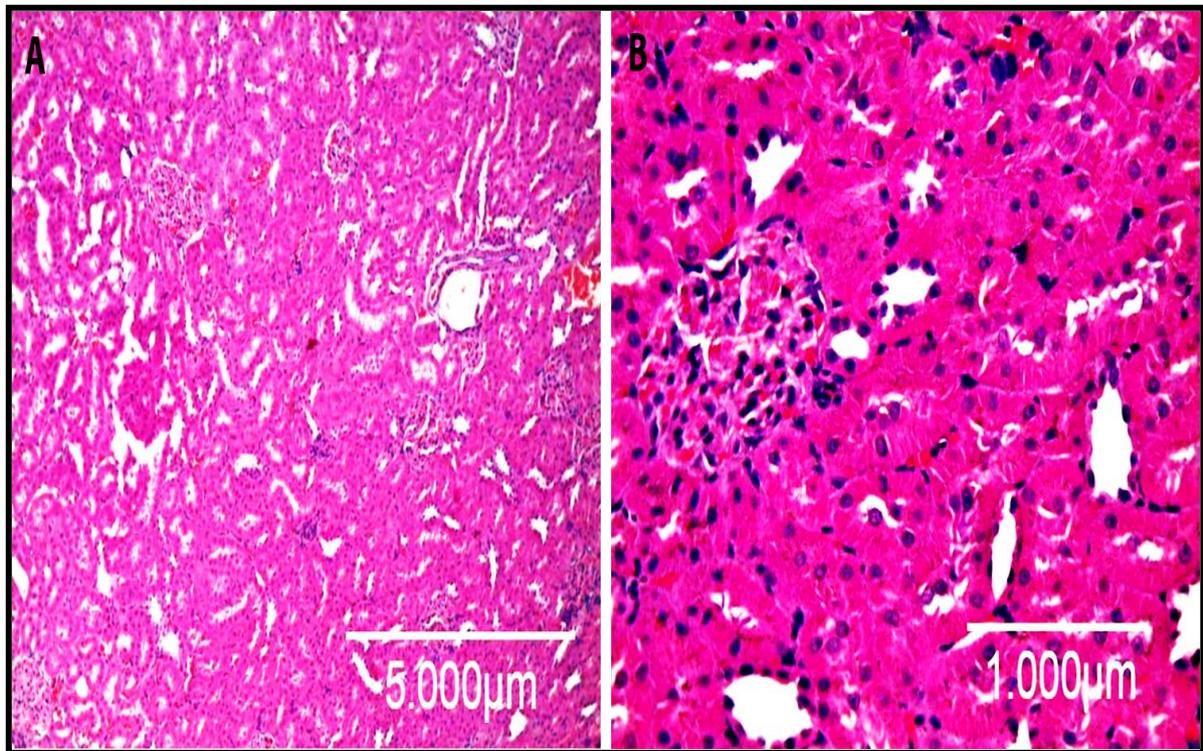
Source: Authors' research data (2020).

Along the same line, the morphometric analysis of renal tissue slides did not show any treatment-related adverse effect on the average number of renal corpuscles, glomerular cellularity (average number of cells per glomerulus), Bowman capsule area, glomerulus area and capsular space area (Table 2).

Table 2 - Morphometric analysis of renal corpuscles from control rats (0.9% NaCl solution, Saline IP, 4 mL/kg bw ip) and from rats injected with BC hydrogel (200 mg/kg bw) under the skin (BC-SC) or into the peritoneal cavity (BC-IP). Kidneys were removed 20 days after treatment and tissue sections were stained with HE.

Groups	Sex	N	Bowman's capsule area (μm^2)	Glomerulus area (μm^2)	Capsular space§ (μm^2)	Mean number of renal corpuscles per unit of area
BC-SC	F	9	7.6±1.0	5.8±0.8	1.8±0.3	15.2±4.8
	M	9	8.5±0.9	6.5±0.6	1.9±0.4	12.9±3.5
	F+M	18	8.1±2.4	6.2±0.8	1.8±0.3	14.0±4.4
BC-IP	F	9	7.5±0.7	5.9±0.6	1.6±0.2	13.9±4.8
	M	9	8.8±0.8	6.8±0.5	2.0±0.3	11.0±5.1
	F+M	18	8.1±1.0	6.3±0.7	1.8±0.3	12.4±5.2
Saline IP	F	6	7.3±0.7	5.5±0.6	1.7±0.2	14.6±4.0
	M	6	8.9±0.7	7.0±0.8	1.9±0.2	14.2±3.2
	F+M	12	8.1±1.1	6.3±0.9	1.8±0.2	14.4±3.6

Data are means ± SD. N, number; BC-SC, Bacterial Cellulose-Subcutaneous; BC-IP, Bacterial Cellulose-intraperitoneal. § Capsular or Bowman's space. For comparisons within the same sex (M, male or F, female), statistical analysis (ANOVA and Bonferroni's post hoc test) did not find differences (P>0.05) between treatment groups (BC-SC, BC-IP or Saline IP).

3.3.2 Histopathology of the subcutaneous tissue at the BC implantation site

In the treated male and female rats, eight out of nine BC hydrogel implants were found immediately beneath the dermis layer of the skin, i.e., in the interstitial space between the subcutaneous tissue and the muscle fascia (Figure 4).

Figure 4 - Photomicrography of the skin on the 20th day after implantation of BC in rats. A - BC implant (asterisk) immediately below the dermis layer (long arrow). B - Neovascularization (short arrows) and fibroblasts (arrowheads). Picrosirius red dye.
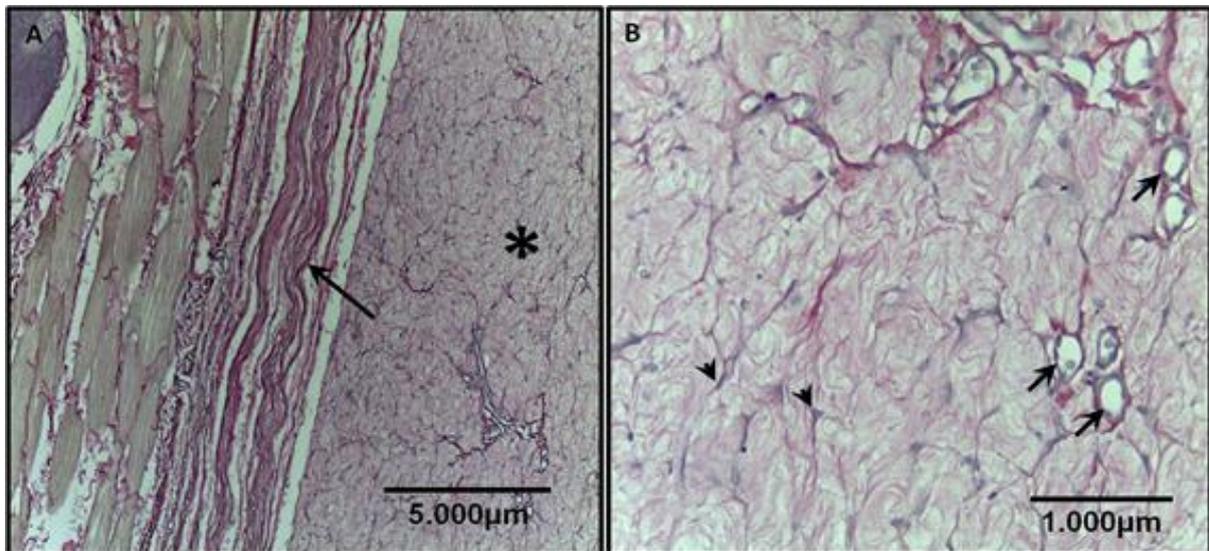
Source: Authors' research data (2020).

It is plausible to think that in the remaining two animals (one male and one female) the implanted BC hydrogel was completely reabsorbed before the 20th day of the experiment (euthanasia day). The histological examination also revealed that the implanted BC gel was continuous (homogeneous) in eight males and fragmented in one male, and continuous in eight females (Table 3). A slight inflammatory response was present in all eight males and females (100%) in which BC implants were localized under the skin, while fibroblasts were absent in four (62.5%) males and one female (12.5%) rat. No giant mononuclear (GMN) cells were found in six (75%) males and three females (77.5%), while four females exhibited a slight score (5-25%) and one female had a mild score (25-50%) for predominance of MNG cells (Table 3).

The implantation of BC hydrogel under the skin led to formation of new vessels (neovascularization) within the implant area in 5 (62.5%) males and 7 (87.5%) females the intensity of which ranged from mild to intense (25%) among males, and from slight (25%) to intense (37.5%) in females (Table 3).

Table 3. Main findings from the histopathologic examination of the skin at the site of subcutaneous injection of BC (200 mg/kg bw) in 9 females and 9 male Wistar rats.

Parameter		Females	Males
Identification of a BC implant under the skin	Present	8 (88.9%)	8 (88.9%)
	Absent	1 (11.1)	1 (11.1)
Continuity (homogeinity)	Continuous	7 (77.8%)	8 (88.9%)
	Fragmented	1 (11.1%)	0 (0%)
	Absent	1 (11.1%)	1 (11.1%)
Giant Multinuclear (GMN) Cells	Absent (0%)	3 (37.5%)	6 (75.0%)
	Slight (0-5%)	4 (50.0%)	2 (25.0%)
	Mild (25-50%)	1 (12.5%)	0 (0%)
	Intense (>50%)	0 (0%)	0 (0%)
Inflammatory response	Absent (0%)	0 (0%)	0 (0%)
	Slight (0-5%)	8 (100%)	8 (100%)
	Mild (25-50%)	0 (0%)	0 (0%)
	Intense (>50%)	0 (0%)	0 (0%)
Presence of fibroblastos	Absent (0%)	1 (12.5%)	5 (62.5%)
	Slight (0-5%)	7 (87.5%)	3 (37.5%)
	Mild (25-50%)	0 (0%)	0 (0%)
	Intense (>50%)	0 (0%)	0 (0%)
Neovascularization	Absent (0%)	1 (12.5%)	3 (37.5%)
	Slight (0-5%)	2 (25.0%)	0 (0%)
	Mild (25-50%)	2 (25.0%)	3 (37.5%)
	Intense (>50%)	3 (37.5%)	2 (25.0%)

Note: The inflammatory response, presence of fibroblasts, and neovascularization were not visualized in the region where a BC implant under the skin was absent (one animal). BC, Bacterial Cellulose.

The laparotomy of the peritoneal cavity revealed that the hydrogel implant was not present in the cavity. No phlogistic signs or implant adherence to the cavity were found. Accordingly, intraperitoneal tissue was not excised and histopathological evaluation was not processed.

4. Discussion

The objective of this work was to present the results of a set of non-clinical safety tests, including toxicological evaluation, histopathological and chemical analysis of the BC hydrogel obtained from sugarcane molasses at 0.8% when administered via the routes subcutaneous and intraperitoneal in Wistar rats.

The proportional elementary composition, except for a small content of nitrogen, corresponds to that expected for carbohydrates, the minimal formula of which is $(CH_2O)_n$, where n is the number of units of carbon. If BC films and BC hydrogels (from which BC films are formed) were constituted by glucose-based polysaccharides only, they should have a slightly different proportional composition (i.e., carbon 40%, hydrogen 6.7%, and oxygen 53.3%) (Pavia et al., 2010). Based on this small deviation from this proportion, it seems fair to think that polymeric carbohydrate molecules other than only glucose may also be present in BC polysaccharides. The small yet significant content of nitrogen, on the other hand, suggests that analyzed BC film samples may also contain a small amount of polysaccharides with a nitrogen side-chain or other nitrogen compounds.

Actually, the BC hydrogel has high content of water and thus ^{1}H NMR spectra result is consistent with polysaccharide-based hydrogels that contain various hydroxyl groups and a few methylene groups (Pavia et al., 2010).

Bacterial cellulose (BC), a product of natural origin, has a chemical structure that consists of polymerized sugars. This biopolymer is stable, safe and is not digested by the surrounding tissues (Pita et al., 2015; Pinto et al., 2016).

In this article, the discussion of the results was presented from a set of non-clinical safety tests, including a toxicological evaluation, histopathological and chemical analysis of the BC 0.8% hydrogel when administrated by the subcutaneous and the intraperitoneal routes.

No abnormal behavior and no clinical signs of toxicity were noted between treated and control groups, on the 20th post-treatment day. These injections did not alter the levels of hepatic injury biomarkers (AST, ALT, and ALP), in the blood serum. The results of the histopathological examination and morphometric analysis of the kidneys, therefore, lend support to the conclusion that BC subcutaneous and intraperitoneal injections caused no systemic adverse effects. The results obtained in this assay are consistent with those of previous studies. An in vitro cytotoxicity test evaluated the cell viability on the bacterial cellulose membrane by the MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide thiazolyl blue) assay and the authors observed 100% cell viability in the control

group; 91.2% in the PTFE (Polytetrafluoroethylene); 43.6% in the polypropylene mesh; 95.3% in the biopolymer group, demonstrating that the sugarcane biopolymer membrane presented a high biocompatibility (Castro et al., 2004). The BC was also safe when administered orally in rats at 2000 mg/kg body weight in a single dose. BC was not cytotoxic and the data also suggest that BC exerts a protective effect against CP-induced myelotoxicity and genotoxicity (Pinto et al., 2016).

BC hydrogel subcutaneous implants were found beneath the hypodermis layer of the skin, a slight inflammatory response was present and the formation of new vessels (neovascularization) was present in the majority of the animals. These effects are supported by previous studies (Rangel et al., 2006; Carvalho Junior et al., 2012; Cordeiro-Barbosa et al., 2012; Lima et al., 2015; Pita et al., 2015; Lima et al., 2017; Cavalcante et al., 2018; Gonçalves-Pimentel et al., 2018).

The bacterial cellulose remains at the injection site (anorectal region), promoted neovascularization and the implant area was colonized by multinucleated giant cells, fibroblasts and dense conjunctive tissue associate to collagen fibers (Cavalcante et al., 2018).

The gel from cellulose produced by Zoogloea sp from sugarcane molasses was shown to be integrated, biocompatible, and nontoxic to the orbits of rabbits (Cordeiro-Barbosa et al., 2012).

The cellulosic exopolysaccharide was present in the implant site (rabbit urinary bladder), exhibited low inflammatory response, presence of neovascularization and integrated with the host tissue better than dextranomer microspheres in the long-term follow-up (Lima et al., 2015).

The cellulose exopolysaccharide was shown to be biocompatible in primary cultures with respect to cell viability, adhesion, growth and cell function (calcium imaging), compared with the Polystyrene or Matrigel® matrix (Gonçalves-Pimentel et al., 2018). The Bacterial Cellulose membrane also showed adequate biocompatibility properties, with no immune reaction, nor chronic inflammatory response and absence of neurotoxicity signals when tested to evaluate the dural defect repair in rats (Lima et al., 2017).

Review studies discuss the most recent key developments and challenges regarding bacterial cellulose or nanocellulose-based hydrogels that have been successfully tested. Therefore, the growing interest in BC derived materials establishes it as a great promise to enhance the quality and functionalities of the current generation of biomedical materials. The diversity of this material allows targeting of many very different applications (Foresti et al., 2017; Picheth et al., 2017; Donini et al., 2018; Athukoralalage et al., 2019).

In synthesis, nanocellulose hydrogels have been demonstrated for 3D cell culture, mimicking the extracellular matrix (ECM) properties with low cytotoxicity. For wound dressing and cartilage repair, nanocellulose gels promote cell regeneration while providing the required mechanical properties for tissue engineering scaffolds. The encapsulation of therapeutics within nanocellulose allows the targeted delivery of drugs. Currently, cellulose crosslinking to peptides and proteins enables a new generation of renewable smart materials used in diagnostics. Last, the organized mesh of fibers contained in hydrogels drives applications in the separation of biomolecules and cells (Curvello et al., 2019). However, the bacterial cellulose hydrogel has emerged as a highly engineerable platform for multiple biomedical applications, providing renewable, safety and low cost.

5. Conclusions

In conclusion, the absence of detrimental effects on body weight and organs (liver, kidney and spleen) associated with lack of signs and symptoms of clinical toxicity indicated by subcutaneous or intraperitoneal injections of BC did not cause any systemic adverse effects in rats. In contrast to a conspicuous presence of BC remaining in the subcutaneous implant area, BC was not found within the peritoneal cavity 20 days after injection. Data also indicated that, regardless of the parenteral route used for BC application, treated rats exhibited a post-treatment weight gain similar to that of the control group and did not present any behavioral abnormalities and clinical signs of systemic or local toxicity. Signs of a slight inflammatory response, fibrosis, and neovascularization were noted in the subcutaneous tissue at the BC application site on the 20th post-treatment day. Overall, the findings are consistent with previous studies and suggested that BC has rather low toxicity and that its uses in surgical procedures are safe.

References

Albuquerque, P. C. U. C., Santos, S. M., Aguiar, J. L. A., Filho, N. P., Mello, R. J. V., Costa, M. L. C. R., Olbertz, C. M. C. A., Almeida, T. M. S., Santos, A. H. S., & Silva, J. C. (2011) Comparative macroscopic study of osteochondral defects produced in femurs of rabbits repaired with biopolymer gel cane sugar. *Revista Brasileira de Ortopedia, 46, 577–584.*

Athukoralalage, S. S., Balu, R., Dutta, N. K., & Roy Choudhury, N. (2019) 3D Bioprinted nanocellulose-based hydrogels for tissue engineering applications: A Brief Review. *Polymers, 11*(5), *898-910.*

Barros-Marques, S. R., Marques-Lins, E., Albuquerque, M. C., & Andrade-Aguiar, J. L. (2012) Sugarcane biopolymer patch in femoral vein angioplasty on dogs. *Journal of Vascular Surgery, 55, 517–521*.

Carvalho Junior, A. M., Barkokébas, B. B., Aguiar, J. L. A., Lima, S. V. C., & Dambros, M. (2012) Characterization of the deposition of collagen fibers and Lithogenic potential in bladder of rats submitted to a sugar cane biopolymer graft. *International Brazilian Journal of Urology, 38, 544-551*.

Castro, C. M. M. B., Aguiar, J. L. A., Melo, F. A., Silva, W. T. F., Marques, E., & Silva, D. B. (2004) Citotoxidade de Biopolímero de Cana-de-Açúcar. *Anais da Faculdade de Medicina da Universidade Federal de Pernambuco, 49, 119-123*.

Cavalcante, A., Lima, R. P., Souza, V., Pinto, F., Campos Júnior, O., Silva, J., Albuquerque, A. V., & Aguiar, J. (2018). Effects of bacterial cellulose gel on the anorectal resting pressures in rats submitted to anal sphincter injury. *Heliyon*, *4*(12), e01058. https://doi.org/10.1016/j.heliyon.2018.e01058.

Cordeiro-Barbosa, F. A., Aguiar, J. L. A., Lira, M. M. M., Pontes Filho, N. T., & Bernadino-Araújo, S. (2012) Use of a gel biopolymer for the treatment of eviscerated eyes: experimental model in rabbits. *Arquivos Brasileiros de* Oftalmologia, *75*(4), *267-272*.

Curvello, R., Raghuwanshi, V. S., & Garnier, G. (2019) Engineering nanocellulose hydrogels for biomedical applications. *Advances in* Colloid *and* Interface Science, *267, 47-61*.

Donini, I. A. N., Salvi, D.T. B., Fukumoto, F. K., Lustri, W. R., Barud, H. S., Marchetto, R., Messaddeq, Y., & Ribeiro, S. J. L. (2018) Biosynthesis and recent advances in production of bacterial cellulose. *Eclética Química Journal,* 35(4), *165-178*.

Foresti, M. L., Vázquez, A., & Boury, B. (2017) Applications of bacterial cellulose as precursor of carbon and composites with metal oxide, metal sulfide and metal nanoparticles: A review of recent advances. *Carbohydrate Polymers, 157, 447–467.*

Fragoso, A. S., Silva, M. B., De Melo, C. P., Aguiar, J. L., Rodrigues, C. G., De Medeiros, P. L., Branco Junior, J. F., Andrade, C. A. S., & Oliveira, M. D. L. (2014) Dielectric study of the adhesion of mesenchymal stem cells from human umbilical cord on a sugarcane biopolymer. *Journal of Materials* Science*: Materials in* Medicine, *25, 229–237*.

Gonçalves-Pimentel, C., Moreno, G. M. M., Trindade, B. S, Isaac A. R., Rodrigues, C. G., Savariradjane, M., Albuquerque, A. V., Aguiar, J. L. A., & Andrade-da-Costa, B. L. S. (2018) Cellulose exopolysaccharide from sugarcane molasses as a suitable substrate for 2D and 3D neuron and astrocyte primary cultures. *Journal of Materials* Science *Materials in* Medicine, *29*(9), *13*9.

International Organization For Standardization. Recuperado de https://www.iso.org/standard/68426.html: Access September 10, 2020.

Lima, S. V., de Oliveira Rangel, A. E., de Melo Lira, M. M., Pinto, F. C., Campos Júnior, O., Sampaio, F. J., & de Andrade Aguiar, J. L. (2015). The Biocompatibility of a Cellulose Exopolysaccharide Implant in the Rabbit Bladder When Compared With Dextranomer Microspheres Plus Hyaluronic Acid. *Urology*, *85*(6), 1520.e1–1520.e15206.

Lima, S. V. C., Machado, M. R., Pinto, F. C. M, Lira, M. M. M., Albuquerque, A. V., Lustosa, E. S., Silva, J. G. M., & Campos Jr., O. (2017). A new material to prevent urethral damage after implantation of artificial devices: an experimental study. *International braz j urol*, *43*(2), 335-344.

Lima, F. M., Pinto, F. C., Andrade-da-Costa, B. L., Silva, J. G., Campos Júnior, O., & Aguiar, J. L. (2017). Biocompatible bacterial cellulose membrane in dural defect repair of rat. *Journal of materials science. Materials in medicine*, *28*(3), 37. https://doi.org/10.1007/s10856-016-5828-9

Lucena, R. G., Vilar, F. O., Andrade, R. T., Pinto, F. C. M. & Lima, S. V. C. (2015) Experimental use of a cellulosic biopolymer as a new material for suburethral sling in the treatment of stress urinary incontinence. *International Braz J Urol (Online).* 41:1148-1153.

Lucena, M. T., de Melo Júnior, M. R., de Melo Lira, M. M., de Castro, C. M., Cavalcanti, L. A., de Menezes, M. A., Pinto, F. C., & de Andrade Aguiar, J. L. (2015). Biocompatibility and cutaneous reactivity of cellulosic polysaccharide film in induced skin wounds in rats. *Journal of materials science. Materials in medicine*, *26*(2), 82.

Martins, A. G. S., Lima, S. V. C., Araujo, L. A. P., Vilar, F. O. & Cavalcante, N. T. P. A. (2013) Wet dressing for hypospadias surgery. *International Brazilian Journal of Urology,* 39(3), *408-413*.

OECD (2000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints. Organisation for Economic Co-operation and Development, Paris, France (OECD Series on Testing and Assessment, No. 19)

OECD (2002). Guidance Notes for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies. Organisation for Economic Co-operation and Development, Paris, 32 France (OECD Series on Testing and Assessment No. 35).

Oliveira, G. M., Vieira, J. M. S., Silva, J. G. M., Albuquerque, E. L. M. S., Albuquerque, A. V., Aguiar, J. L. A., & Pinto, F. C. M. (2019). Curativo de celulose bacteriana para o tratamento de lesões por pressão em pacientes hospitalizados. *Revista Enfermagem Atual In Derme*, *87*(Edição Esp).

Pavia, D. L., Lampman, G. M., Kriz, G., & Vyvyan, J. (2010) Introdução à Espectroscopia, 4ª Edição, *Cengage, 733p.*

Picheth, G. F., Pirich, C. L., Sierakowski, M. R., Woehl, M. A., Sakakibara, C. N., de Souza, C. F., Martin, A. A., da Silva, R., & de Freitas, R. A. (2017). Bacterial cellulose in biomedical applications: A review. International journal of biological macromolecules, 104(Pt A), 97–106.

Pinto, F., De-Oliveira, A., De-Carvalho, R. R., Gomes-Carneiro, M. R., Coelho, D. R., Lima, S., Paumgartten, F., & Aguiar, J. (2016). Acute toxicity, cytotoxicity, genotoxicity and antigenotoxic effects of a cellulosic exopolysaccharide obtained from sugarcane molasses. Carbohydrate polymers, 137, 556–560.

Pita, P. C. C., Pinto, F. C. M., Lira, M. M. M., Melo, F. A. D., Ferreira, L. M. & Aguiar, J. (2015) Biocompatibility of the bacterial cellulose hydrogel in subcutaneous tissue of rabbits. *Acta Cirúrgica Brasileira,. 30*, *296-300*.

Rangel, A. E. O., Aguiar, J. L. A., Lima, S. V. C., & Vilar F. (2006) A new biomaterial for the treatment of vesicoureteral reflux: Experimental study. *International Brazilian Journal of Urology. 32, 184*.

Reitman, S., & Frankel, S. (1957) A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *American Journal* of *Clinical Pathology, 28, 56-63*.

Silveira, F. C. A., Pinto, F. C. M., Caldas Neto, S. S., Leal, M. C., Cesário, J., & Andrade, J. L. A. (2016) Treatment of tympanic membrane perforation using bacterial cellulose: a randomized controlled trial. *Brazilian Journal of Otorhinolaryngology*, *82*(2), *203–208*.

Silveira, R. K., Coelho, A. R., Pinto, F. C., de Albuquerque, A. V., de Melo Filho, D. A., & de Andrade Aguiar, J. L. (2016). Bioprosthetic mesh of bacterial cellulose for treatment of abdominal muscle aponeurotic defect in rat model. *Journal of materials science. Materials in medicine*, *27*(8), 129.

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