https://doi.org/10.31925/farmacia.2026.1.21 **ORIGINAL ARTICLE**

IN VIVO TESTING OF BACTERIAL CELLULOSE IN CORNEAL AFFECTIONS MANAGEMENT

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Abstract

In dog and cat traumatic corneal ulcers are the most common. Corneal ulcers in such pets are locally treated, or surgery can be performed (third eyelid flap). In many corneal ulcers the eyeball enucleation is the unique option caused by complications. For six cases (three common breed dogs and three European breed cats) diagnosed with corneal ulcers, an experimental product obtained from bacterial cellulose was used for the first time. The experimental product was obtained from horticultural by-products through a bioprocess using the bacterial strain *Acetobacter xylinum* DSMZ-2004. The bacterial cellulose membrane (BCM) was applied on the surface of the cornea, similar to an anatomical bandage and after the performed third eyelid flap. The third eyelid flap sutures were kept for three weeks. The treatment was unique and no additional therapies were used. Following the study the healing of corneal ulcers was achieved with corneal scars, but with vision preservation.

Rezumat

La animalele de companie (câine și pisică), ulcerele corneene traumatice sunt cele mai frecvente. Ulcerele corneene la câine și pisică se tratează medicamentos sau chirurgical (flap de pleoapă a treia). In foarte multe cazuri se recurge la enucleerea de glob ocular urmare a complicațiilor. Pentru șase cazuri (trei câini rasă comună și trei pisici rasă Europeană) diagnosticate cu ulcere corneene s-a folosit în premieră un produs experimental obținut din celuloză bacteriană. Produsul experimental a fost obținut prin valorificarea unor subproduse horticole printr-un proces biotehnologic utilizând tulpina bacteriană *Acetobacter xylinum DSMZ-2004*. Pentru aceste șase cazuri s-a aplicat pe suprafața corneei, membrana de celuloză bacteriană (MCB) asemănător unui pansament anatomic și s-a efectuat flapul de pleoapă a treia. Firele de sutură de la nivelul pleoapei a treia au fost menținute timp de trei săptămâni. Tratamentul a fost unic și nu s-au folosit alte terapii adiționale. În urma studiului s-a constatat că vindecarea ulcerelor corneene s-a realizat cu cicatrice corneeană, dar cu păstrarea vederii.

Keywords: *Acetobacter xylinum*, bacterial cellulose membrane (BCM), corneal ulcer

Introduction

Corneal diseases are a significant cause of blindness globally, as highlighted by the World Health Organization (1). Corneal infections pose considerable challenges in the field of ophthalmology (2), often leading to scarring and cloudiness of the cornea (3), which may necessitate corneal graft transplantation (4) or alternative therapeutic approaches (5). One promising treatment option is pirfenidone (6), a broad-spectrum agent that has shown potential efficacy in treating corneal scars in animal models, such as rats and rabbits (2).
Innovative approaches in corneal tissue engineering (7) include the use of advanced biopolymers as substrates that promote corneal epithelial cell proliferation (8,9). Recent studies indicate promising outcomes in leveraging these materials for more effective and sustainable corneal repair. Furthermore, the development of innovative and facile techniques (10) enhances the accessibility and efficiency of these methods, paving the way for improved clinical applications in vision restoration.
Bacterial cellulose membranes (BCM) and silk fibroin nano-composites represent promising materials in the field of corneal tissue engineering. Recent studies have shown that the incorporation of silk fibroin into BCM can enhance the properties of the composite, making it suitable for medical applications. Specifically, nano-composites with a 50% fibroin content combined with BCM have demonstrated non-cytotoxicity, stability, and biocompatibility (11).
On the other hand, biomaterials like collagen gel, collagen chondroitin sulphate or fibrin-agarose were tested in natural or synthetic scaffolds but with no clinical use (7).

However, these materials often fail to fully meet the critical requirements for corneal regeneration, particularly in terms of optical transparency, permeability to water and nutrients, mechanical strength, and long-term stability (12). Consequently, the search for alternative scaffold materials with improved physicochemical and biological properties remains a crucial focus in the development of effective therapies for corneal diseases.

In recent years, bacterial cellulose (BC), a natural polymer produced by bacteria, has attracted much attention for biomedical applications due to its high mechanical strength and elastic modulus, high water holding capacity and porosity, high crystallinity and polymerization, fine web-like network, and good biocompatibility (13-17).

Bacterial cellulose offers several advantages that make it suitable for corneal tissue engineering. Its high optical transparency ensures minimal interference with light transmission, while its mechanical robustness enables resistance to both surgical suturing and intraocular pressure. In addition, its nanofibrillar architecture mimics aspects of native stromal organization and supports cell adhesion and proliferation. Taken together, these properties highlight BC as a particularly promising scaffold material for corneal regeneration (16-17). They are produced by the fermentation of certain bacteria, including *Acetobacter xylinum* (now known as *Gluconacetobacter xylinus*), which is a gram-negative bacterium. During fermentation, the bacteria synthesize cellulose, which forms a pellicle (or membrane) at the air-media interface (11, 18,19).

This biomaterial consists in a micro fibrils network, distributed in random directions, with translucent gelatinous film appearance (20) and with cosmetic, food or biomedical applications (9, 20) BCM is well known as a marketable innovative biomaterial especially as skin wound dressing, drug transport agent, or cell scaffold (21). The native bacterial cellulose was preferred in many *in vitro* and *in vivo* (3, 22, 23) corneal management studies (24) due to its biocompatibility (25) and capability of reducing the rejection (20). In ophthalmology, BCM has been preferred for corneal management because of its excellent biocompatibility, which minimizes the risk of rejection and supports the healing process in both *in vitro* and *in vivo* studies. Its properties offer significant advantages for maintaining ocular health and enhancing patient outcomes (26-29).

Overall, the versatility and beneficial properties of bacterial cellulose make it a valuable biomaterial with promising potential in various applications. *In vivo* studies were usually performed on small animals and less on pets (like dog, cats). Pure nano-cellulose and its polycaprolactone composites, were tested on rabbit superficial corneal ulcer with good tissue integration and no inflammatory reaction (4). Tubular nano-cellulose structures implanted in rats or pigs (20) demonstrated the regeneration of the damaged femoral nerve; tested on sheep carotid arteries BCM biomaterials revealed a long-term stability bypass (22). By now the ophthalmological BCM benefits has a limited testing area, especially for *in vivo* studies in small pets. In companion animals, corneal ulcers exhibit distinct etiologist, standard treatment strategies, and prognostic outcomes compared with human patients, with the third eyelid flap commonly used as a temporary protective measure, thereby highlighting the need for alternative corneal bandage materials such as bacterial cellulose.

The aim of this study was to evaluate the feasibility and preliminary clinical outcomes of BCM used as a temporary corneal bandage in the treatment of corneal ulcers in companion animals.

Materials and Methods

Microorganism

Acetobacter xylinum DSMZ-2004 bacterial strain (German Collection of Microorganisms and Cell Cultures), known for its ability to produce cellulose under specific conditions, was used.

Production and preparation of bacterial cellulose membranes

Nano-cellulose membranes were obtained by a biotechnological process using *Acetobacter xylinum* DSMZ 2004 on a mixed carbon source from horticultural sources and glycerol. All reagents have been acquired from Scharlau Chemie. The microorganism was maintained on a Schramm-Hestrin agar medium which constituted the preinoculum phase. The inoculum phase medium composition was composed of (%, w/v): glucose 1, corn steep liquor 1, and mono potassium phosphate 0.15, citric acid 0.1. Because *Acetobacter xylinum* DSMZ 2004 is a strict aerobic microorganism, the oxygen supply was ensured by the working flask capacity. The incubation at 30°C for 48 hours was performed static culture. The bioprocess and the pure bacterial cellulose isolation and characterization were carried out according with RO Patent 201000566 (31) and Casarica et al., 2013 (30). The static incubation regime was performed at 30 °C for 14 days. The BCM developed on the air-liquid surface was collected and purified by basic treatments, neutralized with low acetic acid solution and microbiologically preserved by sterilization. The yield of bacterial cellulose production was estimated by gravimetric assay.

Endotoxin contamination study

The test for bacterial endotoxins has been used to detect or quantify Gram-negative bacterial endotoxins in bacterial cellulose (BC) samples,

using the Gel-clot method based on the LAL (*Limulus Amebocyte Lysate*) reagent from the horseshoe crab (*Limulus polyphemus or Tachypleus tridentatus*). The standard endotoxin stock solution is prepared from a reference standard for endotoxins that is calibrated against the International Standard, such as the BRP (Biological Reference Preparation) standard endotoxin.

The endotoxin limit of 5 EU/kg body weight/hour or 350 EU for a 70 kg adult has been scientifically established to prevent febrile reactions and hypotension resulting from the intravenous or intramuscular administration of pharmaceutical products contaminated with bacterial endotoxins. Time is a factor taken into consideration as there are mechanisms in the liver and blood to neutralize bacterial endotoxins. One EU (Endotoxin Unit) is a unit of biological activity of the standard endotoxin reference.

For medical devices, following the recommendations regarding extraction volume, the endotoxin limit is 0.5 EU/mL or 20 EU/device for products that come into direct or indirect contact with the cardiovascular system and the lymphatic system.

For devices that come into contact with cerebrospinal fluid, the limit is 0.06 EU/mL or 2.15 EU/device.

For devices that come into direct or indirect contact with the intraocular environment, a lower endotoxin limit may be applied.

Samples of bacterial cellulose for testing the levels of bacterial endotoxins have demonstrated a level of bacterial endotoxins below the acceptable limits for the category of medical devices to which they belong.

Endotoxin concentrations were quantified and reported in endotoxin units per milliliter (EU/mL) and per device (EU/device). All tested BC samples demonstrated endotoxin levels of 0.05–0.08 EU/mL (corresponding to 0.2–0.3 EU/device), which are below the maximum allowable limit of 0.5 EU/mL (or 1 EU/device) established for medical devices intended for ocular surface contact. These results confirm that the BC samples meet the endotoxin safety requirements for ophthalmic applications**.**

Evaluation of BC cytotoxicity

To assess the cytotoxicity of the native, purified, and sterilized bacterial cellulose (BC) product, two types of experiments were conducted: the indirect "extract" method and the direct contact method.

The qualitative assessment of cytotoxic effects was conducted by examining cellular morphology, degree of spread, vacuolization and detachment, cellular lysis, and membrane integrity under a microscope.

The quantitative assessment of cytotoxic effects was performed by determining cell viability using the MTS method [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazole] with the *CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, USA).*

A reduction in cell viability of more than 30% leads to the classification of the sample as having cytotoxic effects.

Evaluation of cytotoxicity – Extraction Method

Testing the cytotoxic effect of "medium 1" and "medium 2" (application of extraction medium over fibroblasts at 70% confluence)

Murine fibroblasts (NCTC clone 929 [L cell, L-929, derivative of Strain L] ATCC) were seeded in 96-well plates. When the confluence reached approximately 70%, the cells were treated: (i) with complete culture medium ("control") and "medium 1" for 24 hours (Figure 1); (ii) with complete culture medium ("control"), "medium 1," and "medium 2" for 25 hours and 30 minutes (Figure 2).

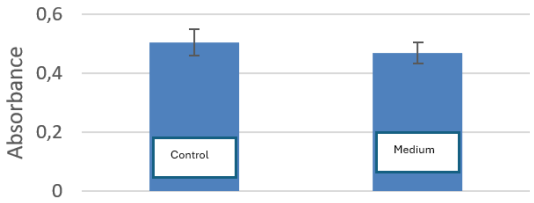


Figure 1.
Evaluation of BC– cytotoxicity (exposure time 24 hours, 96-well plate), *extract method*

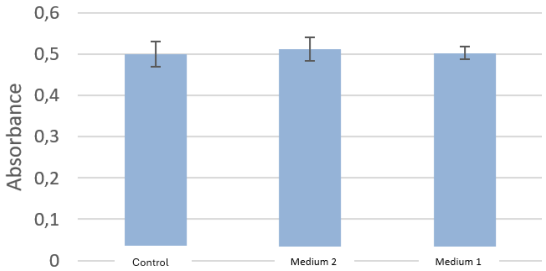


Figure 2.
Evaluation of the cytotoxicity of BC– (exposure time of 25 h 30 min), extract method

The medium was removed and replaced with MTS diluted in MEM-α medium, and cell viability was assessed using the MTS assay [3-(4,5-dimethyltiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], utilizing the *CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, USA).*

The blank was determined in a well where no cell culture was performed, but MTS solution was applied.

The statistical analysis of the results is summarized in Table I and Table II. The interpretation of the data regarding cytotoxicity was conducted using a scale

based on statistical interpretations, according to which the effects are classified as follows: $p < 0.01$ – strongly cytotoxic; $0.01 < p < 0.05$ – cytotoxic; $0.05 < p$ – moderately cytotoxic; and $p > 0.1$ – practically non-cytotoxic.

Table I

BC cytotoxicity evaluation (exposure time 24 hours, 96-well plate), extract method

	Average DO 490 nm	Standard deviation	Cell viability (%)	p (t-test)
Blank 24 h, N = 5	0.505	0.045	100	-
Medium 1, Treatment 24 h, N = 8	0.469	0.036	92.8	0.14 > 0.05

Table II

Cytotoxicity BC Assessment – (exposure time 25 h 30 min, 96-well plate), extract method

	Average DO 490 nm	Standard deviation	Cell viability (%)	p (t-test)
Control 25 h 30', N=8	0.5005	0.031	100	-
Medium 2 Treatment 25 h 30', N=10	0.5139	0.028	102.6	0.355 > 0.05
Medium 1 Treatment 25 h 30', N=10	0.5034	0.015	100.5	0.798 > 0.05

Visual observations: medium 1 had a slightly yellowish colour compared to medium 2 and the control

According to these data, the tested product, native bacterial cellulose, purified and sterilized (BC), is free of cytotoxicity under the conditions of exposure for 24-25.5 hours to the test extracts (medium 1 and medium 2).

The results from the quantitative determinations are supported by qualitative analysis, as the microscopic examination of the cultures did not reveal any morphological changes in the cells.

Cytotoxicity evaluation – Contact Method

The contact test allows for the clarification of the following aspects: cytotoxicity, variation in cell adhesion, and variation in cell proliferation.

Application of the sample BC (initial form, unwashed/unconditioned), 24-groove plate, 40% confluence

The absorbances recorded for both the control and the sample, at 24 and 48 hours, are presented in Figure 3 and Table III.

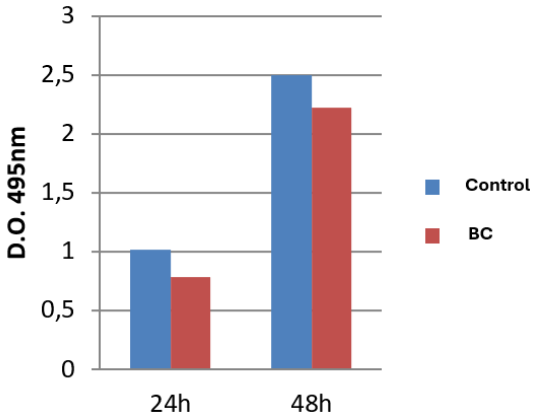


Figure 3.

Evaluation of BC cytotoxicity (initial, unwashed) – contact method, 24-well plates, exposure for 24 and 48 hours

Table III

Evaluation of the cytotoxicity of the initial BC sample (unwashed), applied at a cell confluence of 40% (exposure times of 24 and 48 hours), contact method

	Average DO 490 nm	Standard deviation	Cell viability (%)	p (t-test)
Blank 24 h, N = 5	1.0136	0.013	100	-
BC 24 h, N = 5	0.7842	0.082	74.4	0.011246
Blank 48 h, N = 5	2.499	0.132	100	-
BC 48 h, N = 5	2.221	0.113	88.8	0.054892

From the data analysis, it can be observed that there is a reduction in cellular viability, particularly after 24 hours of treatment. In this case, we consider that it is more likely an inhibition of cellular proliferation rather than a cytotoxic effect. In this experiment, upon applying BC in the culture plates, a change in the colour of the medium was observed; therefore, in subsequent experiments, the BC pieces were washed/conditioned in the growth medium before being administered to the cells, in accordance with point 1 (*Sample Preparation*).

At the same time, based on the experimental observations made, a system involving 96-well plates and samples smaller than the well surface was chosen.

For the next stage, we recommend optimizing the washing method for the cellulose film.

Application of washed/conditioned BC sample, 96-well plate, immediately after seeding of fibroblasts

Seeding conditions: 20,000 cells/well - 96-well plate.

Fibroblasts from 10 wells were treated with a piece of washed/conditioned cellulose (as described in

point 1. Sample Preparation). Another 10 wells containing fibroblasts were maintained as "controls" with complete medium. After 24 and 48 hours of treatment, a qualitative analysis of the cells (attachment, spreading, shape) was performed, and photographs were taken (Figure 4, Figure 5 Figure 6 and Figure 7).

24 hours post-seeding and treatment – representative image are shown.

48 hours from seeding, respectively treatment – representative photos.

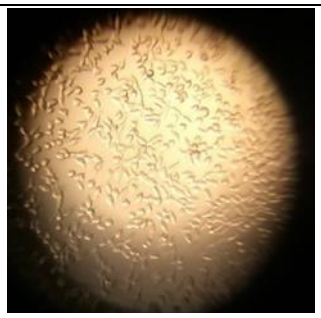


Figure 4.
Appearance of the control culture of L929 fibroblasts, *contact method,* 96-well plate, exposure time 24 hours (Inverted microscope, Obj. 10 X)

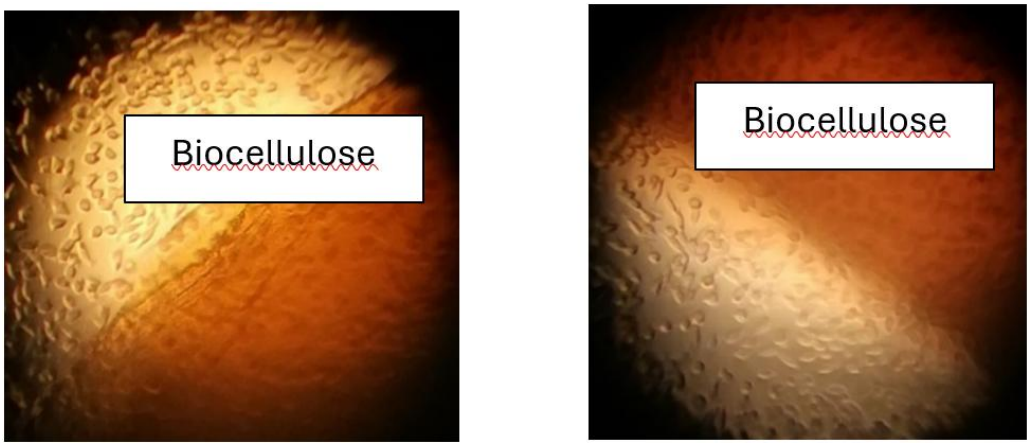


Figure 5.
Appearance of L929 fibroblast culture – contact method, Sample BC, 96-well plate, exposure time 24 hours (Inverted microscope, 10 X objective)

Figure 6.
Appearance of the L929 fibroblast control culture, contact method, 96-well plate, exposure time 48 hours (Inverted microscope, Obj. 10 X)

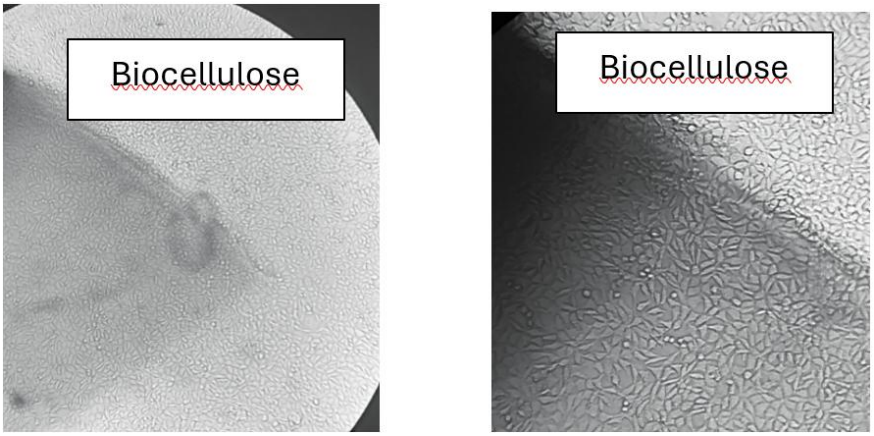


Figure 7.
Appearance of the L929 fibroblast culture – contact method, Sample BC, applied immediately after seeding, 96-well plate, exposure time of 24 hours (inverted microscope, 10 X objective)

The morphological analysis revealed that the cells surrounding the piece of biocellulose looked similar to the control cells, both after 24 hours and 48 hours of exposure to native, purified, and sterilized bacterial cellulose (BC).

Following the MTS test, obtain results a presented in Table IV.

Table IV
Evaluation of the cytotoxicity of the washed BC sample applied immediately after seeding the fibroblasts (exposure times of 24 and 48 hours), contact method

	DO 490 nm	Standard deviation	Cell viability (%)	p (t-test)
Blank 24 h, N = 10	1.164	0.0526	100	-
BC 24 h, N = 10	1.257	0.1810	107	0.114
Blank 48 h, N = 10	1.095	0.0495	100	-
BC 48 h, N = 10	0.944	0.0559	86,2	0.082

Application of washed/conditioned BC sample, 96-well plate, confluent cells 80%

Data regarding cell viability are shown in Table V.

Table V
Evaluation of the cytotoxicity of the washed BC sample applied over fibroblasts that reached 80% confluence (exposure time 24 hours), contact method

	DO 490 nm	Standard deviation	Cell viability (%)	p (t-test)
Blank 24 h, N = 5	0.505	0.044	100	-
BC 24 h, N = 5	0.435	0.053	86.1	0.136

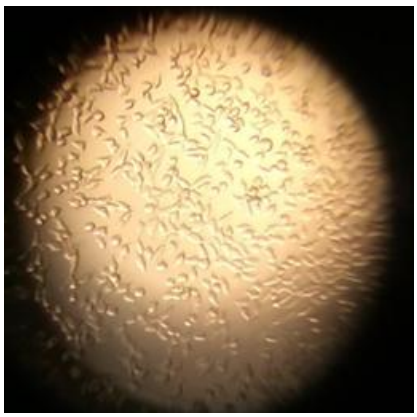


Figure 8.
Appearance of the L929 fibroblast control culture – contact method, 96-well plate, exposure time 24 hours (Inverted microscope, Ob. 10 X)

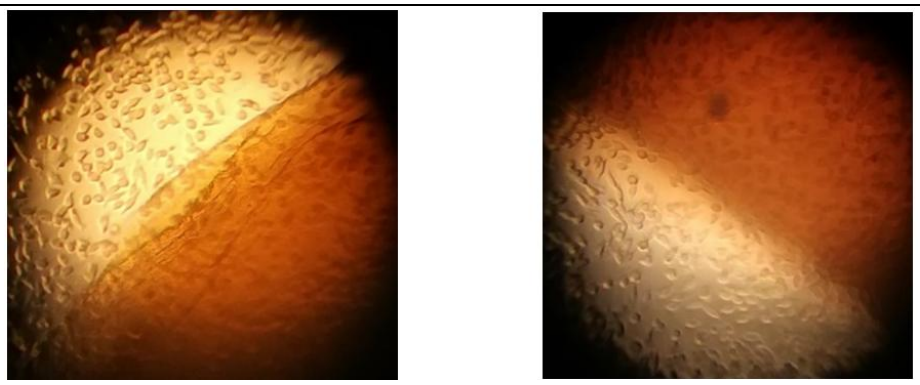


Figure 9.
Appearance of the L929 fibroblast culture - contact method, BC test, applied at 80% confluence, 96-well plate, exposure time of 24 hours (Inverted microscope, 10X objective)

In the case of the experiments, the MTS test was performed after extracting the discs from the wells, which resulted in the detachment/removal of some of the cells adhered to the surface of the disc, explaining the lower number of cells compared to the control wells. The qualitative and quantitative observations made during the three types of "contact" experiments allow the classification of the product, *Native Bacterial Cellulose, Purified and Sterilized (BC)*, as being practically free of cytotoxicity.

The native, purified, and sterilized bacterial cellulose (BC) sample was tested for cytotoxicity using the methodology recommended by ISO standard 10993 – the extraction method and the contact method. The results of the cytotoxicity tests conducted using the extract method reveal that under the described experimental conditions, the BC sample is non-cytotoxic when cells are exposed for 24-25.5 hours to the test extracts ("medium 1" and "medium 2"). The findings from quantitative assessments are supported by qualitative analysis, as microscopic examination of the cultures did not reveal any morphological changes in the cells. The contact method indicates that when fibroblasts are exposed for 24/48 hours to the washed/conditioned form of bacterial cellulose (BC), no cytotoxic effects are observed. For the next stage, we recommend introducing an additional washing step for the cellulose film in the conditioning process.

Testing of skin irritation

The test involved native, purified, and sterilized bacterial cellulose presented as a whitish-yellow film/membrane, sized 6x6 cm, which was cut into pieces suitable for application on an area of skin measuring 2 x 3 cm.

Preparation of laboratory animals

Although rabbits are commonly used for evaluating skin irritability, guinea pigs were selected in this experiment, as literature suggests that they can be chosen due to the similarity of their skin reactivity to that of human skin. Furthermore, with guinea pigs, the study could be expanded to include an analysis of delayed contact sensitization.

Guinea pigs weighing between 400 and 600 g were used, provided by "Cantacuzino" National Institute and with a health certificate. Three animals were used for both the test and control groups, as the technical data received indicated that the material does not contain substances with potential irritant action. An area of 25 cm² on the dorsal region of the trunk of the animals used in the test was shaved approximately 24 hours prior to the start of the test. In the flank region, areas were established for applying the test material and control areas. In the control areas, corresponding 2x3 cm samples soaked in distilled water were applied.

The activity involving the use and maintenance of laboratory animals in the Vivarium of INCDCF is conducted under conditions that comply with the provisions and regulations in the field issued by FELASA (Federation of European Laboratory Animal Science Associations) and adopted by ARSAL (Romanian Association for Laboratory Animal Science), in force: EU Directive 63/2010, Law 43/April 11, 2014, regarding the protection of animals used for scientific purposes.

Description of Material Application

A total of 3 skin exposure sessions were conducted for the test/control substances. The materials to be tested and the control were applied to a skin area of 6 cm² that had been moistened with distilled water, then covered with a dressing and secured with semi-occlusive adhesive tape for approximately 4 hours. After this period, the test and control materials were removed, and the exposed areas were washed with distilled water and then visibly marked. Examination of the exposed areas, both test and control, was carried out at intervals of 4 hours +1 hour, 24 hours, 48 hours, and 72 hours after the first exposure. Following the 72-hour observation period for the first exposure, 2 additional 4-hour exposures were conducted on two successive days.

Evaluation of Results

Under the conditions of this experiment regarding the testing of acute and repeated skin irritation as part of the biological evaluation of medical devices in accordance with the standard SR EN ISO 10993-10:2009, it was concluded that the test material - Native, purified, and sterilized bacterial cellulose presented by the Department of Pharmaceutical Biotechnologies resulted in negligible skin irritation after 3 exposure sessions.

Testing the sensitizing potential of the product native, purified, and sterilized bacterial cellulose

Sensitization reactions typically occur as a result of repeated or prolonged contact with a chemical substance that interacts with the immune system. Because such reactions to biomaterials are of the cell-mediated type rather than humoral or involving antigen-antibody interactions, testing is conducted on the skin of laboratory animals. Sensitization reactions caused by materials are due to chemical substances that can be extracted from these materials, and in laboratory animals, they are evidenced by erythema and oedema.

Description of the material

The material for testing was presented by the Department of Pharmaceutical Biotechnology, referred to as: Native, Purified, and Sterilized Bacterial Cellulose. The testing of the materials was conducted according to the method of the Occlusive Dressing Test (Buehler test). The material presented as a whitish-yellow film/membrane of bacterial cellulose, measuring 6 x 6 cm, packed sterile in glass containers (Berselius beakers), was cut into pieces of 2 x 3 cm.

Description of the Test Animals

Young guinea pigs weighing a minimum of 200-300 g provided by the Cantacuzino Institute were used. The animals were grouped with 10 for the test batch and 5 for the control group. The number of animals was determined based on technical data received and the results of the primary skin irritation test, concluding that the material does not contain substances with potential irritating action.

The fur from an area of 25 cm² on the dorsal region of the trunk of the test animals was removed approximately 24 hours before the start of the test. One group of animals was exposed to the materials, while another group served as the control by being exposed to a dressing with distilled water. Throughout the study, it was necessary to repeat the fur removal at intervals of approximately 7 days.

Description of the Material Application

Induction Phase

The test material, cut into pieces of 2 x 3 cm, was placed in intimate contact with the skin, moistened by the application of a dressing, and then immobilized with a semi-occlusive adhesive tape for approximately 6 hours. After this period, the occlusive systems were removed along with the test materials.

The application procedure for the test materials was repeated for 3 consecutive days within a week over a period of 3 weeks. The control group underwent similar manoeuvres, using only the occlusive systems and distilled water.

Challenge Phase

Fourteen days after the last induction application, both groups of animals were subjected to the challenge. The test material was applied to the previously depilated skin, maintained in contact for 6 hours. After this period, the semi-occlusive dressing was removed, and the surface was gently washed with distilled water.

Observation Recording

Animal observations were conducted in a natural light environment to detect reactions present on the exposed skin. An observer not involved in the experiment was utilized to minimize subjective evaluation errors.

The recording of skin reactions was carried out approximately 2 hours after the removal of the occlusive dressings. The depilated area was gently washed (with warm distilled water) and dried.

Reactions of intensity below 1 were reported according to the Magnusson and Klingman scale, both in the tested batch with native, purified, and sterilized bacterial cellulose, as well as in the control batch during the "challenge" period.

The reactions with an intensity below 1 observed in both the tested samples and in the control batch after the challenge phase led us to conclude that the product native, purified, and sterilized bacterial cellulose to sensitization testing in accordance with the biocompatibility evaluation of medical devices under the standard SR EN ISO 10993-10:2009 does not exhibit sensitizing potential, under the conditions of this experiment.

In vivo testing of bacterial cellulose membranes (BCM) in corneal ulcer

The animal study was approved by the Ethics Committee of the University of Agronomic Sciences and Veterinary Medicine of Bucharest (no. 082/2024) and was conducted in accordance with Directive 2010/63/EU and national regulations on the protection of animals used for scientific purposes.

The owners of the animals included in the study were thoroughly informed about the objectives and experimental procedures and signed an informed consent form prior to the application of the treatment with bacterial cellulose membrane (BCM). All procedures were carried out in a manner that minimized discomfort and suffering for the animals, in compliance with the 3Rs principles: Replacement (using alternative preclinical models where possible), Reduction (reducing the number of

animals to the minimum necessary for scientific validity), and Refinement (improving procedures to minimize pain and stress). The severity of a corneal ulcer is assessed based on the depth of the lesion and stromal loss, with superficial ulcers affecting only the epithelium and deep ulcers involving most of the stroma, potentially leading to the formation of a descemetocele. In the *in vivo* study, bacterial cellulose membranes (BCM) were used in six patients with corneal ulcer (three cross breed dogs, and three European cats). The pure and dried BCM were sealed and steam sterilized for 20 minutes at 120°C. Hydration of sterile BCM was accomplished ten minutes before to be applied on the cornea.

Its reconstitution was realized using sterile saline solution. The parchment and busty appearance of bacterial cellulose had turned into an elastic, opaque, easy-to-cut and melded structure. The ophthalmological examination was performed. In all the cases fluorescein test was positive, Siedel test was negative and the intraocular pressure (IOP) was in normal range (15 mmHg-20 mmHg). The examination of ocular fundus cannot be performed due to the corneal ulcer and oedema, but the eye ultrasound highlights no retinal detachment. General anaesthesia using isoflurane and local anaesthesia using alkaline were performed. The sterile hydrated BCM was prepaid on round shape according to the size of the corneal ulcer. The suture of the BCM was not performed to avoid damage of the applied cellulose membrane. One cat had a corneal foreign body (grass own) and before surgery it was removed. In all the cases the BCM was applied on the cornea and the third eyelid flap was performed. The Elizabethan collar was mandatory and the sutures were removed after three weeks.

Evaluation of Corneal Ulcers and Clinical Scores

To monitor the progression of corneal ulcers, each lesion was assessed based on two main criteria: epithelial healing and the degree of corneal opacity. Healing was rated according to the extent of corneal epithelial regrowth, on a scale from 0 (no signs of healing) to 5 (complete epithelialization), while corneal opacity was evaluated from fully transparent (0) to completely opaque (5). The scores were color-coded-green for good results, yellow for moderate, and red for poor-to enable quick and intuitive interpretation. This approach provided a clear overview of each patient's progress and highlighted the effectiveness of the treatments applied, including the use of bacterial cellulose membranes (BCM), in a safe and reproducible manner.

Results and Discussion

Bacterial cellulose membranes (BCM) preparation

Variety of exogenous and endogenous factors can lead corneal scar formation. There are some researches about innovative treatment of corneal scar such as corneal mesenchymal stem cell or tissue engineering cornea (1). Recent studies in tissue engineering scaffold on human corneal stromal cells demonstrated that BCM 3D reticulated network is a nonimmunogenic, biocompatible biomaterial, that favours water/nutrition transport, and the corneal stromal cells proliferation in internal of BC scaffold (20, 6). BCM resulted as homogeneous, flexible fine membrane, with a translucent aspect. BCM characterization assays were performed according with (30) and (31). Therefore, cellulose proved insoluble in water, acetone, ethyl alcohol, toluene, low acidic solutions, drying loss and ash 5.72%, respectively 20.34%.

Physicochemical Properties

The analytical results describing the physicochemical properties of the biocellulose samples are presented in Table VI.

The macro-morphology of the bacterial cellulose membranes can be noticed in Figure 10.

Figure 10.
Macro-morphology of hydrogel BC - based dressings

Table VI

Analytical results for biocellulose samples

Determination	Results
Appearance:	thin film/powder, without any particular smell, and light beige colour
Solubility: • In water, acetone, anhydrous ethanol: • In ammonia solution containing tetraamminecopper (Schweizer's reagent)	 insoluble soluble
pH of the aqueous supernatant	6.7
Weight loss on drying, %	17.42
Mineral residue (sulfated), %:	34.48
IR Spectra	Corresponding to structure
Degree of polymerization	542.2
Minerals and heavy metals (ICP-MS), ppm - Li - Mg - Ca - V - Fe - Co - Ni - Cu - As - Cd - Pb	 0.083 105.266 375.581 2.217 55.680 0.210 4.231 5.667 0.980 0.022 1.587

Endotoxin contamination study

The endotoxin limit of 5 EU/kg body weight/hour or 350 EU for a 70 kg adult has been scientifically established to prevent febrile reactions and hypotension resulting from the intravenous or intramuscular administration of pharmaceutical products contaminated with bacterial endotoxins. Time is a factor taken into consideration as there are mechanisms in the liver and blood to neutralize bacterial endotoxins. One EU (Endotoxin Unit) is a unit of biological activity of the standard endotoxin reference.

Table VII

Results of the endotoxin contamination study

The solution	Endotoxin concentration / The solution to which it is added.	The thinner	Factor of dilution	Initial endotoxin concentration	The number of replicates
A	The extract to be tested from cellulose sample	-	-	-	4
B	2λ/The extract to be tested from cellulose sample	The extract to be tested from cellulose sample	1 2 3 4	2λ 1λ 0,5λ 0,25λ	4 4 4 4
C	2λ/ apyrogenic water for BET	Apyrogenic water for testing	1 2 3 4	2λ 1λ 0,5λ 0,25λ	2 2 2 2
D	Nul/apyrogenic water for BET	-	-	-	-

For devices that come into direct or indirect contact with the intraocular environment, a lower endotoxin limit may be applied.

Thus, samples of bacterial cellulose have demonstrated a level of bacterial endotoxins below the acceptable limits for the category of medical devices to which they belong.

Results of Cytotoxicity Evaluation

The samples of native, purified, and sterilized bacterial cellulose (BC) were tested for cytotoxicity using the methodology recommended by ISO standard 10993 – the extraction method and the contact method. The results of the cytotoxicity tests conducted using the extract method reveal that under the described experimental conditions, the BC samples were non-cytotoxic when cells are exposed for 24 - 25.5 hours to the test extracts (“medium 1” and “medium 2”). The findings from quantitative assessments are supported by qualitative analysis, as microscopic examination of the cultures did not reveal any morphological changes in the cells.

The contact method showed an inhibition of cell spreading and proliferation when exposed for 24/48 hours to native, purified, and sterilized bacterial cellulose (BC), in its initial (unwashed) form.

The contact method indicated that when fibroblasts are exposed for 24/48 hours to the washed/conditioned form of bacterial cellulose (BC), no cytotoxic effects were observed.

Testing of skin irritation

Under the conditions of this experiment regarding the testing of acute and repeated skin irritation as part of the biological evaluation of medical devices in accordance with the standard SR EN ISO 10993-10:2009, it was concluded that the test material - purified, and sterilized bacterial cellulose resulted in negligible skin irritation after 3 exposure sessions.

Testing the sensitizing potential of the product - native, purified, and sterilized BC

The reactions with an intensity below 1 observed in both the tested samples and in the control batch after the challenge phase led us to conclude that the product native, purified, and sterilized bacterial cellulose subjected to sensitization testing in accordance with the biocompatibility evaluation of medical devices under the standard SR EN ISO 10993-10:2009 does not exhibit a sensitizing potential, under the conditions of this experiment.

In vivo (BCM) evaluation for animal corneal ulcer

The aim of the study was to evaluate *in vivo* the corneal repair potential of BCM in corneal ulcer in dog and cat. In this regard, our studies used BCM as a sterile anatomical dressing for the treatment of corneal diseases. Purified, analysed and sterilized BCM were *in vivo* tested, to assess the ability to repair the corneal ulcer in dog and cat.

The BCM was prepared by packing, sealing and steam sterilization (Figure 11). The BCM was hydrated using sterile saline solution (Figure 12).

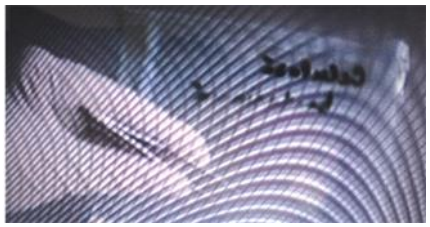
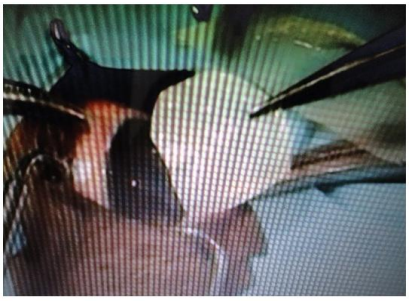


Figure 11.
Packaging and sterilization of bacterial cellulose

Figure 12.
Bacterial cellulose transformation after hydration with physiological serum

Figure 13 presents the shaped nanocellulose membrane application on the affected cornea. For two cases, over the applied rehydrated bacterial cellulose, a therapeutic contact lens system was applied and the suture was maintained for 2 - 3 weeks.



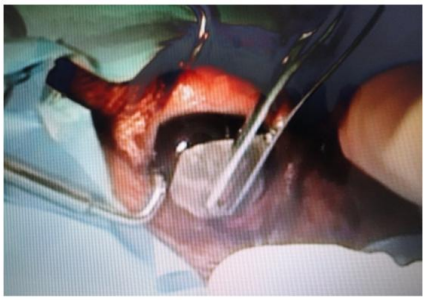
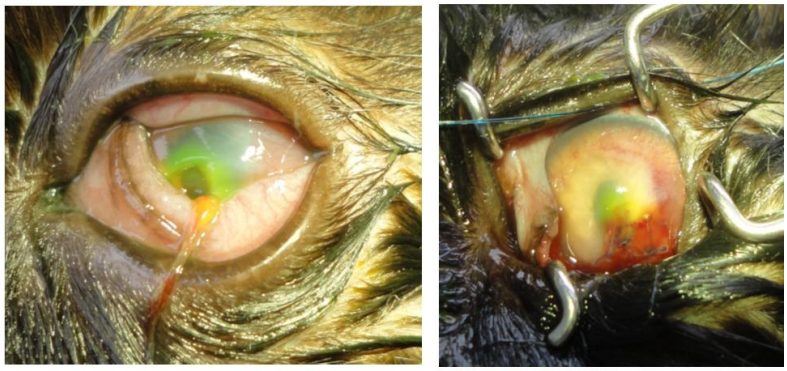


Figure 13.
Modelling bacterial cellulose according to the size cornea affected and introducing it to the internal face of the third eyelid

The BCM was cut according to the size of the cornea (Figure 13), due to it softness it was easy applied on the cornea (Figure 14). The suture of the BCM was not performed to avoid damage of the applied cellulose membrane.

Figure 14 shows the effect of bacterial cellulose membrane on the cornea suffering a secondary trauma caused by the presence of a foreign body. Rehydrated bacterial cellulose was fixed between the cornea and third eyelid. At the end of the treatment period, suture threads were removed. It was observed that the cornea appearance was significantly improved, with a transparent area in super-external dial. The presence of a unique small and very fine scar on the lower dial was observed. Using the cellulose product in corneal treatment led to a ratinated appearance and it was observed that it did not adhere to performed cornea suture. Thus applying bacterial cellulose to corneal secondary trauma (caused by the presence of a foreign body), it has been observed a benefic effect by improving and healing the damaged cornea.



a. **b.**

Figure 14.
Cat European breed, 3 years old, with corneal trauma secondary to foreign body (a, b)

In a cat (Figure 14) with corneal foreign body (grass awn) the cornea was sutured using vicryl 7/0 three simple sutures. Sterile rehydrated BCM was applied on the surface of the cornea, the third eyelid flap was performed using simple interrupted suture. In one case a temporary blepharroraphy was performed (Figure 16).

One of the cases was a perilimbal desmethocele in the corneal outer dial (Figure 15). For such a condition, antibiotics and corneal scarring treatment are usually administered and the suture is done at different points. The use of bacterial cellulose as a non-traumatic anatomical dressing has been applied to the entire surface of the cornea. At the end of the treatment, corneal healing and only a fine scar were observed. Following the bacterial cellulose corneal treatment applied, all tested pets maintained the ability to see and also corneal trauma was fully healed.

In a dog with corneal ulcer in the external quadrant of the cornea (figure 15) the sterile BCM was easy to apply and the third eyelid flap was performed.

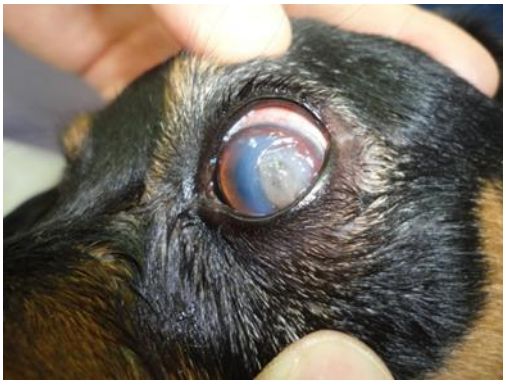


Figure 15.
Common dog breed with perilimbal descemethocele

Treatment of severe conditions such as deep corneal wounds with bacterial cellulose has also been performed. The results of fluorescein test were positive (Figure 16) and the debridement of the anterior corneal epithelium was performed. In this case, the bacterial cellulose was used also as an anatomic dressing and it was applied to the entire surface of affected cornea, and tarsoraphy was performed. After removing the suture threads, it was observed a significant cornea improvement by a transparent appearance. Maintaining ability to see of tested animal was observed.

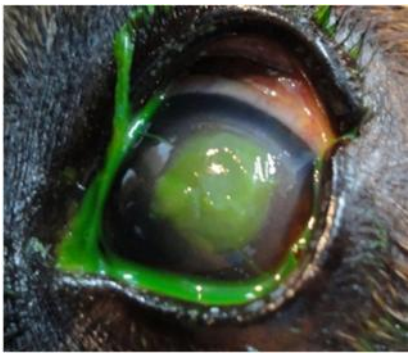


Figure 16.
Common dog breed, 7 years old, with deep corneal wound and positive fluorescein test

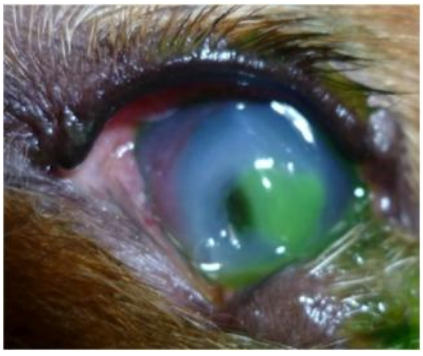


Figure 17.
Common dog breed (3 years old) with corneal deep wound and with central high lack of substances

The treatment of a deep central wound with lack of substance (Figure 17) was performed by applying

the bacterial cellulose to the entire corneal surface and tarsoraphy was performed by temporarily suturing the edges of the upper and lower eyelid.

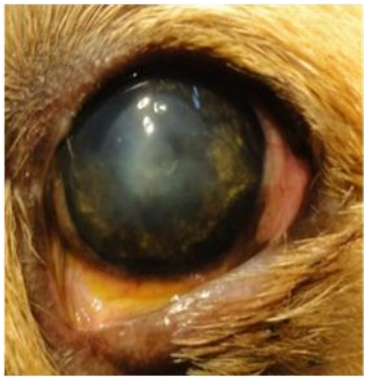


Figure 18.
Corneal appearance of cured cornea, after 3 weeks treatment

Local maintenance of bacterial cellulose was for three weeks like in the rest of tested cases. After 3 weeks the sutures were removed. The healing of the cornea and only a small transparent central scar was observed (Figure 18).

The study results show that the severity and size of corneal ulcers directly influence the healing process. Superficial ulcers, which affect only the epithelium, healed quickly, while deep ulcers with significant stromal loss and a risk of descemetocele required additional support. The size of the ulcers was objectively measured in mm², and the healing progress was monitored through fluorescein testing and complete epithelialization assessment. The application of bacterial cellulose membranes (BCM) provided this support, facilitating complete epithelialization and accelerating corneal recovery. These observations suggest that BCM is a safe and effective option capable of supporting corneal regeneration even in more severe cases, offering a treatment that is adaptable and easy to implement in practice. The clinical characteristics of the corneal ulcer cases treated with BCM are presented in Table VIII.

Table VIII
Characteristics of corneal ulcer cases treated with BCM

No.	Species/ Breed	Corneal ulcer type	Cornea location	Size	Treatment applied	Sutures	Third eyelid flap	Healing time	Final result/ Scar	Visual function	Healing score	Corneal opacity grade
1.	Dog, mixed breed	Superficial ulcer	External quadrant	Small	Rehydrated BCM	No	Yes	3 weeks	Fine scar	Visual	🟢 4/5	🟢 1/5
2.	Dog, mixed breed	Central ulcer	Central zone	Large	Rehydrated BCM	No	Yes	3 weeks	Larger scar	Visual	🟡 3/5	🟡 2/5
3.	Dog, mixed breed	Superficial ulcer	External quadrant	Small	BCM + therapeutic contact lens	Yes	Yes	3 weeks	Fine scar	Visual	🟢 4/5	🟢 1/5
4.	Cat, European	Ulcer + foreign body	External quadrant	Medium	Debridement + BCM + third eyelid flap	Yes	Yes	3 weeks	Small scar	Visual	🟢 4/5	🟢 1/5
5.	Cat, European	Perilimbal descemetocele	External quadrant	Small	BCM applied on entire cornea	No	Yes	3 weeks	Fine scar	Visual	🟢 5/5	🟢 0/5
6.	Cat, European	Deep ulcer + substance loss	Central zone	Large	BCM + tarsorrhaphy	No	Yes	3 weeks	Central transparent scar	Visual	🟢 4/5	🟢 1/5

Colour legend: 🟢 Green = Good/Excellent (healing score 4 - 5, opacity 0 - 1); 🟡 Yellow = Moderate (healing score 3, opacity 2); 🔴 Red = Poor (healing score ≤ 2, opacity ≥ 3)

Following the application of cellulose as an anatomic dressing to treat lack of substance, the pet has maintained ability to see, and the corneal affection was resolved.

In a dog with central corneal ulcer (Figure 15) the BCM was applied more difficult due to the big area of lack of the corneal anterior epithelium and stroma. After three weeks the sutures were removed and the corneal ulcers were healed with scars because the cornea has big area with lack of corneal anterior epithelium and stroma (Figure 16 and Figure 17). The ophthalmological examination performed three weeks after the surgery revealed corneal scars, fluorescein test negative, IOP in normal range and eyes were visual. The goal was achieved to keep the eye visual and to not perform the eyeball enucleation. Due to the success of the corneal healing, no histopathological exams were performed.

Surgical treatment of the corneal ulcer using BCM like a corneal bandage and third eyelid flap proved to be an option to obtain the healing of the cornea. The studies now focus on cytokines influence to corneal scar formation, such as PDGF, FGF, EGF, including TGF-β which is confirmed to have close association with corneal scar formation. Cornea tissue engineering scientists investigated the therapeutic effect of pirfenidone (an anti-

inflammatory drug) applied after alkali burn of rat cornea, demonstrating the corneal epithelial healing, decreasing corneal opacity, inhibiting the corneal scar formation and also the post traumatic inflammatory reaction (1). Considering the pharmacologically severity, our research has focused on development of an non-immunologic and biocompatible biomaterial, such as bacterial cellulose.

According Jia et all. BCM have advantageous in simple preparation, easy sterilization, with stable properties, and most important with a promising potential corneal scaffold (6). Starting from this hypothesis our studies focused on BCM innovative development as ophthalmic atraumatic scaffold. *In vitro* advantages include its ability to adhere and enhance the proliferation of the retinal pigment epithelium and keratinocytes, and *in vivo* studies showed that BCM grafts might potentially reduce the rejection rates of transplanted corneas and improve the treatment of eye diseases (6). Our studies of corneal ulcer in dog and cats support this aspect: BCM leaded and favoured the healing of corneal scars and keeping the eye visual and no eyeball enucleation. According to reported data, this result can be explained by BCM ability to support the growth of corneal stromal cells while preserving a full vision, and by the great biocompatibility as a tissue substitute. Other *in vivo* research demonstrated that bacterial cellulose/ polycaprolactone composite (BC/PCL) tested in rabbits' (20) or rats cornea (20, 2) had an acceptable inflammatory response, but with no effective performance as cornea tissue substitute. On the other hand, pure BCM corneal bandage are able to be used as eye scaffolds and replace the less biocompatible poly(methyl)methacrylate (PMMA) or hydroxyapatite systems currently utilized in the clinic or even those BC-antibiotic complexes for eye drops for ameliorate the recovery of ocular burns (2, 6, 20).

The novelty of our research consists in treating corneal diseases on other types of experimental models than those so far, respectively on pets, dogs and cats. The approach of the studies focused on the treatment of corneal ulcers in these animals by applying a proven biocompatible BCM material, which provides a favourable environment for wound healing, scars and vision preservation, without side effects. The data obtained agree and are supported by studies carried out on other animals such as rabbits, mice, or pigs, from biocompatibility and the effects followed point of view. To avoid the use of classic treatments with antibiotic or anti-inflammatory medication, our study proposes a non-invasive treatment, based on a cornea substitute, with an anatomical dressing role, which can be obtained by low cost biotechnology techniques, regarding the capitalization of vegetated by-products, ensuring an efficient circular economy.

Conclusions

The purpose of this study was to evaluate the *in vivo* effects of an anatomic non-traumatic bacterial cellulose membrane (BCM) obtained from our own bioprocess, using like a corneal bandage in corneal ulcers of dogs and cats. The application of the sterile rehydrated BCM as an anatomical bandage to the selected patients was easily accomplished due to the anatomy of the eye, the presence of the third eyelid. Using the third eyelid flap to maintain on the surface of the cornea the BCM was achieved without suturing the membrane to the cornea. The BCM after hydrating is soft and the sutures tear it on the edges. For all the cases used in the experiments, the healing of the cornea was achieved with scars secondary to the big area of the corneal ulcer.

The *in vivo* study demonstrated that the bacterial cellulose membrane (BCM) can be effectively used in the management of corneal ulcers in dogs and cats, acting as a non-toxic, hydro-active anatomical bandage and a microbial barrier, thereby reducing the risk of local superinfections. The experiment also showed excellent biocompatibility and stability of BCM at the corneal level, suggesting significant potential for the use of bacterial cellulose as a scaffold in tissue engineering of the artificial cornea. However, further studies are required to optimize its structure and functionality.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval

The animal study was approved by the Ethics Committee of the University of Agronomic Sciences and Veterinary Medicine of Bucharest (no. 082/2024) and was conducted in accordance with

Directive 2010/63/EU and national regulations on the protection of animals used for scientific purposes.

AI use disclosure

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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