The Biocompatibility of a Cellulose Exopolysaccharide Implant in the Rabbit Bladder When Compared With Dextranomer Microspheres Plus Hyaluronic Acid

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OBJECTIVE To evaluate biocompatibility of a cellulosic exopolysaccharide (CEC) as bulking agent in rabbit urinary bladder.

MATERIALS AND METHODS The experimental study was developed at the Nucleus for Experimental Surgery or UFPE. The new agent was injected into the bladder of the adult rabbits using a small abdominal incision. Animals were injected with 0.2 mL of dextranomer microspheres (Dx) plus hyaluronic acid and CEC. The animals were studied after 3 days (G1), 90 days (G2), and 11 months (G3). The biocompatibility was evaluated according to the histologic parameters (presence of blood vessels, inflammatory reaction, and collagen deposition) by a quantitative analysis. The Student paired *t* test was used for continuous variables, and the scores were compared through the chi-square test.

RESULTS Both materials were structurally homogeneous and free from inflammatory cells or blood vessels (G1). In 3-month samples (G2), CEC areas were densely invaded by fibroblasts and blood vessels. Dx areas were fragmented but still homogeneous and free from cells or blood vessels. Samples from 3 and 11 months showed a significant difference in favor of CEC especially concerning preservation of material in the implant site, as well as the presence of neovascularization. This experimental study represents a positive outcome in terms of reflux resolution in the long term. Further studies may be necessary to confirm its efficacy when in clinical use.

CONCLUSION The CEC exhibited low inflammatory response and integrated with the host tissue better than Dx in the long-term follow-up. UROLOGY ■: 1.e1–1.e6, 2015. © 2015 Elsevier Inc.

Vesicoureteral reflux (VUR) is a common urologic anomaly, and its management has been a matter of controversy because of its multifactorial nature.[1] Current treatment options for the treatment of this disease include prophylaxis (long term) with antibiotics to prevent pyelonephritis and await spontaneous resolution. However, high grades of reflux have a low rate of spontaneous resolution. On the other hand, the lack of involvement of caretakers in providing patients' medications may represent a complicating factor in dealing with the problem.[2,3] Surgical reimplantation of ureters, by open or laparoscopic approach, has been considered as the gold standard modality of treatment. Although both medication and reimplantation have a high success rate, surgery is technically invasive and involves higher costs.[4]

In recent years, subureteral transurethral injection has become very popular for the therapy of patients with VUR owing to its significant success rate, besides causing little or no postoperative complications.[5] Arguments for the best bulking agent for endoscopic therapy in patients with VUR are controversial.[6,7]

A major challenge to be reached is to find an agent that is safe for treatment and at the same time efficacious

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and effective in the long run. However, one must consider that the ideal filling material may differ depending on what is proposed with its application. It is known that this material should be nontoxic, biocompatible, nonmigratory, and nonantigenic and should cause the least possible inflammation at the site of implantation. Many materials have been used to treat VUR including collagen, polytetrafluoroethylene (PTFE or Teflon [DuPont]), silicone microimplants, polyvinyl alcohol, bovine collagen with glutaraldehyde-preserved autologous chondrocytes, calcium hydroxyapatite, polydimethylsiloxane, injectable autologous materials, autologous fat, and dextranomer–hyaluronic acid (HA; dextranomer microspheres [Dx]).[8,9]

Dx have been the most studied filling material and have shown the best known short-term effects, becoming the most popular bulking agent used for the treatment of VUR. It is composed of Dx and HA, mixed to form a consistent gel. Both components are made of polysaccharide molecules.[9] Despite the reported high success rate after endoscopic correction of VUR using the Dx, there is a shortage of evidence-based literature about the long-term effects. The limited available data, however, clearly demonstrate that there is a significant rate of recurrence over the long term after injection, which requires careful observation of the consequences of this procedure.[9]

The new agent proposed here is cellulosic exopolysaccharide (CEC), obtained by biotechnological synthesis via bacterial action on sugarcane molasses, which is a renewable byproduct from the sugar production process. In vitro cytotoxicity of the CEC was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The rate of adhesion of nitric oxide and cell viability of rat alveolar macrophages was similar to PTFE and displayed toxicity negative.[10]

Thus, this study sought to investigate the biocompatibility of a CEC implant in the bladder of a rabbit when compared with Dx plus HA because the new agent has special features that may represent a new option for the treatment of VUR and related anomalies such as urinary incontinence.

MATERIALS AND METHODS

Animal Model and Experimental Design

Thirty adult rabbits of California race, averaging 6 months of age, were used as an experimental model.

The groups were classified as G1 (killed after 3 days, n = 09), G2 (killed at 90 days, n = 11), and G3 (killed at 11 months, n = 10).

The surgical procedure was performed using an abdominal incision of 10-cm length, which allowed full exteriorization of abdominal organs. The bladder central venous plexus was taken as a landmark for the injection of the gel implants. CEC was injected on the left side of the bladder and Dx gel implants on the opposite side. Thus, each animal received a total of 4 implants, 2 of CEC and 2 of Dx. The implants were injected horizontally and parallel as markers of these regions; 4-0 PROLENE (Ethicon US) stitches were placed to allow identification.

Synthesis of the Cellulosic Exopolysaccharide

CEC was produced from sugars of sugarcane in the laboratory of biopolymers at the Experimental Station of Sugarcane, Federal Rural University of Pernambuco, Brazil. The CEC was obtained by hydration of microcrystalline bacterial cellulose at a ratio of 0.8% cellulose in 99.2% water and sterilization by gamma ray.

Histologic Analysis

Characterization of tissue integrity and validation of implantation technique, by evaluating the location of the implants between the epithelium and the inner edge of the detrusor, were performed starting from hematoxylin and eosin staining. A qualitative analysis was used to ascertain the homogeneity of the structures.

The presence of inflammatory infiltrates was determined from the quantitative analysis, considering the following scores: 0 = no inflammatory cells, 1 = scattered inflammatory infiltrates cell infiltrate within the stroma without lymphoid nodules, 2 = nonconfluent lymphoid nodules, and 3 = large inflammatory areas with confluence of infiltrate.[11]

The assessment concerning collagen deposition was performed using Masson trichrome and picrosirius red (polarized light) staining. The muscle cells and expression of myofibroblasts were identified by immunohistochemical assay using an anti–α-actin antibody.

Analyses were performed with an Axio Imager M2m (Zeiss) light microscope, except for the analysis of blood vessel density, where quantification was performed in 5 selected fields from the cutting area of the histologic section[12] and analyzed using an ImageJ software field to determine the number of blood vessels per mm^2.

Statistical Analysis

For the other analyses, the results of blood vessel density were expressed as mean ± standard deviation and the presence of inflammatory infiltrates as percentage. The means of continuous variables were compared using the Student paired *t* test, whereas scores were compared using the chi-square test. Statistical significance was set at $P \leq .05$. The statistical tests were performed using the GraphPad Prism 5.0 program (GraphPad Software Inc.).

Ethical Aspects

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 of the Center for Biological Sciences, UFPE according to the process No. 23076.012767/2008-69.

RESULTS

After the injections into the bladder wall, both implants, CEC and Dx, were typically located between the epithelium and the inner edge of the detrusor in the 3 groups G1, G2, and G3 (Figs. 1-3).

In samples from G1 implants, CEC and Dx were structurally homogeneous (Fig. 1). The samples in group G2 were arranged in short beams, suggesting the presence of fibrous tissue. However, the G1 and G2 groups did not indicate the presence of collagen when using Masson trichrome staining. The structure remained homogeneous

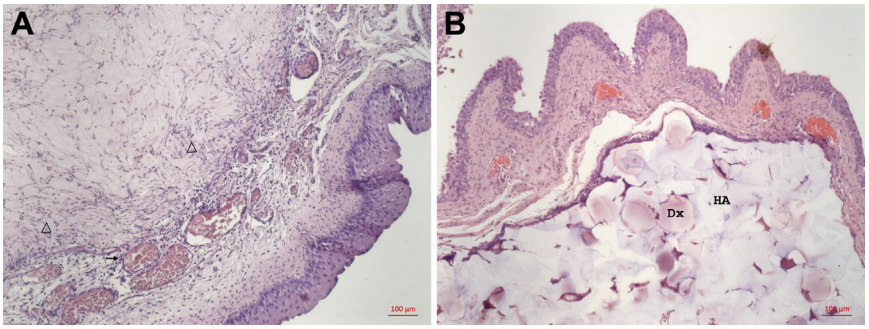

Figure 1. (A) Cellulosic exopolysaccharide implant after 3 days. Staining with hematoxylin and eosin (×10). →: Vascular congestion; Δ: inflammatory cells. **(B)** Dextranomer microspheres implant after 3 days. Staining with hematoxylin and eosin (×10). Dx, dextranomer microspheres; HA, hyaluronic acid. (Color version available online.)

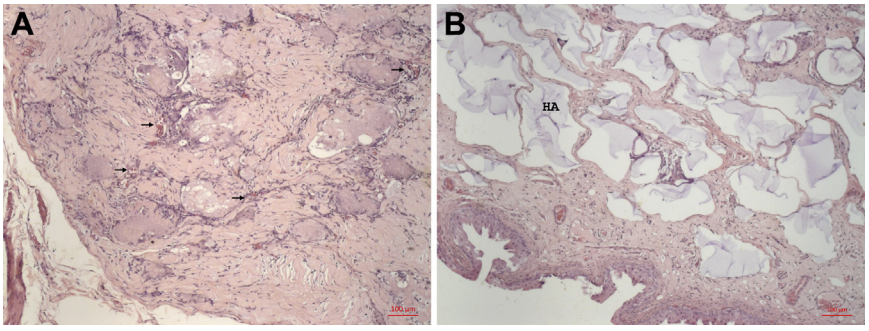

Figure 2. (A) Cellulosic exopolysaccharide implant after 3 months. Staining with hematoxylin and eosin (×10). →: Blood vessels. **(B)** Dextranomer microspheres implant after 3 months. Staining with hematoxylin and eosin (×10). HA, hyaluronic acid. (Color version available online.)

for the CEC implant, but only HA was found in this sample for the Dx group. No Dx were detected (Fig. 2). In the G3 group, from the animals evaluated, there was an extensive area of remaining CEC implants, and these had become homogeneous. It was noted that in this same group, the implant area was seen to be fully populated by multinucleated giant cells (MNGC) and presence of blood vessels in both the peripheral and the central portion of the implant. At the Dx implant area, no Dx were found. Only a scarce area of HA was observed (Fig. 3).

Concerning the inflammatory infiltrates in group G1 CEC with the implant, an expressive response was not observed. No inflammatory infiltrate was found for the Dx implant. In group 2, few inflammatory infiltrates were observed in the areas of the CEC implant, especially around the vessels, whereas for the Dx implant, a moderate inflammatory reaction around the implant was found. In G3, with the CEC implant, the inflammatory infiltrate was mostly concentrated in the periphery, although inflammatory cells were homogeneously found in the whole implant area. As for the Dx implant in the G3 group, the inflammatory response was less intense.

The curve of inflammatory response was according to the time of permanence (3 or 90 days, or 11 months) of each implant (CEC or Dx).

The inflammatory reaction was not significantly different among the groups ($P = .9999$). However, it was more intense (score 2) in G2 (CEC, 72.7%; Dx, 81.8%) and lighter (score 1) in G3 (CEC, 70.0%; Dx, 60.0%). Table 1 shows the score variation by groups.

In the G1, implants CEC and Dx were essentially unchanged and no blood vessels were found (Fig. 1). In the CEC implants in G2, however, many blood vessels were found (Fig. 2A). The G3 group showed blood vessels starting from the area of transition between normal tissue and CEC implant with blood vessels progressing from normal tissue toward the area of the implant (Fig. 3A). No blood vessels were found with the Dx implant in the 3 groups.

When comparing groups, the CEC implants in G2 (23.86 blood vessels/mm^2) and G3 (16.0 blood vessels/mm^2) were significantly different from the groups receiving the Dx implant (absence of blood vessels; $P = .0001$ and $P = .0012$, respectively), with evident biocompatibility and tissue integration with the CEC (Figs. 1A, 3A for CEC and Figs. 1B, 3B for Dx).

The processes of tissue remodeling and integration can be observed in groups G1 and G2 in the CEC implant. Sections of implants in G1 CEC were mainly restricted to normal epithelium, especially in their basal layer, which is expected for this type of tissue pattern. Samples from the

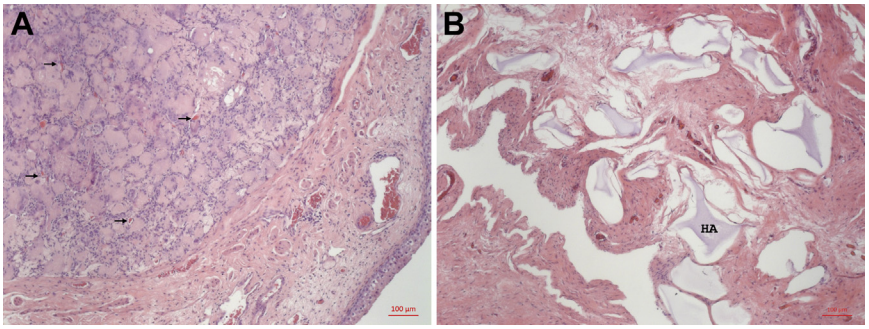

Figure 3. (A) Cellulosic exopolysaccharide implant after 11 months. Staining with hematoxylin and eosin (×10). →: Blood vessels. **(B)** Dextranomer microspheres implant after 11 months. Staining with hematoxylin and eosin (×10). HA, hyaluronic acid. (Color version available online.)

Table 1. Inflammatory response by group

	CEC			Dx		
	G1	G2	G3	G1	G2	G3
Scores	N = 09	N = 11	N = 10	N = 09	N = 11	N = 10
0	5 (55.6)	0 (0)	0 (0)	0 (0)	0 (0)	2 (20.0)
1	4 (44.4)	0 (0)	7 (70.0)		2 (18.2)	6 (60.0)
2	0 (0.0)	8 (72.7)	3 (30.0)		9 (81.8)	2 (20.0)
3	0 (0.0)	3 (27.3)	0 (0)		0 (0)	0 (0)

CEC, cellulosic exopolysaccharide; Dx, dextranomer microspheres.
Values expressed in n (%).
Chi-square test, considered significant if $P \leq .05$, to CEC ≠ Dx.

G2 CEC implant were densely invaded by fibroblasts and blood vessels, whereas the remainder of the CEC islets were seen as homogeneous surrounded by inflammatory cells. In the G3 CEC implant, the process of integration and remodeling was verified by the presence of high MNGC with vacuolization and formation of collagen fibers involving the same pattern throughout the duration of the implant. In animals with a remnant area of CEC, slight signs of MNGC were found, as well as the presence of collagen in the maturation stage, when stained with picrosirius red and Masson trichrome. This process of tissue remodeling and tissue integration was not observed in the groups that received the Dx implant.

The early analysis in both groups showed no difference between them. Samples from 3 and 11 months showed a significant difference in favor of CEC especially concerning preservation of material at the site of the implant, as well as the presence of neovascularization (Fig. 3), evidenced by immunostaining for smooth muscle actin immunohistochemistry (Supplementary Fig. 1A) when compared with groups receiving the Dx implant (Supplementary Fig. 1B).

COMMENT

Endoscopically injected materials represent an alternative to the treatment of VUR, Dx being the most commonly used filling material. Dx is composed of Dx and HA. This material remains virtually unchanged when implanted without inducing changes in bladder tissue, fulfilling the function as a bulking agent, expanding the bladder wall. However, there are no consistent data in the literature on the use of this agent over the long term. It should be noted that there are reports of a significant rate of recurrence in long-term follow-up, which requires careful observation of the consequences of its use.[9,13]

CEC is a natural product obtained from molasses, a byproduct of the sugar production process. Its chemical structure consists of polymerized sugars, which become stable and are not digested by the surrounding tissues. CEC was used in the form of gel, which has a high coefficient of elastic deformity, adapting itself to the variations of functional deformity of organic tissues. Recent studies show that CEC is biocompatible and nontoxic.[10,14-17] In a recent study using the CEC gel as implant in eviscerated rabbit eyes, it was shown that CEC was similar to PTFE and polypropylene (PROLENE) in compatibility, integrating adequately with the surrounding tissues.[17,18] Likewise, when implanted in the bladder wall, CEC is integrated uniformly, preserving its function as a bulking agent.

The CEC caused a remodeling process at the site of injection, fully replacing normal bladder tissue, inducing the formation of new tissue and the extracellular matrix with new vessel formation. The incorporation of the CEC tissue was clearly demonstrated in animals of the G3 group that spent more time in the experiment. The neovascularization showed characteristics starting at the periphery and moved to the center, until almost the entire implant was incorporated to the bladder tissue.

This process of remodeling by the CEC was validated, and the adhesion of mesenchymal stem cells was tested using electrical impedance spectroscopy on a CEC film as a means to assess the ability of the films to act as a substrate for cell culture.[19] These results showed that the films can be considered as matrices suitable for cell culture, representing a promising biomaterial for tissue engineering (Figs. 1A, 2A, 3A).

With respect to neoangiogenesis, it was noted that the reduction in the density of blood vessels in the CEC implants in the period between 3 and 11 months can be explained by the maturation process of these vessels that became larger, evidencing the incorporation and remodeling of the implant to the tissue.

The process of tissue remodeling and integration of the implant with the tissue that was observed with the CEC implant did not occur with the Dx throughout the study period. Although, in the G1 group, the Dx implant was homogeneous, in the other groups (G2 and G3), the material presented was poorly encapsulated by scarce connective tissue. The Dx were observed to have been absorbed and only remnants of HA were found. This phenomenon may indicate that there was a degradation process of the implant (Figs. 1B, 2B, 3B). Some other authors have reported similar results using Dx or other material for treating VUR.[8,20,21]

There were inflammatory and fibroblastic responses, as well as collagen deposition, in all animals receiving the CEC implant in groups G2 and G3. The presence of giant cells and new vessels were also evident, indicating the invasiveness of these cells in the implanted material in the CEC group.

It is well known that an inflammatory response occurs in all tissues after vascular injury of any kind. By the third day, in the absence of infection, the realignment process is started and the macrophages give rise to so-called foreign body giant cells, which will lead to phagocytosis. We agree that this reaction occurs with all materials that remain for a long period without being eliminated, as in the case of bioabsorbable polymers,[22] and CEC. This finding may explain the results of this study and confirms the absence of inflammatory response (55.5% classified with score 0) and giant cells in the G1 CEC implant.

The reduction of the inflammatory response between the G2 and the G3 groups was evident, mainly for animals receiving the CEC implant. The inflammatory response to the implants with Dx in the G2 and G3 groups was less intense, and this can be related to the degradation process already observed in other studies.[20,21]

The results of this study showed stabilization in the process of cell proliferation in the G3 group, suggesting that implantation of the CEC in the long run no longer experiences a significant remodeling.

In this study, we did not look for distant migration of the material, although we have done it in a previous experimental study in mice (unpublished data), and the results were negative when looking at the liver, the lungs, and the brain as we have compared our results with Dx and they did not look at this item in their original work.[23] We are also aware that only Teflon has shown this migration, and this phenomenon has never demonstrated the development of any disease despite its long-term use.

The study also demonstrated that the physiological integration of the CEC implants in the host tissue and resistance to the degradation process were similar in aspect to those found in previous studies.[15,17,22,24] These results also indicate that CEC may be an option for injectable therapy for VUR or stress urinary incontinence, but this is yet to be evaluated.

CONCLUSION

The results obtained in the present study indicate that the CEC implant exhibited low inflammatory response in the long-term follow-up and was integrated adequately into the surrounding tissues when compared with Dx.

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APPENDIX

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.urology.2015.02.028.