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Effects of bacterial cellulose gel on the anorectal resting pressures in rats submitted to anal sphincter injury

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Abstract

The aim of this study is to evaluate if a gel of bacterial cellulose gel can revert the loss of anal resting pressure after anorectum sphincter injury in rat model, elected as a model to simulate fecal incontinence. Thirty-nine animals were equally divided into three groups: Control (CG), Sphincter injury plus Saline injection (SG) and Sphincter injury plus Bacterial Cellulose Gel injection (BCG). Anal pressure at rest was assessed for all animal in the three groups using anorectum manometry. Saline and Gel groups were subject to anorectum sphincter injury to reduce the anal pressure at rest. Fifteen days later Saline or Gel was injected into the anorectum, according to their groups. Sixty days later first manometry, the anorectum of all animals were removed and processed histologically. The CG group showed maintenance of their mean anorectal resting pressure levels; SG presented a fall in their mean anorectal resting pressure. The BCG presented a significant elevation of the mean anorectal resting pressure levels, surpassing the pressure of CG. The gel of bacterial cellulose remained at the injection site and was neovascularized, colonized by fibroblasts and dense conjunctive tissue.

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Those data suggest that BC can be used as a future filling agent treatment for fecal incontinence in clinical trial protocols.

Keywords: Medicine, Surgery

1. Introduction

Fecal incontinence affects around 15% of population and causes great distress with significant social and economic impact [1, 2]. Fecal incontinence can greatly affect lifestyle as the inability to control liquid or solid stool loss tend to cause embarrassment and low self-perception. Many scores that assess its intensity also evaluates its burden on daily life [3].

There are many possible approaches to address fecal incontinence, such as pharmacotherapy, biofeedback therapy, sacral nerve stimulation, anal plugs, radiofrequency administration, anal sphincteroplasty, dynamic graciloplasty and colostomy. Those options range from suitable treatment for the mildest symptons only to therapeutics for last line refractory fecal incontinence [1, 4].

For mild to moderate incontinence one available approach is the bulking agents [5]. They are locally injected into the sphincter complex to cause the enlargement of the anal canal pads, promote reduction of lumen in the injection location and pose as a promising way to ameliorate this problem. They may also work by filling defects in the anal internal sphincter thus reestablishing anal canal symmetry [4, 6, 7].

Injection of bulking agents is a minimally invasive procedural treatment that achieves best results when the patients have internal sphincter defects associated or not to small defects in the external sphincter. This procedure is best prescribed for patients with mild to moderate incontinence who are unwilling or not candidates to undergo surgical treatment after failing medical management [1, 5].

Several products have been used as bulking agents, and they all show different rates of success [8, 9, 10, 11, 12]. But there is still a need for a new product with long durability and low cost, besides the expected characteristics of, biocompatible, non-immunogenic, non-migratory, non-erosiven and non-carcinogenic substance [13, 14].

The bacterial cellulose has demonstrated low toxicity, high biocompatibility, tissue remodelling at the application site and higher durability than others injectables [15, 16, 17, 18].

This study evaluated the effects of bacterial cellulose gel as an anal bulking agent in rats submitted to sphincter injury considering the histopathological local reaction, agent stability and anorectal manometry after the implantation of bacterial cellulose.

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2. Material and methods

2.1. Ethical procedures

This study followed the principles governing the Code of Experimental Ethics and Laws for Protection of Animals, according to the standards in Brazil, receiving full approval from the Ethics Committee on Animal Experimentation (equivalent in Brazil to Institutional Animal Care and Use Committees, IACUCs) of the Center of Biosciences, UFPE, process No 23076.018170/2013.

2.2. Animal model

Thirty-nine Wistar rats (*Rattus norvegicus albinus*), from both sexes, aging from 12 and 14 weeks old, were equally and randomly divided into three groups: Control group (CG), sphincter injury followed by saline injection (SG) and sphincter injury followed by bacterial cellulose gel injection (BCG). The mean body weights of the CG, SG and BCG groups were 310g; 275g, 262g, respectively, with no statistical difference after the experimental procedure.

2.3. Procedure

The procedures were carried out under anesthesia with atropine sulfate (0.44 mg/kg IM) as a pre-anesthetic, ketamine chloral hydrate (5mg/100g) IM and xylazine chloral hydrate (2mg/100g) IM. The anesthesia was considered proper when the animals presented regular breathing and absence of both podal and ciliary reflex. The anorectal resting pressure was measured by gauge manometry in all groups (CG, SG and BCG) before the surgical procedure.

Anorectal manometry was obtained by a U-Hg manometer coupled to a graduated cylindrical balloon with a diameter of 1.7 cm whose 2cm length was introduced into the anorectal segment. The pressure in mmHg was measured for every 0.1 mL water injected into the system. These values were then plotted to make a gauge level curve ranging from 0 mL to 1 mL water (Fig. 1). From these values, were obtained both the individual mean pressure per volume from two consecutive measures for each rat, and the groups (CG, SG or BCG) mean pressure per volume. These evaluations were carried out at the three previously determined times (D0, D15 and D45) in days.

Anorectal resting pressure for groups SG and BCG was measured immediately before and after the sphincter resection procedure, at D0.

The anorectal pressure of rats was measured and recorded at day 15, before and after injection of either 0.6mL physiological Saline, for SG, or 0.6mL Bacterial Cellulose Gel, for BCG. The anorectal resting pressure for all the groups was recorded also on day 45.

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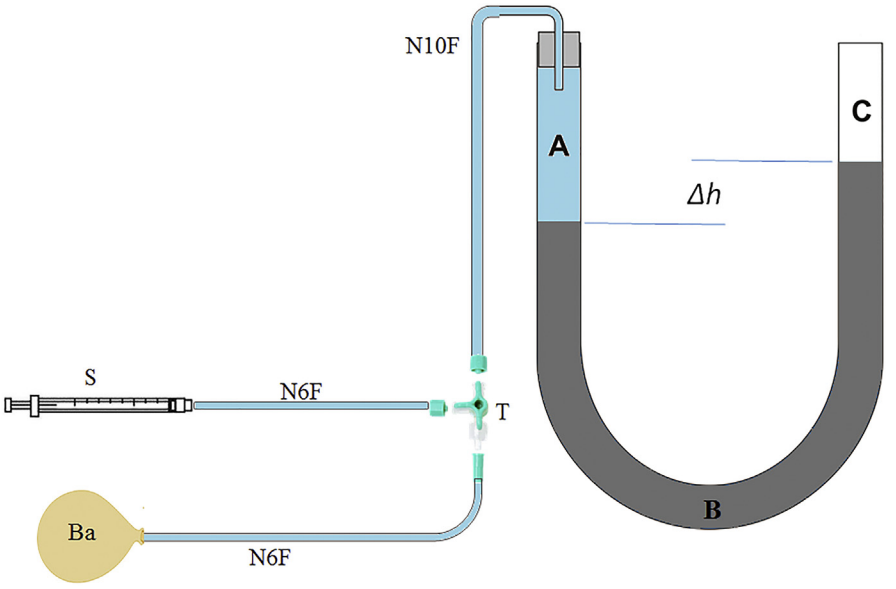

Fig. 1. Design of perfusion system used for the assessment of anal pressure. A: water; B: mercury; C: air; ΔH: difference between the heights; N10F and N6F: Nelaton probes number 10 and 06, respectively; T: three-way stopcock; Ba: latex balloon and S: 1ml syringe.

On D60 all animals were euthanized. A 2cm segment including anus and rectum was collected, weighed and fixed in formaldehyde for histological analysis.

2.4. Surgical technique

The sphincter surgical resection of groups BCG and SG was done according to Yamaguchi's model [19]. The rats were placed in a dorsal decubitus position and both internal and external anal sphincters were removed through a semicircular incision in the left hemicircunference of the anus, (Fig. 2A−C). The surgical resection progressed until it reached rectal mucosa. The incision was closed with separate points of chromed catgut 000.

Injection of saline and bacterial cellulose were done under direct vision with the aid of a speculum at day 15 (Fig. 2D). The saline and BCG were injected with a 1.0mL

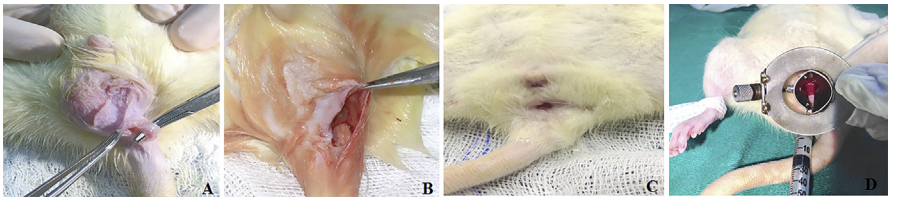

Fig. 2. Animal sphincter resection and BCG injection. A: Rat internal and external sphincter anal canal pulled by Kelly clamp curve. B: Anatomy of the anal sphincter after resection of the left semicircle of sphincter complex. It is possible look at the rectum and ischiorectal fat. C: Appearance of the anal canal on the fifteenth post-operative day (D15). And D: BCG injection under direct view and use of speculum.

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syringe with 25G hypodermic needle, 0.5 cm lateral to the anal margin. It was applied 0.2mL in three points, i.e. left lateral, right lateral and posterior, up to 1cm depth alongside the anorectal wall.

Since resection of the sphincter results in asymmetry of the anal canal, 0.6mL was the volume needed to fill the anatomical defect.

2.5. Bacterial cellulose gel

The bacterial cellulosic polysaccharide (microcrystalline cellulose) was obtained from sugars of sugarcane in the Laboratory of Biopolymers at the Experimental Station of Sugarcane, Federal Rural University of Pernambuco, Brazil. The gel was produced by hydration of microcrystalline cellulosic at ratio of 0.8% in water and sterilized by gamma-radiation. The gel was ready before the injection and squeezed through the needle for application in BCG animals during D15.

2.6. Histological and stereological analysis

On day 60 (D60), the rats were euthanized by intracardiac administration of 150 mg/kg sodium thiopental, after proper anesthesia. The samples from the anorectal region were collected surgically in a uniform way and volume. Samples were weighed on analytical balance, fixed in 10% formaldehyde, processed to histological study and stained in hematoxylin-eosin and Masson trichrome.

The histopathological analysis was done at 400x using an Axio Imager.M2m/Zeiss microscope coupled to an AxioCam HRc/Zeiss digital camera. The volumetric density of blood vessels as well as cell populations were evaluated using a M42 system [grade] test, projected by ImageJ Software. The pictures were taken and transferred to a computer and analyzed using ZEN 2012 Software/Zeiss.

The collagen presence was analyzed by systematically assessing the newly-formed collagen in a semiquantitative manner. Then, the slides were classified as G0, GI, GII or GIII, based on the percentage of recently formed collagen as compared to total. That percentage was calculated based on classic quantitative morphometry [20, 21].

2.7. Statistics

For the statistical evaluation, the data were analyzed using Prism (version 4.0, GraphPad). Analysis of variance (ANOVA) was used to process the continuous variables adjusted to a parametric normal curve. These variables were compared using the Student t-test. The non-parametric data were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney test.

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3. Results

Five rats were excluded from the experiments due to incomplete anorectal pressures measurements caused by failure in anesthetic management during anesthetic padronization. The final number of animals in each group are 12 animals for CG, 11 for SG and 11 for BCG. The number of excluded and included animals in this study are presented in Table 1.

The mean body weight ($p = 0.1829$) as well as the rectum and anus specimens' weight ($p = 0.5709$) were not statistically different.

3.1. Manometric analysis

The mean of the anorectal resting pressures, weights of animals and of the anorectal specimens' weights, for the three groups at D0, D15 and D45, are shown in Table 2. The mean of anorectal resting pressure according the water injected in the balloon are shown in Fig. 3. Anorectal resting pressure differences in the three analysis times (D0, D15 and D45) can be seen in Table 3.

The difference between D15-D0 revealed that there was a corresponding reduction of -0.2mmHg in the mean pressure in the CG, while for the SG it was -1.5mmHg ($p < 0.0001$). BCG D15-D0 difference was -0.3mmHg, statistically different from SG ($p = 0.0004$) but not the CG ($p = 0.0997$).

The pressure difference between D45-D0 was in CG 0.8mmHg, in SG -0.3mmHg and in BCG 1.5mmHg, with statistically significant differences being between CG versus SG ($p = 0.0003$); CG versus BCG ($p = 0.0198$) and BCG versus SG ($p = 0.0002$). Fig. 4 shows the change in volume per pressure difference.

3.2. Histological analysis

The bacterial cellulose gel remained homogeneous and stable in the areas of the implants, located adjacent to the last muscle layer of the anorectal wall. No signs of

Table 1. Rats excluded or lost during research.

Group	Number	Reason	Final number	Sex
CG	1	Incomplete measurement	12	7 M 5 F
SG	2	Incomplete measurement	11	4 M 7 F
BCG	2	Incomplete measurement	11	5 M 6 F

CG: Control group; SG: Sphincter injury plus saline group; BCG: Sphincter injury plus Bacterial Cellulose Gel group; M: Male, F: Female.

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Table 2. The means of anorectal resting pressures, animals and anatomical sample weights.

Outcomes	CG				SG						BCG					
	D0	D15	D45	D60	D0		D15		D45	D60	D0		D15		D45	D60
					BS	AS	BI	AI			BS	AS	BI	AI		
ARP (mmHg)	9.4± 3.6	9.2 ±3.6	10.2 ±4.1	-	10.6 ±3.6	10.2 ±4.1	9.8 ±1.7	8.7 ±3.3	9.9 ±3.8	-	11.5 ±3.6	10.2 ±4.1	9.8 ±2.6	9.9 ±4.0	11.7 ±4.8	-
BW (g)	310.3 ± 81.1	322.8 ± 86.5	337.1 ± 96.5	344.3 ± 97.8		275.8 ±43.5		278.2 ±55.2	294.0 ± 65.6	296.7 ± 61.8		262.6 ±55.0		269.6 ±64.6	294.1 ± 75.8	305.3 ± 82.4
ASW (g)	-	-	-	2.9 ±1.1		-		-	-	2.4 ±1.0		-		-	-	2.6 ±1.0

CG: Control group; SG: Sphincter injury group plus saline; BCG: Sphincter injury plus Bacterial Cellulose Gel group. ARP: anorectal resting pressures; BW: Body Weight; ASW: anatomical sample weights. BS = before sphincter injury; AS = after sphincter injury; BI = before injection; AI = after injection; D0 = first day of analysis; D15, D45 and D60 fifteen, forty-five and sixth days after the first analysis, respectively.

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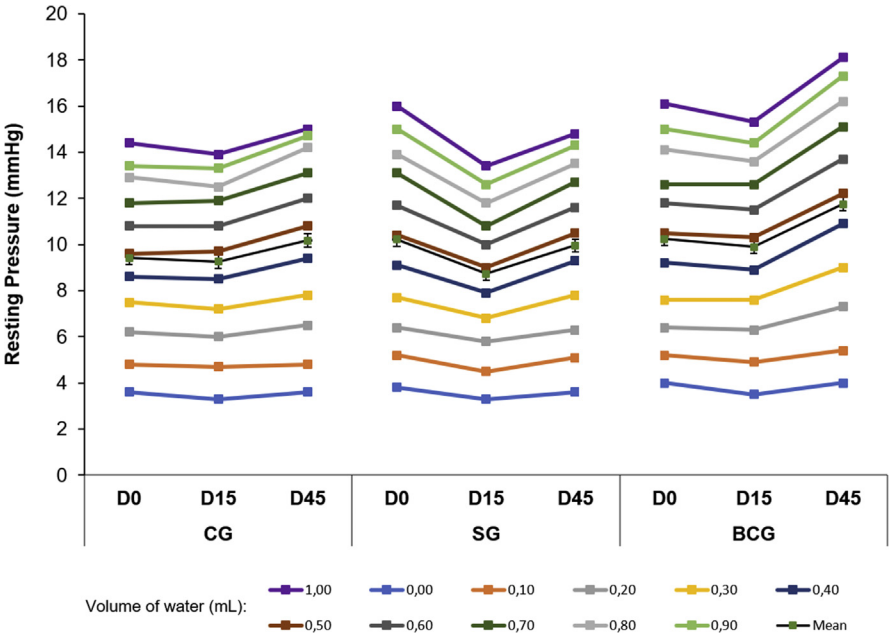

Fig. 3. Curve of anorectal resting pressures for each 0.1mL added in the balloon and for time points D0, D15 and D45 for the three studied groups. D0: day of the beginning of the study, when sphincter injury was done for SG and BCG. D15 and D45: fifteen and forty-five days after the beginning. CG: Control group; SG: Sphincter injury group; BCG: Bacterial Cellulose gel group. D0 pressures were measured after sphincter injury (except for CG); D15 pressures were measured after injection (except for CG). Control Group has 12 animals. Saline Group has 11 animals. BCG group has 11 animals.

extrusion or intense inflammatory process were observed. The rectal mucosa as well as the sphincteric tissue organization were both preserved (Fig. 5A, B).

Giant multinucleated cells were observed in the peripheral area of the implant, extending to the center of the implant. Their presence can be classified predominantly as mild, with an average bulk density of 9.5%.

The characteristics of integration the BCG implants can be observed in Fig. 5 (C, D). Trichrome Masson staining enabled the observation of collagen fibers forming the implant wrap as well as mature fibers in the direction of the central region of the implant. The presence of collagen in the implanted area was classified as grade 0 (0−5%) and grade I (5−25%). In the implant area there were multinucleated giant cells, inflammatory cells, fibroblasts and new blood vessels, from the periphery to the center of the implants with a mean density of 4.5%, confirming its biocompatibility (Fig. 5E, F).

4. Discussion

The use of injectable bulking agents is a procedure involving a simple technique, little discomfort and low morbidity, and may improve the quality of life [22]. This

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Table 3. The means of anorectal difference pressures in mmHg and water volume infused into the balloon.

Group	CG		SG		BCG	
Ballon volume (ml)	**D15-D0**	**D45-D0**	**D15-D0**	**D45-D0**	**D15-D0**	**D45-D0**
0	-0.3	0.0	-0.5	-0.2	-0.5	0.0
0.1	0.0	0.1	-0.6	-0.1	-0.3	0.2
0.2	-0.2	0.3	-0,6	-0.1	0.0	0.9
0.3	-0.3	0.3	-0.8	0.1	-0.1	1.4
0.4	-0.1	0.8	-1.2	0.2	-0.3	1.7
0.5	0.0	1.2	-1.4	0.1	-0.2	1.7
0.6	0.0	1.2	-1.7	-0.1	-0.3	1.9
0.7	0.1	1.3	-2.2	-0.4	-0.1	2.5
0.8	-0.4	1.3	-2.1	-0.4	-0.5	2.1
0.9	-0.1	1.3	-2.4	-0.8	-0.6	2.3
1.0	-0.5	0.6	-2.6	-1.2	-0.8	2.0
Mean ± SD	**-0.2 ±0.2[a]**	**0.8 ±0.5[b]**	**-1.5 ±0.8[a]**	**-0.3 ±0.4[b]**	**-0.3 ±0.3[a]**	**1.5 ±0.8[b]**

Values are mean ± standard deviation. Kruskal-Wallis test followed by Mann-Whitney. CG: Control group; SG: Sphincter injury group; BCG: Bacterial Cellulose gel group. D0 measured after sphincter injury (except for CG); D15 measured after injection (except for CG). a. BCG D15-D0 is statistically different from SG ($p = 0.0004$) but not from CG ($p = 0.0997$). b. Difference between D0-D45 is statistically significant for CG versus SG ($p = 0.0003$); CG versus BCG ($p = 0.0198$) and BCG versus SG ($p = 0.0002$).

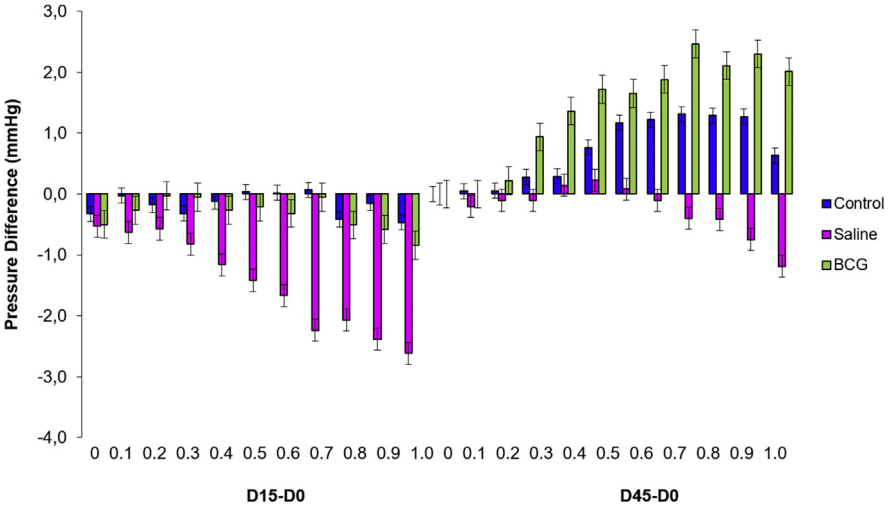

Fig. 4. Difference curve of anorectal resting pressures for the three groups studied between time points D15-D0 and D45-D0. x-axis: Volume of water (mL). BCG: Bacterial cellulose gel injection group.

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approach is best indicated for patients with mild to moderate fecal incontinence who failed medical management [23].

The disadvantage of this treatment is that it involves short term improvement after injections of the substances like dextranomer stabilized in hyaluronic acid, silicone and carbon-coated beads, requiring repeated injections of a high cost material [22, 23].

Our findings show that mean pressure in the control group CG was constant at all the determined times (D0, D15 and D45), SG mean pressure dropped in all the times, whilst the BCG mean pressure increased at the end of the measurements (D45). This change in pressure is higher when the mean pressure difference is observed between the times D15-D0 and D45-D0.

These results of increased pressure in the anorectal segment after the injection of BC indicate the prospect of a clinical study of the administration of this gel for the treatment of fecal incontinence. In BCG, the difference between D15-D0 was -0.3mmHg, statistically different from SG ($p = 0.0004$) but not from the CG ($p = 0.0997$). As previously stated, this may be because the application of the BCG in D15 resulted in a rise in anorectal resting pressure that allowed the maintenance of the pressure level comparable to control. Those significant differences between D15 and D0 when comparing BCG to either SG or CG were similar to Davis' (2003) work in humans [12] corroborating Bacterial Cellulose has potential as a novel agent for fecal incontinence treatment.

Anorectal resting pressure difference between D15-D0 revealed that in the CG there was a corresponding reduction of -0.2mmHg in the average pressure while in the SG it was -1.5mmHg ($CG \neq SG$ $p < 0.0001$). The resting pressure remained lower in D45 only on SG. This set of results demonstrates that the model of sphincter dysfunction was appropriate, as proposed by Yamaguchi *et al* (2013) [22] but opposing to Salcedo's *et al* (2010) work [24].

The analysis of the difference of the anorectal resting pressure between D45 and D0 showed statistical significance for CG versus SG ($p = 0.0003$); CG versus BCG ($p = 0.0198$) and BCG versus SG ($p = 0.0002$). Therefore, the CG group showed maintenance of their mean anorectal resting pressure levels, the SG presented a fall in the mean anorectal resting pressure and BCG presented a significant elevation of the mean anorectal resting pressure levels, even surpassing the pressure of the CG. Those differences for anorectal resting pressure for BCG, and their comparison to the SG and CG resting pressure differences, present statistical significance standing BCG out as a good bulking agent.

Lima *et al* (2015) injected BCG into the bladder wall of adult rabbits to evaluate this material as a bulking agent to treat vesicoureteral reflux [25]. In that study, BCG was compared to Dextranomer Microspheres plus hyaluronic acid according to its

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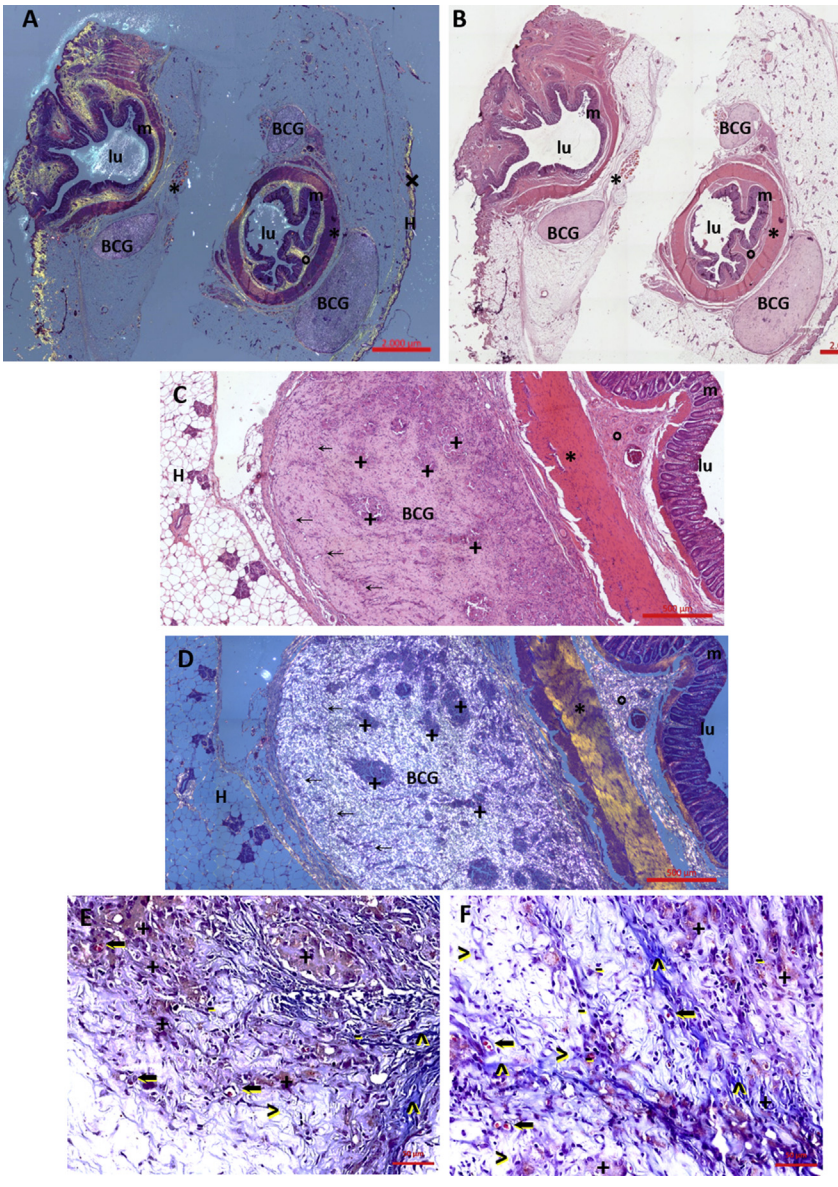

Fig. 5. Photomicrographies of rectus transverse sections of adult Wistar rats that underwent anorectal injury followed of Bacterial Cellulose (BC) Gel implants. A: hematoxylin and eosin staining under polarized light. B: hematoxylin and eosin staining under plain light. C and D: Details of BC implants in a subtle process of absorption and integration under light microscopy and polarized light, respectively. E and F: BC aspects and cells infiltrate with Trichrome Masson's staining. Legends represent epidermis (x); adipose tissue present in hypodermis (H); Bacterial Cellulose (BC); Rectal external muscular layer (*); Submucosal layer (°); Mucosa layer (m); Intestinal light (lu); Multinucleated giant cells (+); Inflammatory cells (-); Fibroblasts (>). New blood vessels, from the periphery to the center of the implants (→). A and B: Scale bars = 2000μm; C and D: bars = 500μm and E and F: bars = 50μm.

biocompatibility and evaluated by histological parameters by the means of morphological and quantitative analysis. In three-month samples areas with BCG were densely invaded by fibroblasts and blood vessels. The areas of Dextranomer

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Microsphere were fragmented but still homogeneous and free of cells or blood vessels. At 11 months after implantation the BCG was remodeled and remained in place with intense cellularity and corresponding vascularization while Dextranomer Microspheres gel presented fragmented without vascular neoformation [25].

Alkan *et al* (2007) observed that the Dextranomer Microspheres decreased over time after injected into the submucosa of bladder rats. The implant reduced the volume by 23% over a period of 12 months [26]. Elzayat *et al* (2008) studied stability in terms of volume changes and local tissue reactions to Dextranomer/Hyaluronic acid (HA) and collagen implants injected subcutaneously in the abdominal area. The implant of Dextranomer reduced the volume by 20% and of collagen by 40% over a period of 12 months [27].

The BCG implants were remodeled and remained at the point of implantation in the anorectal region demonstrating biocompatibility with potential to be an efficient volume agent for the treatment of fecal incontinence. These results are similar to those found in other studies that studied cytotoxicity, biocompatibility and remodeling activity of bacterial cellulose [18, 28, 29].

5. Conclusion

Bacterial Cellulose Gel increases anorectal resting pressures in rats submitted to sphincter injury. The bacterial cellulose remains at the injection site, promoted neovascularization and the implant area was colonized by multinucleated giant cells, fibroblasts and dense conjunctive tissue associate to collagen fibers.

The effects obtained with the bacterial cellulose gel injection showed that this biomaterial presents the ideal characteristics as bulking agent, encouraging clinical trials in the future.

Declarations

Author contribution statement

Aline Ribeiro Teixeira Cavalcante, Rodrigo Pontes de Lima, Veridiana Sales Barbosa de Souza: Performed the experiments.

Flávia Cristina Morone Pinto, Amanda Vasconcelos de Albuquerque: Analyzed and interpreted the data; Wrote the paper.

Olavio Campos Júnior, Jaiurte Gomes Martins da Silva: Contributed reagents, materials, analysis tools or data.

José Lamartine de Andrade Aguiar: Conceived and designed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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