Biocompatibility of the bacterial cellulose hydrogel in subcutaneous tissue of rabbits[1]

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ABSTRACT

PURPOSE: To evaluate the biocompatibility and local sensibility reaction to bacterial cellulose hydrogel (0.8%) implanted in subcutaneous tissue of rabbits.

METHODS: Fifteen New Zeland rabbits were randomly allocated into three groups: T_1, 7 days, T_2, 21 days, and T_3, 84 days. The new material was implanted in the subcutaneous tissue of the ear; on the scalp over the periosteum; and on the outer and inner surfaces of the thighs, in the aponeurosis of the muscle. At 7, 21 and 84 postoperative days, the material was collected for histological study. The clinical signs, inflammatory response, angiogenesis and fibrogenesis were variables used for analysis of the biocompatibility and biological reactivity to BCH. Analyses were performed with an AXIO® Imager. The statistical tests were performed using the GraphPad Prism 5.0 program®

RESULTS: The intensity of the inflammatory infiltrate, considering the different cell types (PMN, LMN and GC), was statistically significant, with group T1 different from groups T2 and T3 ($p = 0.0124$ and $p < 0.0001$, respectively) and T2 different from the T3 group ($p = 0.0007$). Fibrogenesis grade 1 was the most prevalent in groups T1 (55.4%) and T2 (44.6%). The formation of neovascularization in the group was identified in 84.4% of samples.

CONCLUSION: Bacterial cellulose hydrogel (0.8%) is biocompatible, integrating with the subcutaneous tissue of rabbits and inducing tissue remodeling.

Key words: Cellulose. Hydrogel. Biopolymers. Materials Testing. Bulking Agents. Subcutaneous Tissue. Rabbits.

Introduction

Bacterial cellulose hydrogel (BCH) is a natural product obtained from molasses, a by-product of the sugar production process, and its chemical structure consists of stable polymerized sugars[1].

In vitro cytotoxicity of bacterial cellulose was evaluated in rat alveolar macrophages by [3-(4.5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] (MTT) assay, cells adhesion rate and nitric oxide production. The bacterial cellulose presented a high biocompatibility in the three cytotoxicity assays[2].

Bacterial cellulose has demonstrated effectiveness as a conductor cell and inducing the healing process[3-6]. It has also been used in different areas of surgery such as urethral reconstruction[7], bio-sling for treatment of urinary incontinence[8,9], bulking agent in orthopedics, ophtalmology and urology[10-12]. Patches in the femoral vein[13], mesh as an anti-adherent barrier in peritoneal surgery have also been used[14].

This study aims to evaluate the biocompatibility and the local sensibility reaction to the bacterial cellulose hydrogel (BCH) implanted in the subcutaneous space of rabbits.

Methods

This project was approved by Ethics Committee on Animal Research, of the Institution (No. 23076.012705/2012-33.)

Research performed with 15 New Zeland rabbits, adult males weighing between 2.390g and 3,360g. They were housed with cycles of night and day with standard food and water *ad libitum*. The animals were randomly allocated into three groups using research Randomizer® program (Version 4.0 [Computer software] Retrieved on June 22, 2013.), based on post-implant time: T_1, 7 days, T_2, 21 days, and T_3, 84 days.

The anesthetic procedure was performed according to the routine of the Institution. Ten minutes before the onset of anesthesia, atropine sulfate was applied intramuscularly in a dose of 0.44mg/kg. The animals were anaesthetized with a solution of 5mg ketamine hydrochloride® and 2mg of xylazine that were applied intramuscularly on the doses of 0.2ml per 100g of body weight

Under general anesthesia and aseptic and antiseptic conditions 1.0mL of the hydrogel was applied by direct needle puncture 25/7G in 5.0mL syringes at each point of implantation. Regions for the BCH application were defined according to the histological and functional anatomical characteristics. The hydrogel was applied to the subcutaneous space on the cartilage of the ear, the anterior and posterior surface of the shell; the subcutaneous on the scalp, on the periosteum; and in subcutaneous space in the middle third of the external and internal sides of the thighs, on the muscular aponeurosis.

Synthesis of the bacterial cellulose hydrogel

Bacterial cellulose hydrogel (BCH) was produced from sugars of sugar cane in the laboratory of biopolymers at the Experimental Station of Sugarcane, Federal Rural University of Pernambuco, Brazil[5]. The hydrogel was obtained by hydration of microcrystalline bacterial cellulose at a ratio of 0.8% cellulose in 99.2% water and sterilization by gamma ray.

Clinical and histological analysis

The animals were subjected to daily clinical examination by observing the surgical site for the presence of exudates and inflammatory signs, as well as behavior and food consumption. In the post-implant times, T_1, 7 days, T_2, 21 days, and T_3, 84, days, we proceeded to collect material for histological study. The material was fixed in 10% formaldehyde. Histological preparations were performed starting with hematoxylin/eosin (HE) and Masson's trichrome staining.

Histological analysis was carried out to quantify the intensity of the inflammatory response, based on the assessment of cellular infiltrate (polymorphonuclear (PMN), lymphocyte (ULN) and giant cells (GC) of the implant in the structure, the intensity of fibrogenesis (associated with collagen deposition), as well as the occurrence of neovascularization (angiogenesis). These were measured by the score 0-3, with "0" for no occurrence and "3" for more intense[15].

Analyses were performed using an AXIO® Imager .M2m/ Zeiss light microscope and Image J software® (Image Processing and Analysis in Java - 1:46 ImageJ, National Institute of Health, USA).

The clinical signs (animal behavior, food consumption and local signs of inflammation), the cellular inflammatory infiltrate, angiogenesis and fibrogenesis were variables used for analysis substantiated on the biocompatibility and subcutaneous reactivity to bacterial cellulose hydrogel, in accordance with the requirements the RDC/ANVISA No. 56, 2006[16].

Statistical analysis

The means of continuous variables were compared using the Student's paired t test while scores were compared using the

Chi-square test. Statistical significance was set at $p \leq 0.05$. The statistical tests were performed using the GraphPad Prism 5.0 program (GraphPad Software Inc., USA).

Results

The mean weight of the animals showed no statistical difference among the groups: 2.633g (T_1), 2.659g (T_2) and 2.586g (T_3).

In the T_1 group, the histopathological study of BCH implants at the three application sites (ear, scalp and thighs), was characterized by the predominance of polymorphonuclear (PMN) as score 3 (53.8%) and lymphomononuclear (LMN) as score 1 (40%). No giant cells (GC) were found in 43.1% of the samples.

In the T_2 group, the histological response to the BCH implant demonstrated equivalence between the scores: 0 (35.4%), 1 (29.2%) and 2 (35.4%) for the intensity of polymorphonuclear (PMN); a score of 2 (47.7%) for lymphomononuclear (LMN); and score 1 (50.8%) for giant cells (GC).

Polymorphonuclear (PMN) cells were not found in 68.9% of samples from the T_3 group. A predominance of lymphonuclear (LMN) and giant cells (GC) were classified as score 1 (50.8% and 44.6%, respectively) (Figure 1).

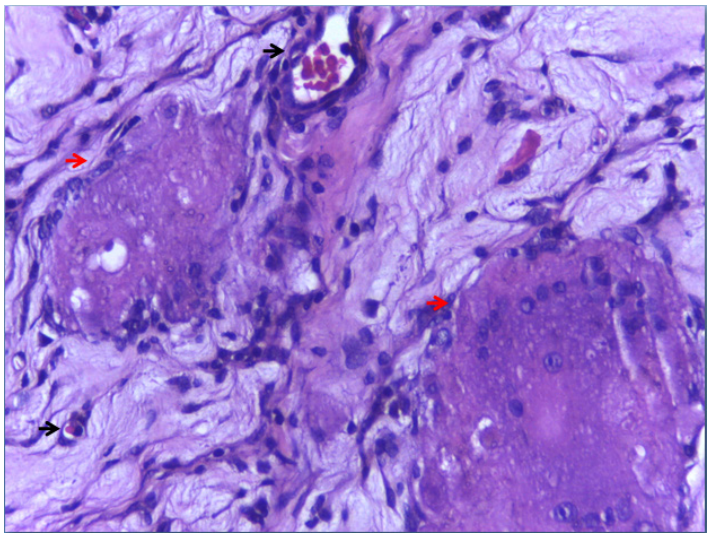

FIGURE 1 - BCH Implant with a slight lymphocytic inflammation, multinucleated foreign-body giant cells (FBGCs) (*red arrows*) and presence of blood vessels (*black arrow*) – T3, 84 days post-implant. HE, x400.

The intensity of the inflammatory infiltrate, considering the different cell types (polymorphonuclear (PMN), lymphomononuclear (LMN) and giant cells (GC)) was statistically significant among groups, with group T_1 being different from the T_2 and T_3 groups ($p = 0.0124$ and $p < 0.0001$, respectively) and T_2 different from the T_3 group ($p = 0.0007$) (Figure 2).

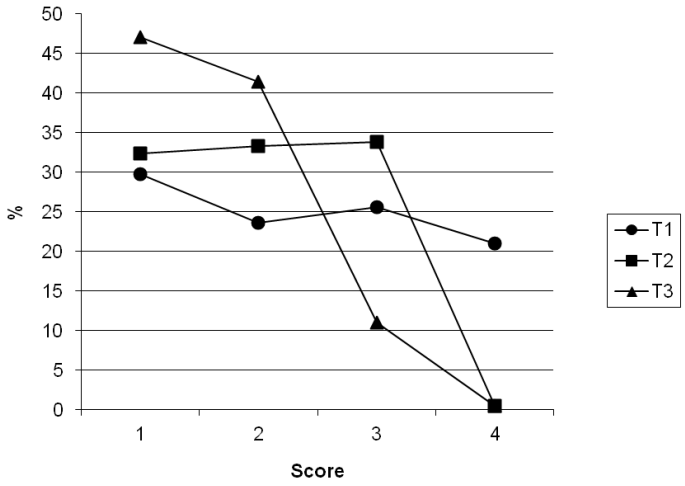

FIGURE 2 - Inflammatory response according to the permanence of the implant (T_1, 7 days, T_2, 21 days, and T_3, 84 days).

Fibrosis with score 1 was the most prevalent finding in groups T_1 (55.4%) and T_2 (44.6%). In the T_3 group, grade 2 fibrosis was present in 40% of the samples. No fibrosis was found with score 3. There were no statistical differences among the groups.

Neovascularization formation in the T3 group was identified in 87.4% of the samples (grade 1, 35.80%, grade 2, 50.0%, and grade 3, 1.60%). For the T1 and T2 groups, this corresponded to 75.4% and 64.6% of the samples, respectively, with no statistical differences.

Discussion

Bacterial cellulose was used in the form of gel, which has a high coefficient of elastic deformity, adapting itself to variations functional deformity in organic tissues. The chemical composition of its physical properties does not induce immune response, featuring a promising material with an extensive range of applications in the biological sciences[17].

In the present study, the Bacterial cellulose used for the implants was a formulation of 0.8% dissolved in water. Concentrations of 0.8% and 1% were used in different translational studies with an eye towards future medical applications. The results seem promising, for example, as a bulking agent in the cartilage of knee deformities, where the osteochondral defects produced in femoral condyles repair of rabbits[10] are observed; or

as in enophtalmics such as for the implant cavity in eviscerated rabbit eyes, with proven orbit compatibility and integrity[11]; or for urologic endoscopic therapy in patients with vesicoureteral reflux and urinary incontinence[12]. In all these studies, the BCH has been demonstrated as stable and resistant to degradation and elimination process, with no variation in end-volume after long permanence time and physiologically integrating with the tissue.

The BCH implant aimed in different anatomical sites at evaluating the permanence of the hydrogel against the local effects of gravity and the compression offered by muscular action in the region. In addition to these variables, the cellular subcutaneous implants remained in contact with the cartilage, periosteum and fascia of the muscle fiber.

All animals tolerated the procedure well, keeping the expected ponderal weight curve. No clinical signs of toxicity or adverse events such as swelling, redness, infection or elimination of the grafted material were found, demonstrating the biocompatibility of the hydrogel.

These results are in accordance with in vitro studies where bacterial cellulose showed low cytotoxicity[2] and in vivo that showed high biocompatibility and bio-integration with the implanted tissue[3-14].

The presence of inflammatory infiltrates, classified as "0" or grade "1", represented 53.3% in T_1 group, 65.3% in T_2 and 88.5% in T_3, characterizing a predominantly low intensity response.

In the T3 group, where elapsed time for the collection of the implants was 84 days, there was a lower incidence of polimorphonuclear and lymphomononuclear (20.0%) cells, which explains the reduction of the local acute inflammatory reaction in the long run, as noted by other authors[18-20]. The giant cells remained stable, showing no increase of foreign-body reactions, despite the longer time elapsed in relation to T_1 and $T_{2\ groups}$.

However, there was a linear trend towards the reduced inflammatory response to the BCH implant over the period studied (T_1, T_2 and T_3) (Figure 2).

Histological analysis of the BCH implants in groups (T_1, T_2 and T_3), showed the presence of neovascularization.

These alterations are characterized by a foreign body reaction, a process expected for any foreign material in the organism. Even materials considered compatible or autologous provoke this type of reaction after the first days of an implant[18-20].

The formation of multinucleated foreign-body giant cells (FBGCs) is explained as part of the repair process, necessary for BCH tissue bio-integration and the induction of the tissue remodeling. This repair process may be regarded as physiologically and immunologically inert.

The process of tissue remodeling induced by bacterial cellulose has been validated by a previous study[21], in which adhesion of the mesenchymal stem cells (MSCs) was tested through the use of electrical impedance spectroscopy on a biopolymer film (bacterial cellulose) as a way to assess the biopolymer film's potential as a substrate for cell culture. The results showed that the films may be regarded biopolymer matrices adequate for cells culture, representing a promising biomaterial for tissue engineering.

The neovascularization (angiogenesis) finding in the BCH implant demonstrates the integration of bacterial cellulose tissue. The new vessels were present in the T_1 group (75.4%) and were more frequently observed in the analysis of the $T_{3\ group}$ (84.4%).

The fact that there was no migration, infection or extrusion of the BCH in the implantation sites during the study period demonstrates its low toxicity and high biocompatibility with the tissues. Similarly, the neovascularization found in the BCH after the 7th day (T_1) and the preservation of the implant during the entire study period (T_2 and T_3), demonstrated the successful integration of implants at different sites, which encourages further research using BCH as a biomaterial for bulking agents for tissue expansion.

Conclusion

Bacterial cellulose hydrogel is biocompatible, integrating the subcutaneous tissue of rabbits and inducing tissue remodeling.

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