Original
ISSN 2413-0516

Comparing the Endoscopic Sub Ureteral Injection of Lyophilized/ Micronized Decellular Prepuce with Urodex: Histological Findings

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(Submitted: 10 February 2021 – Revised version received: 25 February 2021 – Accepted: 06 March 2021 – Published online: 26 June 2021)

Abstract

Objectives In the present study, a new engineered bulking agent called lyophilized micronized prepuce was examined as a natural scaffold to compare its safety and efficacy with the Urodex®.

Methods For an in vivo study, 12 rabbits were divided into two groups. In the first group (n = 6), 0.2 cc of lyophilized and micronized prepuce, and in the second group, 0.2 cc of Urodex® was injected into the seromuscular wall of the bladder. The biopsy was provided from all animals for histological evaluation in 3 and 6 months' post-surgery and for each timeline 12 animals were assigned. The biopsies were stained with H&E and trichrome Masson. IHC staining was also performed with anti-LCA$^+$, anti-CD34$^+$, and anti-CD68$^+$ antibodies.

Resluts Microscopic examination of acellular prepuce compared with normal tissue demonstrated the success of this process, and ECM and collagen fibers were preserved with no evidence of cellular remnants in the acellular tissue, Immunohistochemistry staining with CD68 and LCA revealed a higher inflammation grade in Urodex as compared with Prepuce. However, no significant difference was detected in CD34 staining between Prepuce and Urodex experimental groups SEM analysis detected the micronized particle size varying between 2–5 µm. MTT assay revealed that cell proliferation was similar in the presence of control group and acellular prepuce.

Conclusion The results of this study disclosed that lyophilized and micronized prepuce could be an operative alternative to Urodex® as a natural and non-synthetic bulking agent in the treatment of children with vesicoureteral reflux (VUR).

Keywords Vesico-ureteral reflux, acellular scaffold, prepuce, bulking agent

Introduction

Vesico-Ureteral Reflux (VUR) is one of the most prevalent anomalies of the urinary tract among children. Persistent VUR can prompt progressive kidney impairment and, eventually, kidney failure so that it can be considered As leading reasons for disorder development, high blood pressure, and renal failure in childhood.[1] The main methods of treatment in children with reflux are long-term administration of prophylactic antibiotics, open surgery, or injection of submucosal biomaterials with endoscopic techniques.[2] Given the long-term necessity of antibiotic therapy and linked problems, as well as possible complications of open surgery, so endoscopic treatments have been presented for vesicoureteral reflux treatment from the early 1980s. The application of different bulking agents for the treatment of VUR has a low-cost, noninvasive approach with fewer side effects.[3] It is a practical procedure in outpatient under local anesthesia. Preventing phagocytic activity and migration of the injected substance, reducing its toxicity, resisting biodegradation, eliminating or minimizing inflammatory responses are characteristics of an ideal bulking agent. Current materials including collagen,[4] Vantris[5] carbon-coated particles[6,7] Teflon, DAM+[8] polyacrylamide hydrogel[9] polydimethylsiloxane,[10,11] calcium hydroxyapatite.[12] Urocol is synthetic augmentation agent, and the side effects of these substances are considerable.[13] Dextranomer / hyaluronic acid (HA) copolymer (Deflux®), known as a synthetic, non-migratory, non-allergenic, non-mutagenic, non-immunological and biodegradable material that contributes to the growth of collagen and fibroblasts.[14] HA can also aid in the strength of implants based on Dextranomer and commonly are used in children. Urodex® is a sterile, adherent gel consisting of a non-originating animal-derived cross-linked doctanomeric-microstatic suspension and HA, known as a synthetic material, and contributes to the growth of collagen and fibroblasts.[15] Nowadays, HA is widely used in various forms in tissue engineering for ECM regeneration.[16] There are various types of biomaterials or artificial constructs for reconstructive purposes. Findings from past studies indicated that microspheres could be used independently in the short term, while the simultaneous application of tissue engineering bulking agents with microspheres has a long-lasting and promising effect.[17] Further studies have revealed that collagen is a biodegradable material as a bulking substance and its initial success rate decreases over time. Besides, it may also trigger an allergic reaction. It has also been shown that the injection of autologous chondrocytes through the urethra is an effective and safe low-invasive method for correcting urinary bladder reflux in children.[18] Early endoscopic injection of Deflux® in connected treatment of reflux in urinary bladder obstruction (UPJO), provided promising results in the treatment of reduction of hydronephrosis or spontaneous obstruction.[19] After a Deflux® endoscopy injection successful reflux correction can also be predicted by the grade of preoperative reflux. It was presented that tissue-engineered prepuce bio-scaffold is a collagen-rich matrix with significant mechanical properties and when seeded by mesenchymal stem cells attained acceptable results for bladder restoration.[20] The results of multiple studies

confirmed that the endoscopic injection of autologous collagen could be a safe approach for the treatment of urinary incontinence as well as a low grade of urinary bladder reflux in children.[21] Because it's so vital to reduce the complications in infants and children, therefore, we were looking for a natural scaffold as a promising substitute instead of a synthetic bulking agents. Natural scaffolds are probably more stable and durable. The source and providing a method of scaffolds are important because they can affect the functional efficiency of these scaffolds. One of the objectives of this study was to evaluate the safety viability of this new bulking substance (lyophilized prepuce) at the injection site compared to Urodex® in a rabbit animal model. The golden goal of employing these natural bulking substances (prepuce) was to reduce the number of complications of synthetic bulking agents, including inflammation, ureteral obstruction, and migration to distant organs. Therefore, the present study used a biologically lyophilized and micronized scaffold that appears to have a minimal inflammatory response and to be more effective for future vesicoureteral reflux treatment in male and female infants than the other bulking agents. After circumcision, prepuce specimens can be kept in tissue banks in sterile conditions as a backup resource for the correction of urinary tract reflux. In our study, by applying this material, we aimed to reduce the rate of cystoscopies and frequent interventions to treat the common problem of urinary bladder reflux. In order to achieve this purpose, we compared inflammation as well as the reconstructive role of the biologic decellularized prepuce with Urodex® in short (3 months) and long term follow up (6 months).

Materials and Methods

Decellularization Process

The prepuce was obtained from circumcising normal boys in the pediatric urology operating room in The Children Medical Center of Tehran University of Medical Sciences, with the informed consent of research protocol from their parents. Then the sterilized prepuce tissue was transferred to the laboratory for decellularization. First samples were washed with phosphate-buffered saline (PBS) for 30 minutes and were placed in sodium dodecyl sulfate (SDS) 2% solution for 20 minutes, followed by 1% Triton X-100 solution for one hour. In the next step, the tissues were immersed in a trypsin ethylene diamine tetraacetic acid (EDTA) solution at 37°C for 10 minutes. To ensure the efficacy of decellularization process, samples were then stained with hemotoxylin and eosin (H&E) for general observation, Masson's trichrome for ECM architecture evaluation, and DAPI (4', 6-diamidino-2-phenylindole) to ensure the nuclear residue removal.

MTT Assay

L929 cells were obtained from the Pasteur Institute of Iran and were seeded at a density of 20,000 cells in a 96-well cell culture plate. After 24 hours, a desired portion of the sterile decellularized prepuce tissue was floated in each well and was incubated at 37°C. After 72 hours of incubation, the tissues and medium were removed, 100 µL MTT solution at a concentration of 0.5 mg/ml was poured and incubated for further 4 hours. Then, 100 µL dimethyl sulfoxide (DMSO) was added and was placed in a dark place for 10 minutes. The optical density (OD) was recorded at 570 nm by the ELISA reader (Stat Fax, USA) and relative cell viability (%) was normalized by control group according to the following equation:

$$\text{Viability (\%)} = (\text{average optical density of samples}) / (\text{average optical density of control group}) \times 100\%.$$

The experiments were repeated three times for each specimen, and cell in monolayer culture was considered as a control group.

Recellularization of Decellularized Tissue

To evaluate cell adhesion and cytotoxicity, the recellularization of decellularized tissue was performed with adipose-derived mesenchymal stem cells (ASCs). We isolated ASCs, as described in our previous study.[22] Decellularized tissues were prepared (1cm × 1cm) and were sterilized with PBS buffer containing antibiotics. After washing with PBS for three times, the decellularized prepuce tissues were seeded with ASCs at the density of 1×10^4 and were incubated at 37°C, 5% CO_2 for 3 hours to allow cell adhesion on the surface of decellularized tissues.

Afterward 1 ml of 10% Dulbecco's modified Eagle medium/ fetal bovine serum (DMEM/ FBS medium) was added to the specimens and was stored in an incubator. After 48 hours of incubation, the specimens were transferred to new plates and were kept in an incubator for two weeks. The culture media was changed every two days. After that, the samples were washed with PBS and immersed in 10% NBF for histopathological evaluation.

Scanning Electron Microscopy (SEM)

Native, decellularized and sterilized prepuce were fixed by immersion in 4% glutaraldehyde for 45 min at room temperature. Tissues were dehydrated using a series of alcohol concentrations (40%, 50%, 60%, 70%, 80%, 90% and 100% (2)) in distilled water and dried at room temperature for 24 h. By sputtering, a thin layer of gold was applied on the surface of all samples and then visualized by SEM (VEGA TESCAN Inc, USA) at 30-kV voltage and 500x magnification. In this step, the powder was again subjected to SEM to check the particle size and to prevent excessive coarse or excessive tiny particles being used. The last step sterilization has been done to make it ready for injection.

Bulking Agent Preparation

After assuring the efficacy of the decellularization steps, the scaffolds were lyophilized at –80°C for 48 hours and were micronized. Then micronized prepuce was combined with HA (50 mg/15 mg). After this step, the powder was again subjected to a scanning electron microscopy (SEM) to check the particle sizes and to prevent excessive coarse or excessive tiny particles being used. The last step sterilization has been done to make it ready for injection.

In vivo Study

12 healthy male New Zealand rabbits weighing 2–2.4 kg was selected for this study. The animals were kept under the standard diet and water. All stages of the study and animal protocols have been approved by the local ethics committee of Tehran University of Medical Sciences. Intramuscular injection of Ketamine (120 mg/kg) and Xylazine (15 mg/kg) was

applied to perform bulking agent injection under general anesthesia after suprapubic incision and exposure of the bladder. The animals were divided into two groups (n = 6). The first group received 0.2 cc lyophilized micronized prepuce, and the second group underwent the same procedure with the injection of 0.2 cc Urodex® in the seromuscular layer, under the sterile condition. The injection site was marked with a 0-4 silk suture. Since each time line of study had 12 animals so after 3 and 6 months follow up 4 animals per group were sacrificed, and the desired biopsies were harvested. Histopathological evaluation, including hematoxylin eosin (H&E) and Masson-Trichrome were conducted to determine fibrosis, inflammation, angiogenesis, and collagen integrity. Immunohistochemical tests were also performed with anti-LCA antibodies to assess inflammation, anti-CD31 for angiogenesis, and anti-CD68 for intrinsic immunity.

Statistical Analysis

To capture 15 random images from 15 different fields of each tissue sample at 400X magnification, a Nikon ACT-1 with v.2.70 software was used. The quantification of LCA, $CD34^+$, and $CD68^+$ cells was conducted using ImageJ software version 1.46e (National Institutes of Health). Un paired T test, One-Way ANOVA, mann-whitney U test and Kruskal-Wallis were performed as parametric and non-parametric tests, respectively. All data were analyzed using the SPSS software version 24 (IBM Corporation, Armonk, NY, USA) and then expressed as mean ± (SD) for numerical variables and frequencies and percentages for categorical variables. P values of less than 0.05 were considered statistically significant.

Results

Microscopic evaluation of specimens revealed that detergent-based (DET) decellularization was successfully removed the cells. H&E staining showed that the cellular components were completely removed (Figure 1b). This finding was also proved by DAPI staining (Figure 1d). Masson's trichrome staining confirmed the architecture preservation of the decellularized matrix (Figure 1f). SEM was also performed to evaluate the ECM structure alterations due to the decellularization process (Figure 1g, h). MTT assay findings revealed that the percentage of cell survival was greater than 85%, which indicates that the decellularized tissues have no cytotoxic effect, and there was no significant difference between control and decellularized prepuce groups (Figure 2a and S1). Recelluarization of the acellular prepuce demonstrated that the cell site safely preserved after the cell removal process, and as shown in figure 2b, ASCs are well attached on the surface of acellular prepuce matrix. SEM analysis detected the micronized particle size varying between 2-5 µm (Figure 3a, b, c, and d). During and after the operation, no surgery complication such as infection, bleeding, and perforation was not observed in any of the experimental models. Since the either micronized prepuce or Urodex® agents were placed at the same site of injection in all groups, tracking of these agents revealed no far off relocation in the bulking agent injected groups. After a 3 and 6 months follow up of the post-injection group, the animals were sacrificed, and the target tissue was gathered for

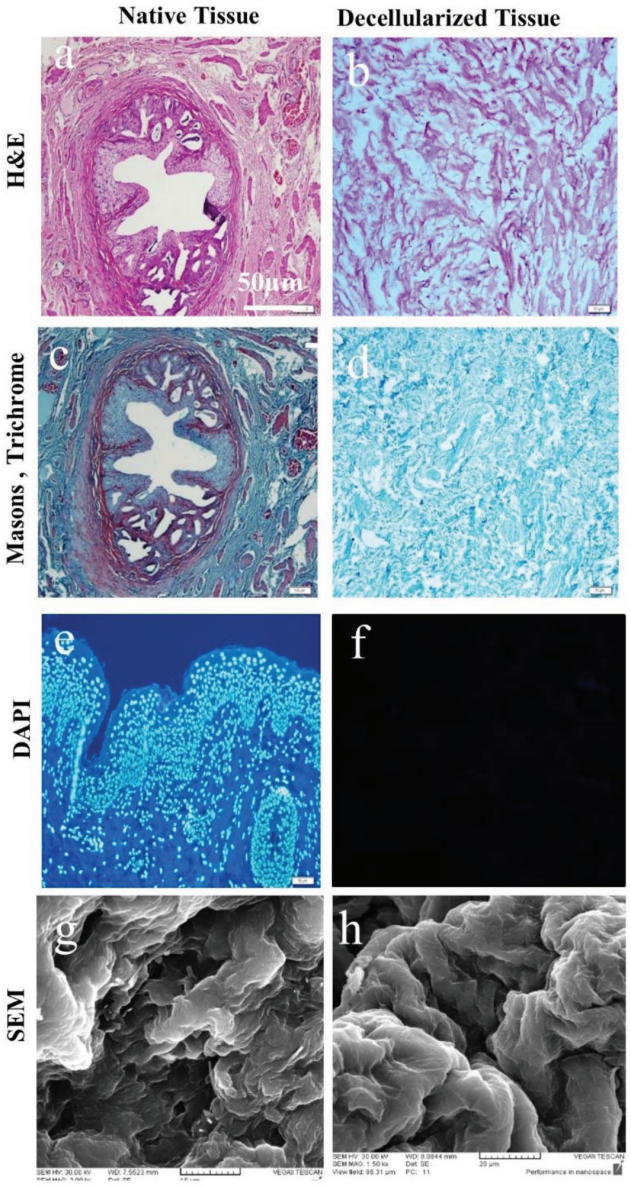

Fig. 1 **Histomorphological evaluation of native and decellularized prepuce tissue. a, b H&E staining, c, d DAPI staining, e, f Masson's Trichrome and g, h SEM imaging represent the normal and acellular prepuce. Scale bar presented in 20X magnification.**

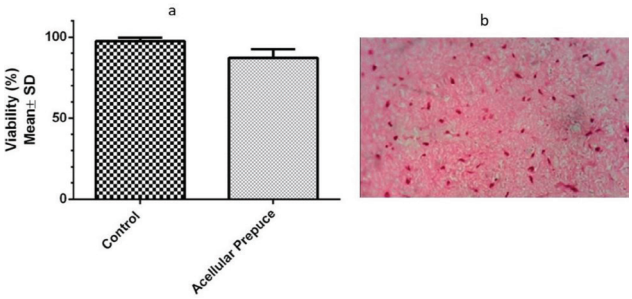

Fig. 2 **Cell viability and recellularization process. a. MTT assay carried out to confirm the cytotoxicity of the decellularized tissues. In the prepuce tissues, the percentage of cell survival was greater than 85%, which indicates that the decellularized tissues are not cytotoxic. Data presented in Mean ± SD. b. Prepuce recellularization performed to evaluate the adhesion potential of the decellularized prepuce. H&E micrograph provided in a:10X magnification.**

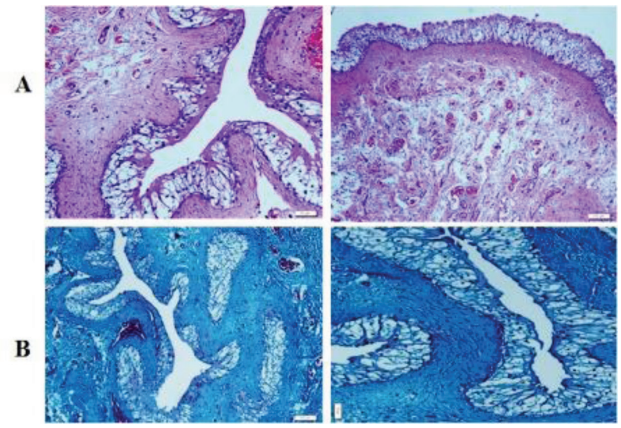

Fig. s1 **Natural histomorphology of Urethra.**

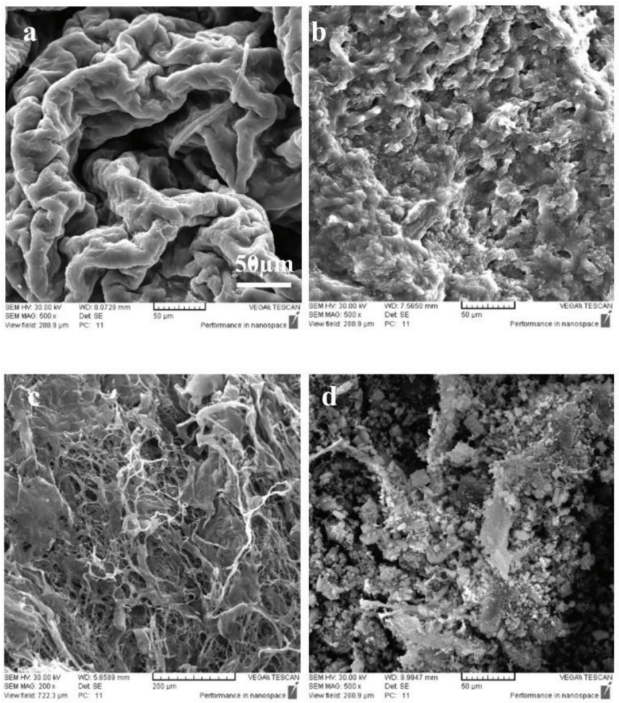

Fig. 3 **SEM imagining of the prepuce in different stages. a. The native prepuce, b. The acellular prepuce, c. Lyophilized prepuce and d. micronized prepuce.**

following histology studies. All rabbits survived during the whole course of the study. No postoperative impediments were detected in either prepuce, or Urodex® injected groups. In the macroscopic and microscopic observations, no sign of allergic reactions such as erythema or swelling and infection or necrosis were detected at the site of injection in 3 and 6 months. Also, no bulking agent migration was detected to the distant organs in any of the groups.

Immunohistochemical sections revealed similar expression level of LCA in following groups Urodex® 6 months (13.81 ± 3.63), prepuce 3 (16.45 ± 2.60) and 6 (10.83 ± 3.27) months after injection but there was a significant difference of LCA expression between Urodex® after 3 months (20.42 ± 4.01) and prepuce after 6 months (10.83 ± 3.27) ($P \leq 0.05$). No significant difference was observed in the level of $CD34^+$ cell marker between Urodex® three months (28.73±7.71), six months (36.21±3.31), and prepuce 3months (23.23±4.27) after injection. However, a significant difference was found between 6months (38.41 ± 8.18) and three months (23.23 ± 4.27) in prepuce injected groups for $CD34^+$ expression ($P \leq 0.05$). The inflammation level was also investigated by immunohistochemical evaluation of $CD68^+$ expression. Our obtained findings showed that there was similar expression level of $CD68^+$ in Urodex® 6, 3 months (28.23 ± 7.51), (19.62 ± 6.81) and prepuce 3months (23.97 ± 5.13) but the difference between Urodex® three months (28.23 ± 7.51) and prepuce after six months (12.25 ± 2.88) was significant ($P \leq 0.05$). (Figure 4,5 and 6).

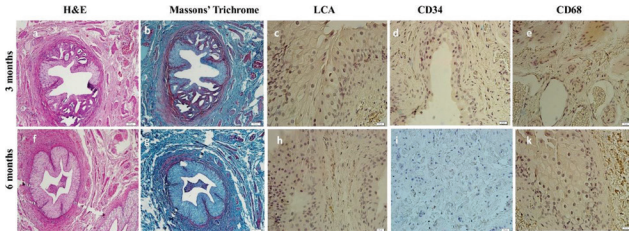

Fig. 4 **Histological and immunohistochemical (IHC) staining of prepuce injected in the seromuscular layer of the bladder in 3 and 6 months. H&E (a, b) and Masson's Trichrome (f, g) staining confirmed the natural histomorphology of bladder. IHC staining of rabbit specimen with anti CD34, LCA, and CD68 in 2 groups being injected by the prepuce. Images were captured with Olympus BX31 microscope.**

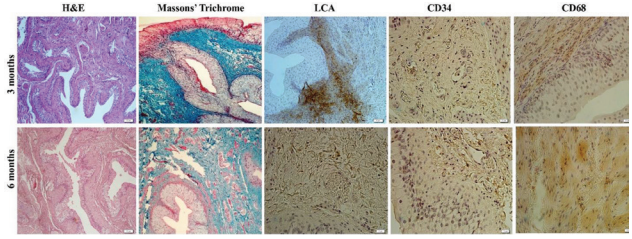

Fig. 5 **Histological and immunohistochemical (IHC) staining of Urodex® injected in the seromuscular layer of the bladder in 3 and 6 months. H&E (a, b) and Masson's Trichrome (f, g) staining confirmed the natural histomorphology of bladder. IHC staining of rabbit specimen with anti CD34 (d, i), LCA (c, h), and CD68 (e, j) in 2 groups being injected by Urodex®. Images were captured with an Olympus BX31 microscope.**

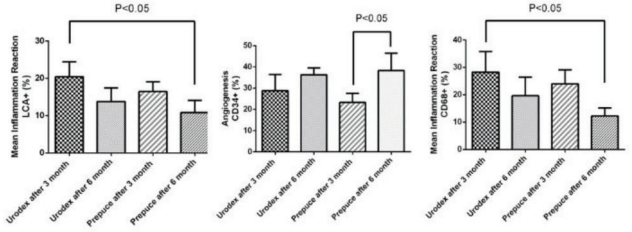

Fig. 6 **Urodex® and prepuce injection in the seromuscular layer in 3 and 6 months follow up. $P \leq 0.05$ statistically considered significant. The data are presented as mean +SD .**

Discussion

Primary VUR predisposes children at risk for renal scarring and recurrent pyelonephritis, and there is an absence of an agreement for ideal monitoring of early VUR diagnosis.[23] The endoscopic injection technique has been introduced as first-line therapy for whole grades of VUR by Matouschek; since then, it has been turned into the main substitute to open operations.[24] Endoscopic repair with rare complications suggests several advantages such as minimally invasive procedure, and also a short hospital stay with minimal postoperative pain.[25] So far, several bulking agents have been examined for the correction of VUR and in some cases combined with tissue engineering. In 1981 Teflon particles were administrated for the treatment of VUR, but because of its synthetic properties such as mobilization to the distant organs or granule formation, the scientists decided to find an appropriate alternative for this method.[26] Several agents such as fat, carbon-coated beads, collagen or silicon with various success rates or side effects have been applied.[27] For the first time in 1995, the copolymer of dextranomer-hyaluronic acid has been applied, and due to its unique characteristics such as biocompatibility, biodegradability, mechanical and non-immunogenic properties it was considered as a promising option for the correction of VUR.[28] In addition, tissue engineering played a major role in urology aims to regenerate injured tissues by uniting cells from the body with the injected native or synthetic scaffolds. The ultimate goal of tissue engineering was developing these bulking agents to mimic anatomical and functional features of native tissue. So the natural scaffold attracts large attention toward itself. Recently, commercialized product provided from decellularized dermal matrix of porcine called Permacol™ has been used for heterologous urethra augmentation for this purpose. Harvested collagen and elastin from acellular scaffold were used to increase the strengths of marix but it was limited in therapeutic potential in the long term follow up with 41.6% successful rate.[29,30] Although, for a long time, acellular dermal matrix was applied for cosmetic surgeries such as breast[31] and abdomen[32] procedures, nowadays, there is a growing tendency to apply these matrices for reconstructive surgeries. In the current study cell-free dermal biological scaffold has been used as a natural construct to restore and conserve normal function of damaged or unhealthy tissues. In the current study, we used tissue-engineered prepuce as a novel cell-free collagen-based scaffold as a urethral bulking agent in a rabbit model. The originality of our technique is that the lyophilized and micronized acellular scaffold of prepuce has been used as a biologic scaffold. Microscopic examination of specimens of the acellular prepuce compared with normal tissue demonstrated the successful acellularization process of the prepuce with designed protocol while maintaining the structure of ECM component and collagen fibers. DAPI staining also presented nucleus elimination on the acellular tissue. In addition, SEM studies revealed that the structure and morphologic features of acellular prepuce tissue had been preserved. No serious complications were observed during surgery, including bleeding, infection, perforation following injection of the micronized prepuce, and Urodex® in rabbit's bladder. Moreover, after injection of both micronized prepuce and Urodex®, no allergic reactions such as redness or swelling were observed in any of the following groups. In addition, there was no significant effect of necrosis at the injection site in the study groups. Results of pretreatment with lyophilized and micronized particles in comparison with Urodex® in rabbit animal model during short and long-term follow up presented no significant inflammation induced by innate immune cell infiltration, which has been proved by IHC staining. 6 months after injection, inflammation in the prepuce injected group. Testing disclosed enhanced augmentation and the scaffold could then be efficaciously implanted after injection. This study, in the long term established exceptional biocompatibility and gradual regeneration with time with no migration to other organs challenging the current bulking agents such as Deflux® which is golden standard for VUR treatment to perform further investigation to compare their curative efficiency. Finally, the main impact is that we describe an off-the-shelf and cost-effective autologous product with an equivalent surgical consequence to the cellular grafts. To the best of our knowledge, this is the first study of prepuce decellularized matrix in combination with HA as a bulking agent for the treatment of VUR.

Conclusion

Although this study focused on the effect of short and long term bulking agents, further investigation is essentials to figure out the main complications in the long term. The findings of current study suggested that injectable compositions comprising biocompatible particles can be applied as a natural bulking agent for the treatment of urinary incontinence or urinary reflux disease.

Acknowledgments

This project was conducted as a PhD thesis with grant number 9211598021. We appreciate Dr. Amirzargar and Dr. Hekmati for proof reading of manuscript.

Financial Support and Sponsorship

None.

Conflicts of Interest

There are no conflicts of interest.■

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