

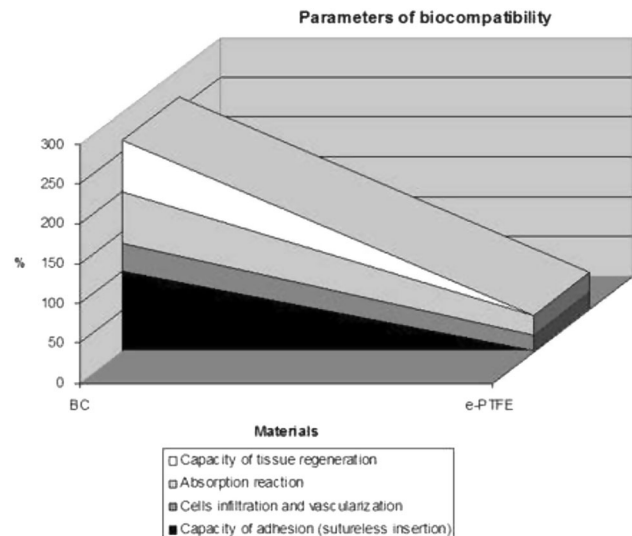
Biocompatible bacterial cellulose membrane in dural defect repair of rat

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Abstract Duraplasty is necessary in nearly 30% of all neurological surgeries. Different tissues and materials have been evaluated in dura mater repair or as dural substitutes in neurosurgery. The aim was to evaluate the biocompatibility of the bacterial cellulose (BC) membranes, produced from sugarcane molasses, for dural defect repair in rats. Forty adults males Wistar rats divided into two groups: a control (ePTFE) and an experimental (BC). Bilateral frontoparietal craniectomy was performed, and a dural defect was created. The arachnoid underlying defect was disrupted with a narrow hook. The animals were observed for 120 days. There were no cases of infection, cerebrospinal fluid fistulae, delayed hemorrhages, behavior disturbances, seizures and palsies. The BC membrane showed to have suitable biocompatibility properties, was not induced immune reaction, nor chronic inflammatory response and absence of neurotoxicity signals.

Graphical Abstract



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1 Introduction

Several conditions can promote damage to the dura mater, which can affect human beings at different stages of their existence. At a neurosurgical procedure, an inadequate dural closure exposes patients to cerebrospinal fluid fistula, infections, neural tissue herniation, hypertensive pneumocephalus, pseudomeningocele, and adhesions [1–3].

In everyday neurosurgical practice, numerous autogenic, allogenic, xenogenic, absorbable, and nonabsorbable synthetic materials have been used with varying degrees of success in the search for the perfect dural graft [3–7].

Different tissues and materials have been evaluated in dura mater repair or as dural substitutes in neurosurgery [8]. However, an entirely satisfactory solution still remains to be found, considering reports of side effects from the use of autologous, heterologous or synthetic substances [9–11].

The use of an autologous graft surgical procedure is an additional variable magnitude and the amount of tissue obtained may be insufficient, especially in children [8]. Furthermore, systemic treatment with synthetic materials increases the risk of wound infection when compared to the use of graft autólogo [10]. The need to employ substitutes for dura mater was identified before dawn neurosurgery as a medical specialty.

Important requirements for using biopolymers in the central nervous system are absence of toxicity of the material, suitable hydrophilic properties, ability to be absorbed by the host tissue and that they permit vascularization, especially due to the poor regenerative capabilities of the brain [12]. Bacteria-synthesized polysaccharides have attracted interest for biomedical applications as unique and promising materials to be used as implants and scaffolds, especially in bone, cartilage, and skin tissue engineering [13]. Some studies using bacterial cellulose have also indicated the compatibility of this type of biomaterial for duraplasty [14–17]. Either in the form of neurotubes, facilitating the process of nerve regeneration [18], or in combination with recombinant proteins or other molecules, bacterial cellulose has been used as substrate for neuronal cell lines or mesenchymal stem cells [19].

Recent evidence has shown that cellulose produced from sugarcane molasses by bacterial synthesis [20] is a non-toxic and low cost biomaterial, useful in a number of biomedical applications, as a support for trypsin immobilization [21], sutures [22], bone healing [23, 24], artery and vein prosthesis [25, 26], abdominal wall mesh repair [27, 28]. In these *in vivo* studies, satisfactory results were obtained, especially considering the ability of this bacterial cellulose to be successfully integration in the different tissues without causing side effects. Recent *in vitro* assays demonstrated its biocompatibility to support the adhesion and growth of human umbilical cord Wharton's jelly's mesenchymal stem cells [29].

Considering the evidence above described, the aims of the present study was to evaluate the biocompatibility of the bacterial cellulose (BC) membranes, produced from sugarcane molasses, for dural defect repair in rats.

2 Materials and methods

2.1 Properties of BC

BC was produced from sugarcane at the Experimental Station of Carpina, Federal Rural University of

Pernambuco, Brazil. The sugar cane molasses is the only raw material used for the synthesis of BC. Molasses are adjusted for the ideal brix in order to facilitate the digestion process. Preparations of BC hydrogel 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) [20].

The BC membranes have produced from the hydrogel to 0.8%. The compact BC membrane with a thickness of 0.2 mm, is manually pressed, during the process of dehydration and air dried. From the compact membrane, if it prepares multiperforated is stamping-holes 16 one millimeter diameter per cm². BC membranes can be standardized in different thicknesses, widths and lengths. The membranes can be preserved in isopropyl alcohol 80% or dried, packed in surgical grade envelope and sterilized by gamma irradiation.

Biomechanical tests with parameters analysis as traction speed, the maximum breaking strength and the specific deformation maximum strength, showed that multiperforated membrane of the BC was better than compact membrane, but with results similar to expanded polytetrafluoroethylene (ePTFE) [27, 28].

The mechanical properties including maximal loading (N), tensile strength (stress, MPa) and elongation (strain, %) of BC patches (previously moistened with isopropyl alcohol) were measured by conducting a standardized tensile test in a United Universal Mechanical Testing Machine (EMIC, model DL 500 MF) with a gap distance of 2.5 cm and a crosshead speed of 250 mm/min. Stress was calculated as F/A , where F is the loading force and A is the area of the cross section of materials. Strain was calculated considering DL/L , where L is the initial length and DL is the difference between the lengths at break and the initial length.

The samples ($n = 10$) were cut into test pieces each 2×7 cm. A digimatic point micrometer (Mitutoyo Series 342, Japan; Resolution: 0.001 mm; Graduation: 0.01 mm) was used to measure the thickness prior to loading the samples into the testing machine.

2.2 Animals

Male adult Wistar rats (40) with ages ranging from 4 to 6 months and weighing 300–440 g were used. All procedures related to the animal care were performed under veterinary supervision according to the institutional rules of Experimental Surgery Center of the Federal University of Pernambuco. The animals were fed a balanced commercial diet (Labina[®]), water was available *ad libitum* and a 12/12 h light/dark cycle (lights on at 6:00 h) was observed.

The animals were divided into two groups according to the material used as dura mater substitute: Group BC ($n = 20$) was treated with bacterial cellulose membranes and

Group e-PTFE ($n = 20$) with membranes of expanded polytetrafluoroethylene (Preclude Membrane[®]) donated by W.L. Gore & Associates, Flagstaff, AZ, USA.

All experimental procedures adopted in this study were approved by the Ethics Committee for Animal Research of Federal University of Pernambuco (protocols #23076.000254/2013-72 and 60/05), in accordance with the Brazilian College for Animal Care guidelines which follows the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA).

2.3 Anesthesia and neurosurgery

The rats were systemically anesthetized with *Chloral hydrate* 10%, (0.4 g/kg; i.p.). Local anesthesia in the region of trepanning was obtained with a subcutaneous injection of 1% lidocaine. Prophylactic antibiotic therapy was done with a single dose of cephalothin sodium (160 mg/kg, i.p.) during anesthesia induction. Before neurosurgery, a trichotomy and asepsis using Povidine-iodine[®] was performed in both groups, in the region along the incision of scalp and retraction of temporal muscle. Using a trephine of 10 mm diameter coupled to a dentistry drill with very low rotation, a fronto-parietal bilateral craniotomy was performed and subsequently the dura mater was displaced under the bone border. A 0.9 cm defect in dura mater was generated with preservation of the superior longitudinal sinus. In one of the cerebral hemispheres, the arachnoid membrane underlying defect was disrupted with a narrow hook to allow better adherence of the sutureless dural substitute [14].

For each group a circular patch of e-PTFE or BC (14 mm diameter) was placed over the exposed encephalic surface. The piece of bone was discarded, keeping the periosteum in contact with the dural substitutes and the scalp was sutured on two planes: the subcutaneous cellular with Vicryl[®] sutures and the skin with mononylon along continuous points. All the surgical procedure was performed using a *D F Vasconcellos*[®] FC 250 surgery stereo microscope.

After the surgical procedure the animals were kept in individual cages and the two groups were compared in the postoperative period with respect to behavioral and motor changes, seizures, presence of liquoric fistula, cicatricial process, hematoma or signs of infection. After 120 days, each animal was weighed and re-anesthetized with 10% *chloral hydrate*. Through a transesternal thoracotomy, the catheterization of the ascending aorta was performed and the inferior vena cava was opened for perfusion of formaldehyde 10% in PBS, pH 7.4. Subsequently, the head was disarticulated and after removal of the skin, it was immersed in a similar solution of fixative for an additional 7 days.

2.4 Histopathological analysis

After fixation, the region of the head where the surgery had been performed was dipped in paraffin wax and coronal sections of 5 μ m were taken and stained in hematoxylin-eosin solution. The histopathological analysis was carried out independently by two individuals using a light Microscope (Nikon Eclipse 50i) coupled to a camera (Nikon DS-Fil) at magnifications of 100x and 400x using planachromatic objectives. A double blind approach was adopted where materials of BC or e-PTFE were not identified.

The systematic study was performed to evaluate the qualitative and quantitative responses of the nervous tissue and bone to the BC or e-PTFE grafts. Tissue cellularity in the external and internal surfaces adjacent to the prosthesis was analyzed according to the following criteria: 1, presence of macrophages only; 2, macrophages associated to giant cells; 3, presence of macrophages, giant cells and granuloma. It was also analyzed the ability of the tissue to biological integration the BC or e-PTFE films and the presence of fibrosis or ossification. A third parameter analyzed was related to the integrity of the prosthesis, taking into account the invasion of each membrane by mononuclear cells, the neovascularization and the presence of fibroblasts. The ability to be absorbed was classified as: absent, mild, moderate, strong or advanced, according to the intensity detected. All the data were transformed into quantitative variables by assigning indices and histological scores.

2.5 Parameters of biocompatibility

The clinical signs, inflammatory response and the capacity of adhesion of the membrane in dural defect (sutureless insertion of the BC), coupled with data from the literature, particularly involving the BC in vitro and in vivo tests that demonstrate toxicity [30, 31], mechanical aspects [27, 28] and performance requirements [29, 32], list the parameters needed for substantiated analysis of the biocompatibility of the BC membrane.

2.6 Statistical analysis

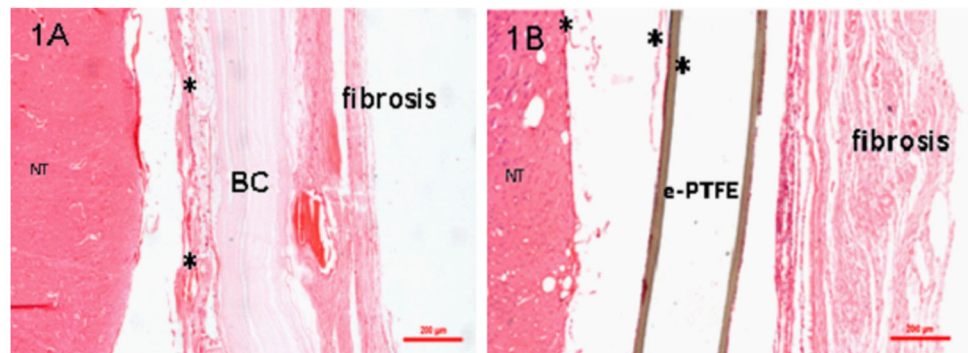
The intergroup comparison was analyzed using Pearson Qui-square, Fisher test and the Mann-Whitney test. Statistical significance was accepted at 95% confidence level $p < 0.05$ for comparison between the groups.

Table 1 Clinical parameters and histopathological analysis

Variables	Outer cell reaction			Inner cell reaction			Absorption reaction				Fibrosis	Calcification	Ossification
	MΦ	MΦ + GC	MΦ + GC + Granuloma	MΦ	MΦ + GC	MΦ + GC + Granuloma	Null	Light	Moderate	Intense			
BC	7	3	10	7	6	7	–	5	13	2	3	4	13
e-PTFE	13	3	4	6	10	4	20	–	–	–	1	9	10
<i>p</i> -value	<i>p</i> (1) = 0.112			<i>p</i> (2) = 0.379			<i>p</i> (1) < 0.001				<i>p</i> (1) = 0.208		

BC bacterial cellulose, e-PTFE expanded polytetrafluoroethylene, MΦ macrophages, GC giant cells, Statistical analysis by (1) Fisher's exact test and (2) χ^2 test of Pearson

Fig. 1 Microscopic views of the BC or e-PTFE implants over the arachnoid membrane and cerebral cortex 120 days after dura mater excision surgery. The presence of fibrosis in the outer surface of both BC **a** and e-PTFE **b** membranes are seen in these representative images (arrows). Examples of ossification process (arrows) are seen in the adjacent outer surface of BC and e-PTFE membranes



3 Results

3.1 Clinical characteristics

Following the dural graft, at the 120 days no signs of toxicity including autonomic (salivation and piloerection) or neurological effects (tremors and convulsions), or even deficiency in the motor or feeding behavior were observed during this period. The healing of the post-operative wound was similar between the two groups. Presence of dehiscence, hematoma formation, leakage of liquor as well as signs of infection, was not observed. Before the surgery, the average body weight of the animals was 380 ± 50 g in Group BC and 395 ± 40 g in Group e-PTFE. On the day of sacrifice, the body weight did not differ between the groups.

3.2 Macroscopic aspect of the surgical wound at the 120th post-operative day

The visual evaluation performed during the removal of the head for histopathological analysis did not detect deformities in the surgical scar, fistulas, focal collections nor granulomas. After withdrawal of the scalp, no cicatricial adhesions in the conjunctive tissue between the scalp and the dura mater substitute were identified. Both the BC or e-PTFE membranes were covered with a thin layer of conjunctive tissue.

3.3 Histopathological analysis

The occurrence of variables between the groups with respect to tissue cellularity in the external and internal surfaces adjacent to the prosthesis of BC or e-PTFE is shown in the Table 1. The ability of the nervous tissue to biological integration the membranes was assessed by the presence of fibrosis or ossification (Table 1) as well as prosthesis integrity, which gave information on the absorption capability (Table 1).

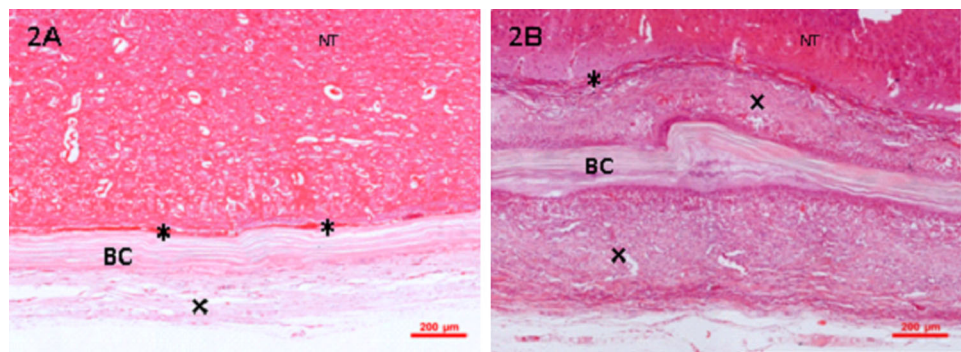
The presence of fibrosis in the outer surface of both membranes can be seen in these images where the absorption of BC was considered mild.

The capability of the BC membrane to be absorbable and invaded by vessels was distinct from the synthetic and non-porous e-PTFE film (Preclude[®]) as dural substitute. In most animals (~65%) the BC membrane absorption was considered moderate, in 25% it was mild, being registered in one case as strong and in another as advanced absorption reaction. e-PTFE absorption was not observed in the animals analyzed in the present study. Representative examples of moderate and strong absorption of BC membrane are shown in Figs. 1, 2.

4 Discussion

The main findings obtained in the present study corroborated the working hypothesis that bacterial cellulose

Fig. 2 Microscopic views of the BC implant 120 days after dura mater excision surgery presenting different degrees of absorption by the host tissue. Examples of moderate **a** and strong **b** absorption. *Thin arrows* indicate the presence of vascularization and *thick arrows* show cell infiltration in the BC



membrane is a versatile biomaterial with potential biomedical and medical applications in neural tissue engineering. The use of e-PTFE as a positive control in this study was based on previous data in the literature showing the biocompatibility of this material as dural substitute without causing adhesion, rejection or absorption, and because of its ease in handling and durability [33–35].

A suitable hydrophilicity, low toxicity, and capacity to adherence were identified in the present study as beneficial features of the BC membranes when they were implanted for 120 days, as observed in previous studies [30–32, 36]. Integration of the BC film to the tissue was biocompatible and induced the tissue remodeling.

Dural closure has been performed with different techniques, materials and protocols, and the optimal combination has yet to be elucidated [37]. It has been recommended that an ideal material for dural repair should be able to protect nerve tissue, inexpensive, easy to handle, without inducing infection or cortical inflammation [38]. Membranes of e-PTFE, chosen here as a control dural substitute, have been widely used in neurosurgery, either in experimental or clinical studies [33–35]. e-PTFE is easily sterilized, keeping its functional properties for long periods of time and is inert, promoting a mild inflammatory reaction characterized by macrophages and giant cells in the inner and outer surface of the implant. Usually they are not rejected by the host tissue and have not been related to malignancy in humans [39, 40].

Despite the different structure of BC and e-PTFE membranes with respect to chemical composition and ability to be absorbed by the tissue, the sutureless insertion of both membranes as a dural substitute did not induce CSF leakage, or local and systemic toxicity. There were no cases of infection, delayed hemorrhages, behavior disturbances, seizures and palsies. The occurrence of a mild inflammatory reaction characterized by the presence of few macrophages and giant cells in the inner and outer surface of the BC membrane was comparable to that found using e-PTFE. Similar reaction was also found when patches of BC and e-PTFE membranes were used in femoral artery [25] or vein [26] angioplasty in dogs for a period of 180 days. In these

studies, both membranes were sutured and despite a mild reaction, none of the animals showed signs of degeneration, infection, rupture, pseudo aneurysm formation or thrombosis at clinical examination.

In the present study, microscopic analysis also demonstrated a satisfactory level of graft acceptance. Moderate extradural fibrosis in the external surface occurred in 15 and 5% of the animals treated with BC or e-PTFE membranes, respectively, enhancing adherence of the implants to the bone fragment. No case of adherence of these membranes to the nervous tissue surface was observed even considering the small incision in the arachnoid membrane performed in the surgical procedure adopted in our study and similar to that reported previously [14, 36].

Our results are comparable to those described using bacterial cellulose membranes synthesized as substitute dural in dogs [14] or in rabbits [17]. In these studies, moderate fibrosis occurred in contact with the bone fragment; but no adherence was seen in the cortical surface. In the dogs, cellulose fibers decreased in thickness until 270 days and were invaded by histiocytes and collagen fibers with low cellular reaction [14].

A recent study using bilayer chitosan scaffolding as a dural substitute allowed fibroblast infiltration and organized collagen fiber deposit in its porous layer [41]. Late favorable results of duroplasty in humans with *Acetobacter xylinum* synthesized cellulose were recently reported although a limitation of this material was the low elasticity of the membranes during suturing [15]. The thickness of BC (~85 µm) is comparatively lower and its elasticity was considered adequate for suturing vein or artery without tearing [25, 26].

In one study about with meshes manufactured from bacterial cellulose (BC), evaluate their organic tissue interaction and compare with an ePTFE's prosthesis used to repair acute defect of muscle aponeurotic induced in rats, the authors observed that there was biological integration and biomechanical elasticity of BC membrane, and therefore, they considered as a new alternative biomaterial for use as a bio prosthesis [28].

Ossification in the external surface of BC or e-PTFE grafts was the most frequent data observed in the present study, regarding the host capacity in retaining the dural substitute (65% for BC vs. 50% for e-PTFE). These results seem to reinforce recent evidence related to biocompatibility of BC for interacting with the bone tissue in the treatment of critical defects in rat skulls [24]. According to this study, the association of collagen membrane of bovine origin (GenDerm[®] Baumer SA) and BC gel as carrier of bone morphogenetic protein (BMP) and lyophilized bovine bone grafts (Gen-ox inorg[®] Baumer SA) was able to induce new bone formation at the edge of the skull defect. The performance of BC graft was better than that using GenDerm[®] alone as BMP carrier for a period of 90 and 180 days after surgery [24].

Integration of biomaterials or implants with surrounding native tissue is determinative for both functionality and long-term performance of the tissue. Capability to be absorbed and integrated into different living tissues has been reported as one of BC's properties, which motivated the present study. The findings revealed that as dural substitute for 120 days, this biopolymer was slowly integrated into the surrounding tissue and a moderate level of absorption was detected in 65% of the animals. In two animals, a higher level of absorption was seen, characterized by the presence of intense cells infiltration and vascularization.

5 Conclusion

These results show that bacterial cellulose membrane is an adequate biomaterial for dural repair. The biocompatible BC was, retained the properties of local tissues and provides adequate mechanical properties, without the need for sutures when implanted for 120 days. The BC membrane not induced immune reaction, nor chronic inflammatory response and absence of neurotoxicity signals.

Furthermore the BC membrane can be easily produced, it has simple sterilization, is easy to handle and application and affordable.

Future studies should be carried out in order to assess the viability of BC as a dural substitute over longer periods of time.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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