Construction and *in vitro* testing of a cellulose dura mater graft

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Introduction: Cerebrospinal fluid (CSF) leaks are a common complication after cranial and spinal surgery and are associated with increased morbidity. Despite continuous research in this field, this problem is far from solved. In this paper, we describe the construction and testing of a bacterial cellulose (BC) membrane as a new dural patch.
Materials and Methods: The synthesis of BC was performed using *Gluconacetobacter hansenii* (ATCC 23769) and films were sterilized by autoclaving. The membranes were seeded with human dural fibroblasts. Growth, shape, and cell viability were assessed after 4 weeks.
Results: Normally shaped fibroblasts were seen on the BC grafts; confocal microscopy showed cells inside the structure of the mesh. Both viable and nonviable cells were present. Cellular attachment and viability were confirmed by replating of the membranes.
Discussion: BC membranes are used in clinical practice to improve skin healing. In the presence of water, they form an elastic, nontoxic, and resistant biogel that can accommodate collagen and growth factors within their structure, thus BC is a good candidate for dural graft construction.

Keywords: Cerebrospinal fluid leak, Dura mater patch, Bacterial cellulose membrane

Introduction

Cerebrospinal fluid (CSF) leaks represent a usual and severe complication in neurosurgery, with an incidence of up to 10% in posterior fossa and cranial base surgery.[1,2] CSF leaks are associated with meningitis, pneumocephalus, prolonged hospital stay, and even death. In many patients, a reoperation is required to achieve closure of the dural gap.[3–5] Endoscopic skull base approaches constitute a major challenge in this matter and closure and new techniques are proposed every year to prevent CSF leaks.[6,7]

Dural implants are used either to complete a dural gap or to reinforce primary closure. An ideal graft should contain CSF and allow for native dural tissue to form; many materials have since been designed and are commonly used.[5] We previously documented the difference between animal and human dural biology differ; thus, testing the interaction between human dural cells and implants is necessary.[8]

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Dural biology has not been extensively studied and few experimental models exist.[8,9] Understanding dural tissue ingrowth into grafts is key in designing implants. We previously demonstrated how selected growth factors increase human cellular migration *in vitro,* and how collagen is not an essential molecule to this procedure.[10] On the other hand, no cellular growth is observed in animal models in the absence of collagen.[9]

Bacterial cellulose (BC) is an extracellular hydrophilic polysaccharide constituted by β-(1→4) glucose chains and biosynthesized on an air/culture medium interface by *Gluconacetobacter hansenii,* among other micro-organisms.[11,12] The unique nanofibrillar structure has a high water content (approximately 99%) and is distinctly pure with a high degree of polymerization (up to 8,000), crystallinity (70–80%), and mechanical stability.[13,14] In particular, BC has been widely studied for wound healing, as well as tissue engineering.[15–17] Yet, it has never been used in dural defect repair.

In this study, we used an *in vitro* model of dural healing to test BC membranes as candidates for dural grafts.

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Initial testing with a human model can answer questions in the pre-clinical phase that may only become apparent in clinical trials when grafts are first exposed to human tissue. BC is easily obtained, impermeable, and can be harvested over large surfaces inexpensively, which makes it a good candidate for dural repair.

Methods

Graft building

Bacterial cellulose production and purification

The synthesis of BC by *Gluconacetobacter hansenii* (ATCC 23769, Manassas, Virginia, USA) was performed in a medium containing (g l^{-1}): 25.0 Mannitol, 5.0 yeast extract, and 3.0 peptone, which was adjusted to a pH of 6.5 with 0.1 M NaOH solution before sterilization. The culture was maintained statically in 96-well plates at 30° C for 6 days. BC films were collected from the plates and washed with distilled water. BC purification was performed by incubating the membranes in 100 mM NaOH at 50° C for 24 h, followed by successive washings with distilled water, after which the pH was adjusted to 7.0. Later, BC films were sterilized by autoclaving (121° C for 20 min).

Thermogravimetric Analysis (TGA)

Dynamic thermogravimetric measurements of native and hybrid bacterial cellulose membranes were performed with a Shimadzu TGA-50 instrument (Columbia, Maryland, USA). Testing was performed from 20 to 900º C at a heating rate of 10º C/min under atmospheric N_2.

X-ray diffraction (XRD)

XRD patterns of cellulose film samples were collected in reflection mode on a glass substrate, and analyzed by Origin and Sigma Plot software. Measurements were performed with an Analytical Expert instrument using Cu-Kα radiation (λ = 1.54 Å) from 2θ = 10 to 70º in continuous mode with a 0.07º step size.

Scanning Electron Microscopy (SEM)

Samples were made by sputtering the surface with gold, using a metalizer (Balzers SCD 030, Schamburg, Illinois, USA), obtaining a layer thickness between 15–20 nm. Film surfaces and morphologies were observed by SEM (Philips SEM 505, Rochester, NY, USA) and the images processed by an image digitalizer program (Soft Imaging System ADDA II, Munster, Germany)

In vitro testing

Specimen collection

Human dural specimens were obtained from decompressive craniectomies, where a small fragment of dura could be resected without changing surgical technique. Informed consent was obtained in every case after protocol approval by our Institutional Review Board. Specimens were maintained at 4° C in sterile saline overnight to be processed the following morning.

Explant culture

Tissue was processed as previously described. Specimens were washed with Dulbecco′s modified Eagle′s medium (D-MEM, Gibco, Waltham, Massachusets, USA) and debrided with a sterilized swab. After debridement, specimens were fragmented into 4 x 4 mm squares that were used to prepare dural fibroblasts explant cultures and subcultures.

Specimens were cultured on 30-mm culture plates (Nunc, Rochester, New York, USA) with 2 ml of D-MEM supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B (Gibco), 200 mM of α-glutamine (Gibco), and 10% of bovine fetal serum (Bioser, Barcelona, Spain) simple plates (Gibco) were used. Cultures were performed in a humidified atmosphere with 5% CO_2 at 37º C.

Subcultures in cellulose membranes

Dural fragments were used to perform subcultures on 30-mm plates (Nunc) under the aforementioned conditions and in the same medium. Tissue specimens were seeded, allowing cells to migrate. When cell confluence was reached, the tissue specimens were removed from the plate and the cells were subcultured. The plate was treated with a 0.25% trypsin solution (Sigma, St. Louis, Missouri, USA) for 3 min. After neutralizing the trypsin with culture medium, cells were centrifuged at 1200 rpm for 10 min, resuspended, and plated onto T-75 flasks (Nunc). Cells were quantified by the trypan blue exclusion method and 100,000 cells were seeded on 12 cm^2 fragments of BC membranes.

Specimens were analyzed after 4 weeks, when cellular growth, shape, and viability were assessed.

Immuno histochemistry

Cultured cells were examined using brightfield microscopy; hematoxylin–eosin and Masson trichromic staining were used. Cells were marked by staining with anti-vimentin primary antibodies (Millipore, Billerica, Massachusets, USA) as previously showed.[8] Membranes were fixed in 4% paraformaldehyde for 1 h, then washed with phosphate-buffered saline (PBS) at pH 7.2; then they were treated with PBS with triton X-100 for 1 h and incubated with PowerblockTM (Biogenex, San Ramon, California, USA) for 30 min to block non-specific binding sites. The primary antibody was incubated overnight at 4º C in a moist chamber. The following day, the BC membranes were washed with PBS and incubated with biotinylated (Multilink™; Biogenex) secondary antibody for 60 min at room temperature, and then with fluorescein streptavidin (Vector Labs) for 60 min; finally, they were washed with PBS at pH 8.2. Last, they were analyzed under a Nikon Eclipse E400 microscope and a Zeiss LSM 510 metaconfocal microscope.

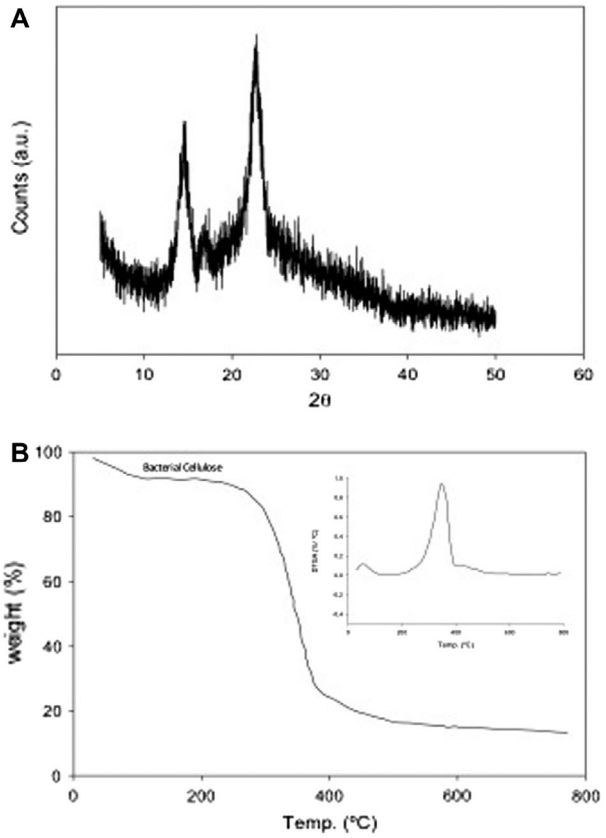

Figure 1 (A) XRD spectra for BC. (B) Thermogravimetric assays of native BC. TGA curve and DTGA curve.

Cell viability

Cultures in cellulose membranes were observed using an inverted microscope (Nikon TMS). Vital staining with acridine orange and ethidium bromide (AO/EB) was performed to count cells that had grown in the collagen meshes, and to assess viability. The total cell count was then established and assessed for viability, with viable cells staining green and non-viable cells staining red when observed under a Nikon Eclipse E400 fluorescence microscope. Membranes containing dural cells were extensively washed and replaced onto new Petri dishes to assess for cellular growth from the membrane.

Handling characteristics of the BC membranes

The BC membranes underwent surgical instrumentation by conventional microsurgical tweezers and were sutured with 3.0 silk surgical sutures, in order to test the mechanical stress produced in a surgical scenario.

Results

Cellulose membranes

Thermal properties of the BC membrane were investigated using thermogravimetric analysis (TGA). A two-step decomposition curve was observed. The unbound water content was responsible for the first step, observed at a range of 30–120° C. At 180° C, the percentage of weight loss was approximately 7%. The second peak was attributed to thermal decomposition of the biomaterial. In (Fig. 2), the decomposition temperature of the films at 350° C can be seen. Other thermal parameters can also be observed, such as a thermal degradation temperature of 345.6°C (T_p as the maximum rate of degradation), which is affected by structural parameters such as molecular weight, crystallinity, and orientation. The amount of residue that membranes left at 750° C correlated with the carbon content of the sample after all degradation processes (13,5%).

As a way of continuing with the structural analysis, XRD assays for BC films were also performed in order to specifically evaluate crystallinity and network properties. Characteristic peaks for the biomaterial at $2\theta = 14.6º$, 16.9º, and 22.8º, indexed as 1 0 0, 0 1 0, and 1 1 0 reflexions planes, evidenced the presence of cellulose I crystals (Fig. 1). Spectra profiles were very similar in comparison to the ones previously reported. A high crystallinity index (71%) was also observed.

BC films were observed by scanning electron microscopy. Images showed the network pattern and the assembly of nanofibrils (Fig. 2).

Growth of dural fibroblasts in three-dimensional cultures

Cellular growth was observed from all explants. After seeding onto cellulose membranes, dural cells could be seen as normal-shaped fibroblasts. Regular staining techniques showed fusiform cells displaced randomly in the center of the mesh, forming a circular pattern on the edges (Fig. 3).

Immuno histochemistry

Vimentin staining showed positive elongated cytoplasms as seen in two-dimensional cultures, pseudopods, and a fusiform arrangement within the mesh (Fig. 4). Confocal scanning showed similar results and proved that dural fibroblasts were growing inside the membrane and not only on its surface (Fig. 5).

Viability

After four weeks, seeded cellulose membranes were assessed for the presence of viable cells. Using a vital technique, mixed red and green nuclei were observed, reflecting the presence of both viable and dead fibroblasts (Fig. 4). To further assess the cells within the mesh, the membranes were extensively washed in saline and replated on new Petri dishes. After one to two days, migration of dural cells could be observed in all the specimens ($n = 5$), reflecting that the fibroblasts were viable and retained their migratory capacity.

Handling characteristics of the BC membranes

BC membranes are soft and, once hydrated, the structure, elasticity, and strength did not change in the presence of

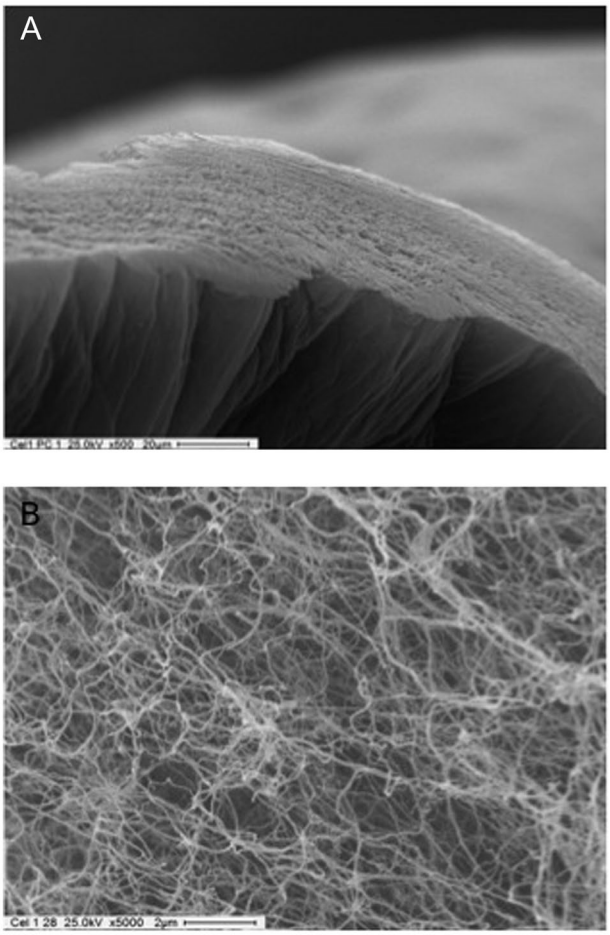

Figure 2 SEM images of BC membrane. 500 × (A) and 5000 × (B). The multiporous structure of the mesh and its nanofibrils can be seen.

blood or CSF. They were easy to handle, resembling the characteristics of synthetic skin. The BC membrane was very easy to suture and preserved its structure, applying and tightening a silk knot (Fig. 6).

Discussion

Despite the development of new dural grafts and sealants, CSF leaks remain a major issue after neurosurgical procedures.[1, 2] Extended endoscopic approaches have significantly complicated this matter, as primary closure cannot be accomplished in this setting.[6, 7] In this study, we developed and tested a cellulose scaffold that may aid in dural closure after surgery.

Recently, much research in scaffold development for dural repair has been performed.[18] BC is a material with good mechanical properties which is a promising candidate for dural repair[19] and published reports state fibroblasts from continuous lines can grow within its structure.[20] However, to our knowledge, there is no evidence that dural cells grow inside this kind of mesh, nor are there reports of the viability, attachment, and shape of this kind of material. Transformed (continuous) cell lines have an outstanding growth capacity and thus their ability to proliferate within a tridimensional scaffold exceeds what it is expected with non-transformed (primary) cell lines. Also, as we previously published human dural fibroblast behave different from rabbit and mice cells. Because of these preclinical tests with human, primary organotipic cultures are important to further develop a dural grafting material.

BC membranes are used in different fields of tissue engineering and current clinical experience is primarily from skin reconstruction.[21] BC membranes have been tested as a scaffold for artificial blood vessels, urethral tissue, cartilage, and as drug delivery devices.[22, 23] BC membranes are hydrophilic, biocompatible, and nontoxic.[23, 24]

The specific use in neurosurgery as a dura mater graft is supported by their structural resistance [25–27]. BC membranes are composed of a network of fibrils that form an elastic and resistant hydrogel when exposed to water. It can be customized and specifically shaped for any defect. They have a smooth surface that minimally interacts with native tissue and a porous one that allows for cellular penetration into its structure. Construction of BC membranes under sterile conditions is a complex but inexpensive process; large membranes can be constructed with minimal cost.[28]

We seeded dural fibroblasts on the porous surface of BC membranes and evaluated cellular growth after 4 weeks. Cells were able to penetrate the scaffold,

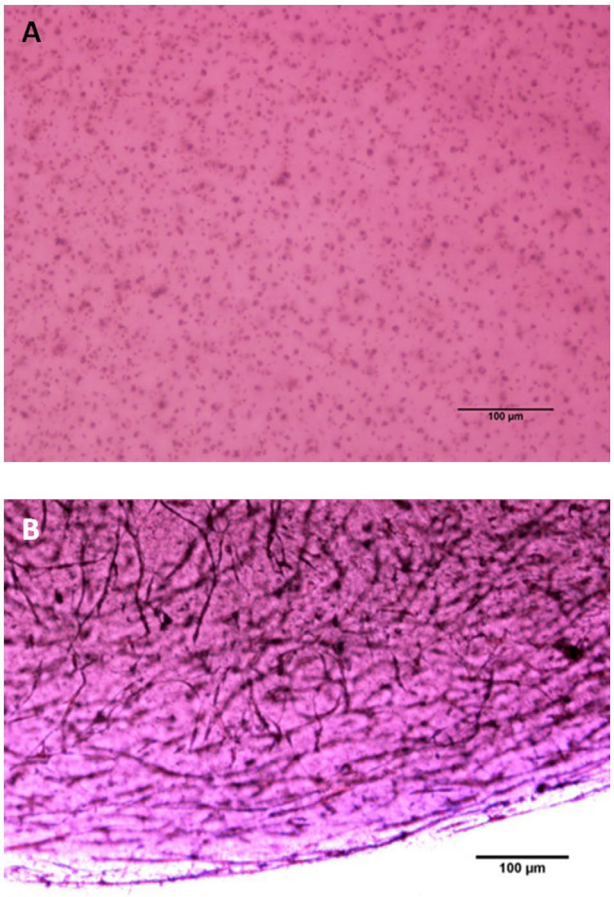

Figure 3 CM with (A) and without (B) dural fibroblasts, Masson´s trichromic staining. Cells enter the membrane and align in radial fashion near the edges of the cellulose network and randomly toward the center.

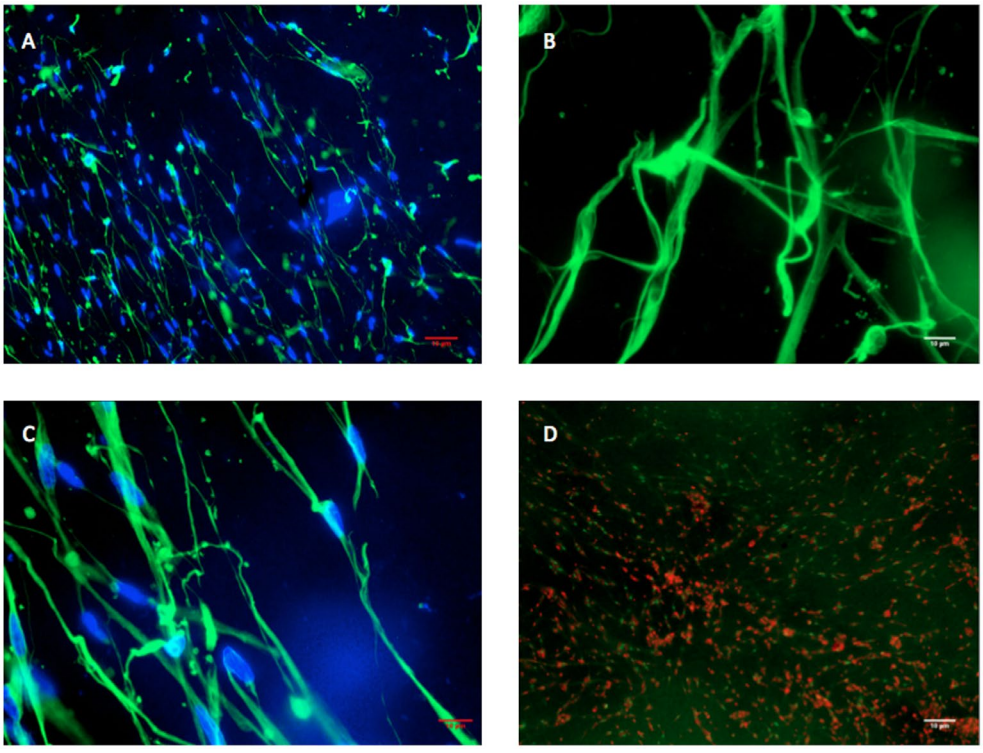

Figure 4 Immunofluorescence for vimentin (A, B, C) shows positive spindle-shaped cells within the cellulose membrane. Nuclei are stained with the mesh technique (A and C), A = 10 X, B and C = 40 X. D shows a mixture of red and green nuclei after staining with a mixed solution containing acridine, a mixture of live (green) cells, and dead (red) cells appearing 4 weeks after seeding.

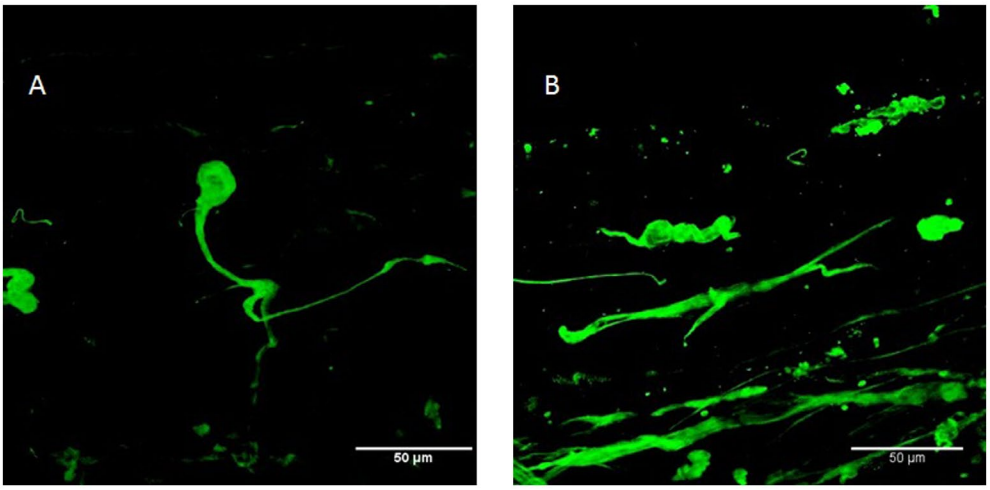

Figure 5 Immunofluorescence for vimentin seen under confocal microscopy (100X) shows typically shaped fibroblasts inside the structure of the mesh. A, 3-D reconstruction; B, axial cut of the CM.

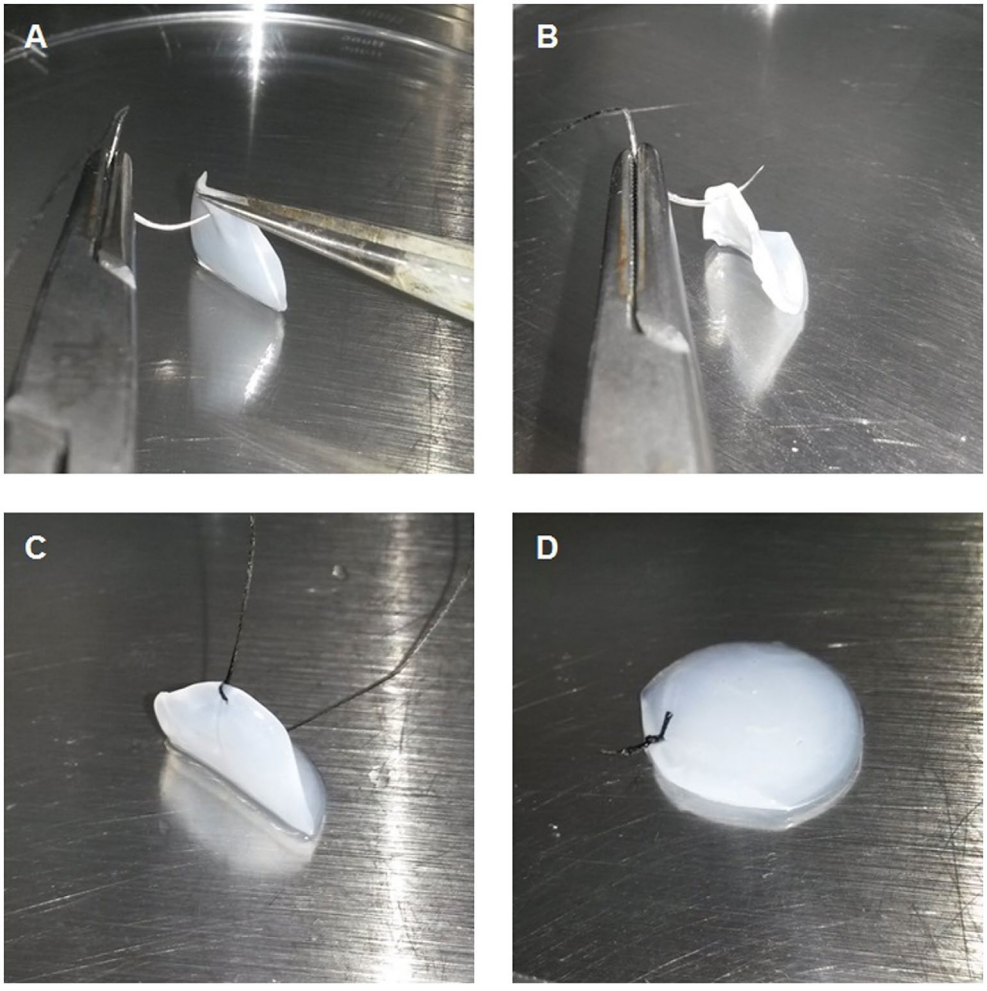

Figure 6 Macro photographs showing the handling features of the BC membranes under surgical instrumentation. A, B, surgical tweezers, and suture needle implementation; C, suture holding; D, suture knot stretching.

preserve their normal membrane structure, and remain viable. Confocal microscopy demonstrated cells to be present inside the BC structure, and not only on the surface. When seeded BC membranes were extensively washed and replated, dural fibroblasts appeared on the Petri dish, implying that cells were attached to the membranes and viable.

Previously, we published that FGF and insulin were the primary growth factors involved in dural fibroblast migration.[10] The BC allows for immobilization of these

molecules; a mesh that contains them and accelerates the healing process can be built using these principles. Also, further experiments will look for the interaction between the BC and hemostatics and dural sealants.

Conclusion

To our knowledge, this is the first report of a BC membrane with dural fibroblast ingrowth. The biomechanics of this material, the capacity to customize its shape, its biocompatibility, and its documented dural fibroblastic ingrowth implies that BC can be a potential candidate for perioperative dural repair.

Notes on contributors

Regenerative medicine and skull base reconstruction for all authors.

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