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UROLOGY - ORIGINAL PAPER

The application of tissue-engineered preputial matrix and fibrin sealant for urethral reconstruction in rabbit model

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Abstract
Background To introduce the role of fibrin sealant and preputial acellular matrix (PAM) as a new source of inert collagen matrix for urethral reconstruction.
Methods A ventral urethral segmental defect was created in 24 male rabbits divided into four groups. In group 1 (G1), urethrotomy was closed in layers. In group 2 (G2), closure was followed by applying fibrin sealant. In groups 3 (G3) and 4 (G4), urethroplasty was performed with a patch graft of PAM, and in G4, fibrin sealant was also applied. Serial urethrography was performed before and after the operation. Then, the animals were euthanized, and their urethra was excised 1, 3, and 9 months postoperatively for further electron microscopic examination, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) technique, and immunohistochemical (IHC) staining with CD34, CD31, desmin, SMA, and α-actin.
Results In G1 and G2, the fistula repair failed in all the time points. In G3 and G4, serial urethrography confirmed the maintenance of a wide urethral caliber without signs of strictures or extravasations. Satisfactory vascularity was observed in G3 and G4 during the whole study, which was more significant in G4 after 9 months of follow-up. The presence of a complete transitional cell layer was confirmed over the graft in G3 and G4 in all time points. IHC staining confirmed the effectiveness of fistula repair in G3 and G4, 3 months postoperatively.
Conclusion This rabbit model showed that PAM combined with fibrin sealant may herald a reliable option for repairing segmental urethral defects.

Keywords Acellular · Fibrin sealant · Fistula · Foreskin · Urethra

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Introduction

Fistula formation or urethral loss after failed hypospadias surgery is continuing challenge in the field of hypospadiology. Several creative techniques have been innovated to prevent and treat these complications. Nevertheless, urethrocutaneous fistula still remains as the most significant complication of hypospadias repair [1]. The application of additional tissue grafts such as buccal mucosa, small intestinal submucosa, amniotic membrane, recently autologous grafts of urothelial cells, and acellular collagen scaffolds can be mentioned as the most sophisticated tissue engineering techniques [2–4]. Acellular matrix derived from bladder mucosa, as an off-the-shelf product, has been used for urethral reconstruction and theoretically seems to

avert problems of a xenograft tissue like SIS or intricacies and autologous tissue culture expense [5]. Although various grafts and methods have been applied to overcome these complications, an acceptable method and reliable grafts have not been identified yet [6]. Commercial fibrin sealant is applied as a hemostatic agent, a urinary tract sealant, and a tissue adhesive in urologic surgeries, and these properties make it effective for better wound healing and managing a variety of genitourinary tract defects [7]. There are also several reports of using commercial fibrin glue and recently single-donor product in hypospadias repair and urethral reconstruction as means to reduce fistula formation [8–10].

In the current study, we tried to compare the techniques of simple multilayer closure with and without fibrin glue and the application of preputial acellular matrix (PAM) with and without fibrin glue for segmental urethral reconstruction in an animal model.

Materials and methods

Acellular prepuce scaffold preparation

Prepuce tissues were prepared from circumcision in 12 children (an informed consent was obtained from parents before the procedure) under the sterile condition and washed with normal saline and gentamicin at 4 °C for 6 h in order to prevent any infection. The outer-layer fat was removed mechanically by microdissection. The tissues were rinsed with phosphate buffer saline (PBS) in 37 °C. Then, cellular components of prepuces were removed by using 5 % sodium dodecyl sulfate (SDS) at 4 °C for 6 h, using a rotator shaker. In the next step, the decellularized prepuces were washed with distilled water for 1 h. Afterward, trypsin (0.05 %)/ethylenediaminetetraacetic acid (EDTA) (0.01 %) was added and incubated at 4 °C for 4 h. Tissue fragments were thoroughly rinsed with Hank's balanced salt solution (HBSS), digested with 1 % Triton X-100 (Biosharp, USA) for 1 h, and then washed in HBSS at 4 °C for 48 h. Acellular tissues were washed and placed in PBS containing cocktail of antibiotics (penicillin and streptomycin) and amphotricin at 4 °C for further implantation. Acellular prepuces were irradiated by ultraviolet light for 24 h, preoperatively.

Characterization of scaffold

In order to evaluate the efficacy of our acellularization procedure, acellular prepuce samples were fixed in 10 % phosphate-buffered formalin for 24 h at room temperature. Then, the samples were paraffin embedded and sectioned to 5 μm thickness. Hematoxylin and eosin (H&E) and Masson's trichrome stainings were applied to visualize extracellular matrices and fibers. The dry–freeze tissues were also prepared to evaluate the orientation of fibers and pore sizes and assure the maintenance of extra cellular matrix (ECM) structure on scanning electron microscopy (SEM). For this purpose, PAMs were placed in 2.5 % solution of glutaraldehyde for 1.5 h at 4 °C. Then, they were placed in PBS for 90 min at 4 °C, and the solution was changed every 30 min. Subsequently, they were placed in graded ethanol–water series for 9 h. A Gatan ion beam coater was applied to coat the dehydrated specimens with Au.

Fibrin sealant preparation

The fibrin sealant was prepared from single-donor products in Iranian Blood Transfusion Organization. Protamine (Sigma Chemical Co., St. Louis, MO) was added to the cryoprecipitate to precipitate the fibrinogen content followed by centrifugation (3,000*g*, 10 min). Mean final fibrinogen concentration was 73 ± 8 mg/mL. For the thrombin part, 10 mL plasma and 4 mL reagent (calcium chloride and ethanol) were added and mixed in a glass tube and incubated for 30 min. Finally, the supernatant was used as recovered human thrombin with 59.6 ± 0.6 NIH activity. All the process was done in a standard clean room, and the products were delivered to operation room in two separated sealed tubes.

Surgical technique

Twenty-four New Zealand white male rabbits weighing approximately 2.5 kg were selected and divided randomly into four groups (6 rabbits in each group). The Animal Ethics Committee of the Tehran University of Medical Sciences, School of Medicine and Education Section of Basic Sciences, approved the experimental protocol. Rabbits did not have access to food and water for 10–12 h prior to operation. General anesthesia was applied with intramuscular injection of ketamine (25–30 mg/kg), xylazine (5 mg/kg), and acepromazine (0.80 mg/kg). After exposing the penile, a ventral segment measuring 5 × 5 mm (approximately one half of the urethral circumference) was excised in all rabbits. Then, the animals were divided into four groups. The animals in G1 underwent simple urethroplasty. Two additional layers of subcutaneous tissue and skin were reapproximated over the urethral defect. No fibrin glue or graft was applied in this group. G2 underwent urethroplasty in which the urethral defect was closed primarily with a single simple suture using 7-0 PDS in the middle of the incision to approximate two borders. Then, fibrin sealant was directly injected through a dual lumen syringe over the suture lines, beneath the skin. Two

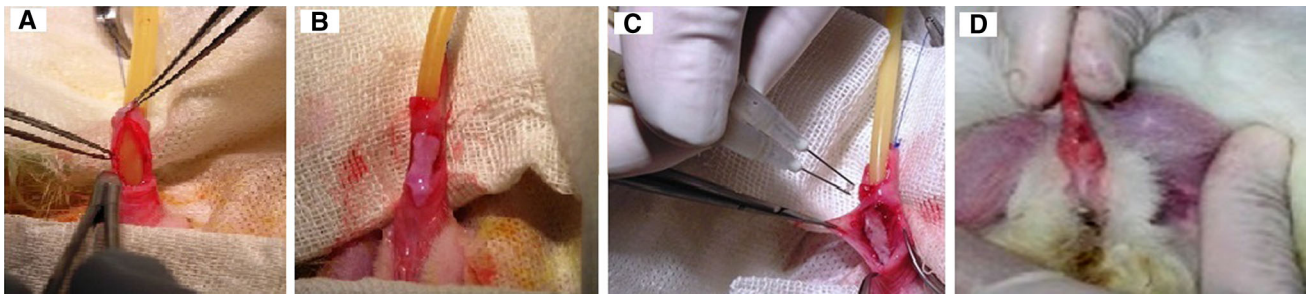

Fig. 1 Surgical procedure: **a** creation of a ventral urethral segmental defect; **b** reconstruction of urethra by tissue-engineered prepuce scaffold; **c** application of fibrin glue; **d** fistula repair in a rabbit of G4, 9 months postoperatively

additional layers of subcutaneous tissue and skin were reapproximated over the reconstructed urethra. In G3, the PAM graft was defatted and anastomosed to the urethral defect. The epithelial side served as the lumen surface, using a continuous running 7-0 PDS suture in onlay fashion. Animals in G4 underwent the same procedure as G3, and fibrin sealant was directly injected through a dual lumen syringe over the suture lines. The proximal, distal, and lateral margins of the graft were marked with a permanent suture (prolene), and the distance between the sutures was measured for future reference. In all groups (G1–G4), two additional pedicled dartos layers of subcutaneous tissue were used to cover the graft, and the skin was reapproximated with 7-0 PDS suture. The urethra was catheterized using a 6-Fr silicon urethral catheter in the distal end of the penis. In order to allow the animals to urinate spontaneously, the catheters were removed 7 days postoperatively (Fig. 1).

All rabbits were euthanized with an overdose of pentobarbital for urethral removal at 1 ($n = 8$), 3 ($n = 8$), and 9 ($n = 8$) months after reconstruction (2 rabbits from each group in each follow-up). The whole urethra and corpora were circumscribed with dissection and removed en bloc for staining and immunohistochemical (IHC) analysis. The permanent sutures (prolene) were referenced to isolate the graft margins.

Histology and immunohistochemistry

Biopsies from each group were obtained after 1, 3, and 9 months of operation for histopathological and IHC evaluation. Smooth muscle fibers were labeled with monoclonal alpha-smooth muscle actin antibodies (Novocastra, Newcastle, UK). The neovascularization of the acellular graft was investigated using anti-CD31 for staining of microvessels and anti-CD34 for the identification of angioblasts and progenitor hematopoietic stem cells. Immunolabeling was performed using the avidin–biotin detection system. Sections were counterstained with hematoxylin.

To study apoptosis, apoptotic nuclei were identified with in situ terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) technique using in situ cell death detection kit. For each sample, five photomicrographs (100×) were used for scoring, and the means of the obtained scores were used as final values for analysis.

Retrograde urethrogram

Serial urethrography was performed before the operation and at 1, 3, and 9 months, postoperatively. All animals underwent a retrograde urethrogram prior to killing. A 8-Fr urethral catheter was secured in the distal urethral meatus after sedation with pentobarbital. Renografin-76 (10–15 mL) was injected, and real-time fluoroscopy was used to obtain static images to document urethral shape.

Results

Hematoxylin and eosin and Masson trichrome staining of PAM showed an acellular collagen-based matrix with fibers orientation similar to the natural preputial tissue. No cellular or nuclear remnants were preserved in scaffolds, while ECM was satisfactorily preserved (Fig. 2a–d). Under a scanning electron microscope, the matrix fibers of the acellular prepuces appeared in a network with no cell fragment in the interstices (Fig. 2e). Furthermore, cells were better seeded in G4 as compared to G3 after 3 months of follow-up (Fig. 2f, g).

All rabbits survived the whole period of the study with an uneventful postoperative course without any manifestation of urinary retention or stone formation. In G1 and G2, the fistula repair failed in all the time points. Histopathological evaluations with H&E and trichrome staining demonstrated no regeneration in G1 and G2 after 3 months (Fig. 2h, i). Serial pre- and postoperative urethrography confirmed the preservation of a wide urethral caliber with no signs of strictures, dilation, or extravasations in G3 and

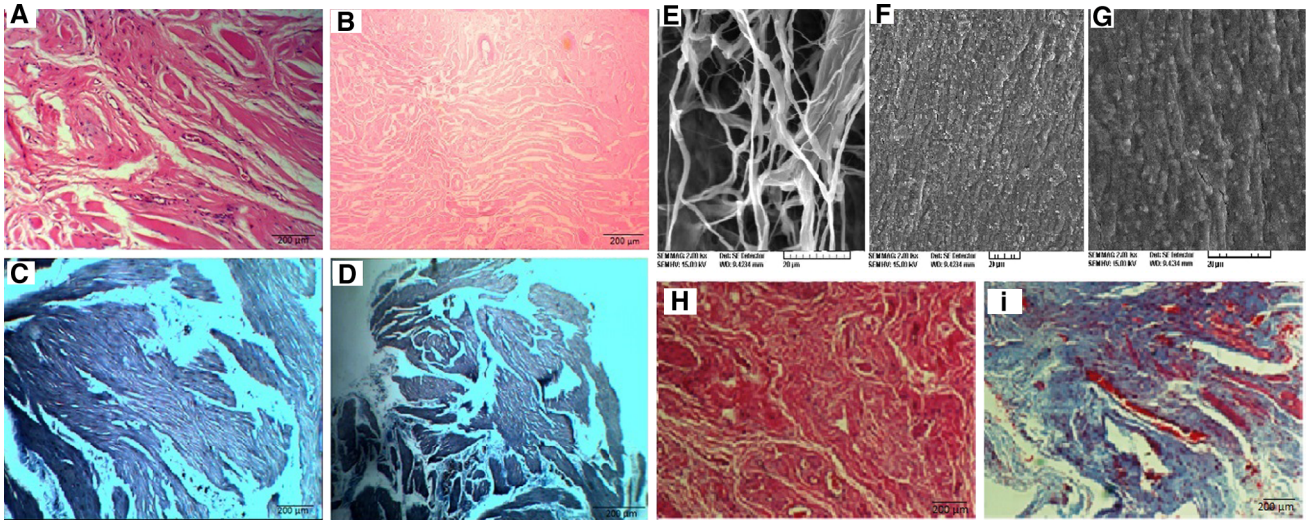

Fig. 2 **a** Hematoxylin and eosin (H & E) staining of natural preputial tissue; **b** acellular preputial matrix. Trichrome staining of **c** natural **d** decellularized preputial tissue demonstrated the presence of ECM and absence of cellular or nuclear remnants in all the scaffolds. Scanning electron microscopy: **e** well-organized porous cytoskeleton and extracellular matrix in acellular preputial scaffold; **f** grafted tissue in G3 after 3 months of follow-up; **g** grafted tissue in G4 after 3 months of follow-up. **h** H&E examination and **i** trichrome staining: No regeneration occurred in G1 and G2 after 3 months. Fibrosis is evident at the edges of the failed fistula tissues

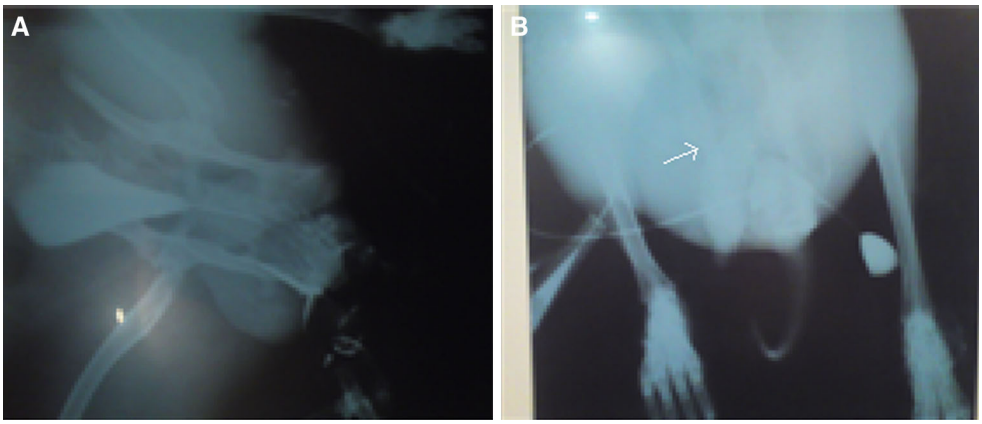

Fig. 3 Retrograde urethrography: **a** urethral repair in G3 and G4 (normally formed tract of urinary stream is shown, and retrograde urethrography showed no discernible difference in the matrix implant and host); **b** urethral repair failure in G1 and G2 (extravasations in the proximal part of urethra)

G4 (Fig. 3). Gross examination at all the retrieval periods showed normally appearing urethral tissue without any evidence of significant fibrosis or scarring in G3 and G4. The junction between the acellular graft and the normal urethral tissue was discernible after one month in G4. At retrieval, the distances between the marking sutures placed at the anastomotic margins remained stable, with no distance varying more than 10 % in any axis, which indicated the maintenance of the initial implant diameter.

The grafts contained host cell infiltration and generous angiogenesis 1 month after surgery.

Satisfactory vascularity was observed during the entire duration of the study in G3 and G4. However, better vascularity was observed in G4, 9 months, postoperatively (Fig. 4a, b).

The presence of a complete transitional cell layer was confirmed over the grafts 1 month after the repair, which was consistent throughout the study in graft groups. Macroscopic examination of the urethra confirmed that the grafted matrices were well incorporated into normal urethra wall and could hardly be discerned by its gross appearance in graft groups, while the incorporation was more prominent in G4.

At three-month follow-up, macroscopic examination of the urethra confirmed the absence of ulceration, fistulae, strictures, diverticula, or calcification. H&E, α-actin

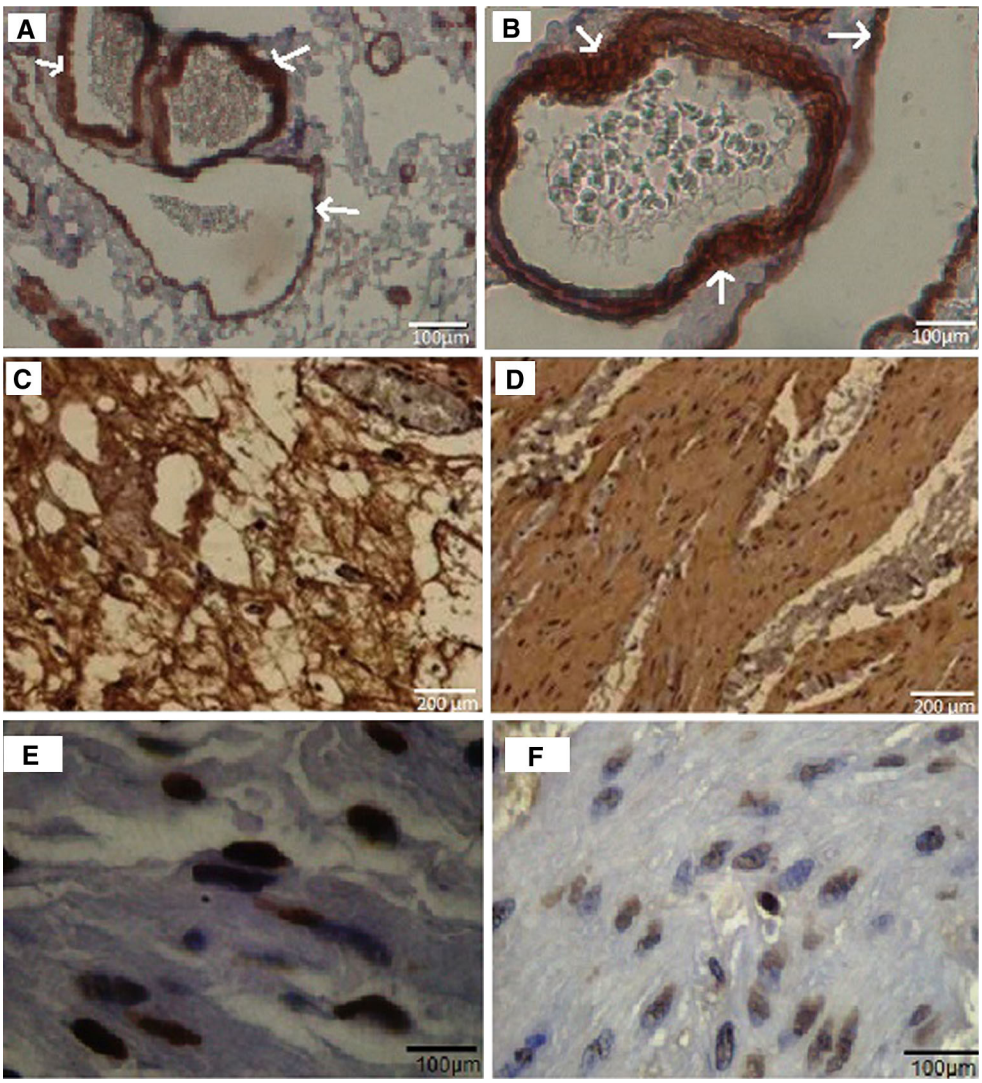

Fig. 4 Vascularization study: **a** CD34+ hematopoietic progenitor cells *arrows* in G4 after 3 months; **b** CD31+ microvessels were found in the regenerating grafted prepuce *arrows* in G4, 9 months after grafting. α-Actin staining in **c** G3 and **d** G4, 9 months postoperatively. Apoptotic nuclei in tissue sections in **e** G2 and **f** G4, 3 months postoperatively

Table 1 Immunohistochemical quantification of G3 and G4, 3 and 9 months after grafting

Markers	Group 3		Group 4		*p* value
	3 months	9 months	3 months	9 months	
SMC	19.5	74.25	48.75	80.25	0.01
Desmin	27.45	84	61.25	93.45	0.03
α-Actin	15.5	87.75	70.45	99.25	0.007
CD 31	21.5	89.5	54.5	98.5	0.03
CD 34	37.25	86.25	62.5	97.75	0.01

Data are means (percent of normal urethral tissue). All markers revealed significant enhancement in biopsies obtained in G4, 3 months postoperatively. $p < 0.05$ is considered statistically significant

positive muscular layer with positive desmin, and SMA staining confirmed the effective fistula repair with inducing normal tissue growth 3 months after surgery in G3 and G4, which was more significant after 9 months of follow-up (Fig. 4c, d). The apoptosis indices were higher at the site of fistula in G1 and G2, 3 months postoperatively (6.14 ± 0.73 and 7.11 ± 0.82) in comparison with those of G3 and G4 (4.58 ± 0.44 and 3.74 ± 0.36; $p < 0.05$; Fig. 4e, f).

However, the number of α-actin positive cells, which demonstrated organized muscle fiber bundles, was significantly higher in G4 after 9 months ($p < 0.05$). Nevertheless, a significant difference was observed in enhancement

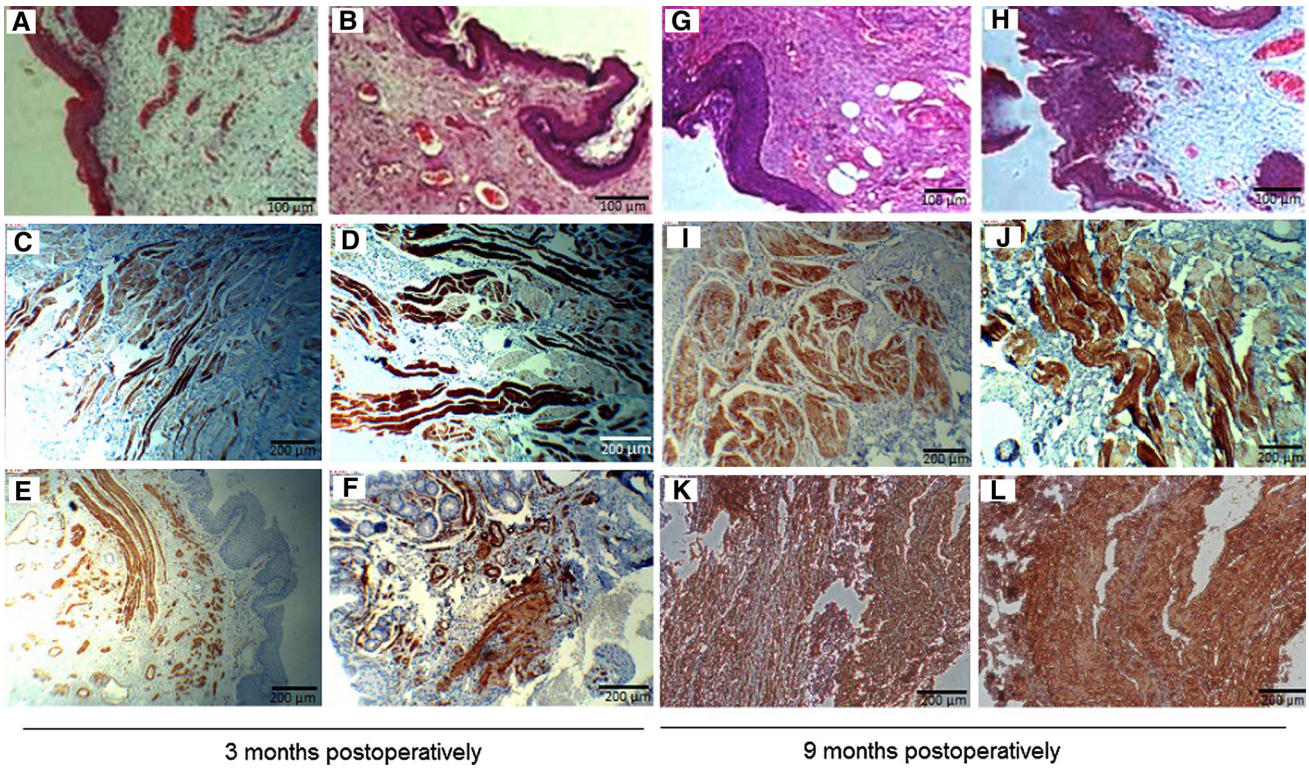

Fig. 5 Histopathological evaluations in G3 and G4, 3 months postoperatively: **a**, **b** H&E staining of the biopsies demonstrated normal tissue appearance at the site of fistula; **c**, **d** immunostaining for desmin; **e**, **f.** SMA staining. Histopathological evaluations in G3 and G4, 9 months postoperatively: **g**, **h** H&E staining of the biopsies demonstrated thick urothelial and muscle layer; **i**, **j** immunostaining for desmin; **k**, **l** SMA staining

of IHC staining between G3 and G4, 3 months after the operation (Table 1; Fig. 5).

Discussion

The outcomes of the current study authenticated the effectiveness of PAM and fibrin glue injection as a promising approach for treating patients with urethral defects. Adjacent tissue grafts, local tissue flaps, or grafts without blood supply from genital or extragenital tissues are usually used in techniques of hypospadias repair [2, 11]. Pedicle skin flaps are still method of choice for severe hypospadias repair [12]. However, when local skin is deficient, grafts without blood supply are needed. Grafts usually experience neovascularization after excision and transfer to the host tissue. The nature of the grafted tissue and quality of the host bed determine the success of this process [13]. Split-thickness grafts, full thickness skin grafts, bladder epithelial graft, and buccal mucosal graft are four types of grafts commonly used in the urethral reconstruction. Although full thickness grafts are more durable, they are not usually acceptable due to the fastidious vascular support in comparison with the split-thickness grafts. Bladder epithelial grafts are composed of superficial and deep plexus with favorable vascular characteristics. However, oral mucosal grafts with a para laminar plexus have recently gained promising outcomes in the field of urethroplasty. These grafts are less likely associated with the complications of the above-mentioned grafts, including strictures, hair growth, and graft contracture. The buccal grafts can be thinned enough without any disturbance of graft vascular characteristics. Pain and oral paraesthesias have been reported as common complications of buccal mucosa grafts [14].

Despite dramatic developments in methods of hypospadias surgery, finding an ideal urethroplastic donor tissue is still challenging. The effectiveness of tissue-engineered materials and tissues brings new hopes for the hypospadias treatment [15, 16]. The availability of acellular tissues was first mentioned by the application of small intestinal submucosa (SIS) and bladder acellular matrix in bladder augmentation [17, 18]. The preliminary results were encouraging with complete luminal epithelialization, considerable angiogenesis, and acceptable smooth muscle ingrowth with no signs of stricture [16]. De Filippo et al. [19] showed that tissue engineering techniques may be used effectively, when extensive urethral reconstruction is

mandatory. They showed that tubularized unseeded acellular bladder resulted in poor tissue formation and leads to strictures, whereas tubularzied urothelium seeded with collagen matrixes showed no signs of obstruction in their animal model. In present series, no sign of stenosis was observed in G3 that could be attributed to more structural and functional similarity of acellular prepuce to the host tissue that had covered the area before surgery. Fossum et al. [20] introduced the tissue culture technique into the realm of hypospadiology. They have noted that in vitro-cultured urothelial cells could be used for the treatment of a selected group of hypospadias patients when there is shortage of preputial and penile skin. The efficiency of this technique is very arguable in view of the 50 % complication rate in the small group of patients treated with cultured urothelial cells. Fibrin sealant is prevailing in different fields of surgery (urology and especially urethral surgery) where sealing of suture line is of utmost importance [21, 22]. In one study in 2004, 18 patients underwent pendulous urethral reconstruction and fibrin sealant was injected along suture line after urethral anastomosis. In 25 other cases, this procedure was applied without the application of fibrin glue. The results demonstrated that in the fibrin sealant group, catheter was removed earlier and penile edema was decreased with enhanced wound healing [22]. Fibrin sealant can be applied in two major areas as a topical agent for hemostasis and as an adhesive for tissue approximation. The authors believe that fibrin sealants may actually work in a double manner for the prevention of urethrocutaneous fistulas.

In this study, we have tried to incorporate the two novel techniques of acellular matrix and fibrin glue for hypospadias repair. Fibrin glue is supposed to enhance the integration of acellular matrix into the host tissue due to its effect in promoting the healing process. Preputium may have potential advantages over previous sources of acellular matrix as it is easy to obtain, and there is no need neither for cadaveric postmortem dissection nor opening the bladder of the patients. It has also a potential of autologous or related donor transplant. Although conservation of the foreskin is being increasingly practiced, it is still the case that the majority of boys have their foreskin removed to create the appearances of a circumcised penis at the time of the primary hypospadias operation. No donor site morbidity also happened following the application of TEP matrix. In this study, the best results were obtained in G3 and G4. However, better incorporation of graft to the host tissue and better tissue regrowth were observed in concomitant fistula repair with PAM and fibrin sealant. This may herald a more important role for fibrin sealants in the territory of hypospadias repair with tissue-engineered scaffolds. These results may strengthen the concept that minute and potentially temporary extravasations in immediate postoperative period may give way to a permanent defect in hypospadias repair. However, despite any effort to provide a sealed water proof, suture line cannot be overemphasized as all fistula repairs failed in G2 when fibrin sealant was applied over suture lines without any preputial graft. Fibrin glue may also have a role in promoting angiogenesis, as markers of vascularity were prominently expressed in G4, and TUNEL-assayed apoptosis indices were also lower in G4 compared with G3 that did not reach to a significant level.

The development of epithelium and even more interestingly smooth muscle in G3 and G4 demonstrated a successful tissue rebuild with minimal to no fibrosis, which is theoretically protective against subsequent delayed stricture formation. Proliferation of smooth muscle showed a corpus spongiosum-like structure, which is conceptually against spongiofibrosis that is considered as a main factor of restenosis after urethral reconstruction.

The outcomes of the current study authenticated that this combination may be safe, effective, and with favorable results without any need for cell isolation, culture, and seeding. Based on biophysical properties and microscopic arrangement of urethral fibers and pores and their similarities to the host tissue, we hypothesized that preputial matrices might be an appropriate biomaterial for fistula repair. To the best of our knowledge, this is the first study in which preputial tissue was used for urethral reconstruction with concomitant use of fibrin glue. These results showed that PAM is a unique tissue to support three-dimensional growth for penetration and growth of urethral cells. In spite of significant IHC enhancement in G4 in short-term follow-up, it has not been proven that these IHC differences translate into a better clinical result because of the fact that G3 and G4 performed equally in terms of successful urethral repair in long-term follow-up. It should be also mentioned that the size of grafts used in this experimental model was small. So, we cannot state that replacement by native host tissue would occur rapidly in the size of graft required for clinical purposes which is the limitation of the current study. Eventually, this study stressed the usage of fibrin glue as an uprising important step in hypospadias repair and offered PAM as safe and effective additional tissue in the field of hypospadiology, which can be applied for the phase of human studies thereafter.

Conclusion

This rabbit model demonstrated that the use of PAM combined with fibrin sealant may indicate a novel option for repairing segmental urethral defects. Prepuce may also provide an ideal tissue source of inert collagen matrix for

complicated cases of urethral reconstruction and hypospadias repair with minimal adverse reaction and excellent recapitulation of host tissue. Fibrin glue may promote healing and ensure higher success and less fistula rate. However, more investigations are required in human phase to demonstrate its ultimate indication in clinical background.

Conflict of interest No conflict of interest exists in relation to the submitted manuscript, and there was no source of extra-institutional commercial funding or funding received from National Institutes of Health (NIH), Wellcome Trust, Howard Hughes Medical Institute (HHMI), and others.

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