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Experimental defect in rabbit urethra repaired with acellular aortic matrix

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Abstract Urethral reconstruction following failed hypospadias repair or post-traumatic chronic stricture requires adequate amounts of tissue. Many surgical techniques utilizing different types of biological tissues have been attempted: (a) vascularized skin flaps from the prepuce, scrotum or penile shaft; (b) full-thickness free skin grafts; (c) vesical or buccal mucosa grafts; (d) ureter; artery; vein and appendix tissue. More recently, biodegradable polymers have also been used as delivery vehicles of urothelial cells in animals. It has been demonstrated that the implant of an acellular tissue matrix in the bladder can guide the regeneration of urothelium, blood vessels, smooth muscle and nerves. The aim of this study was to create an experimental model of urethral defect, and then repair it by implanting homologous acellular aortic grafts as urethral substitutes. An acellular matrix was obtained by detergent enzymatic treatment of rabbit thoracic aorta. The growth of urethral epithelium was verified in vitro, and homologous acellular vessels were then implanted in rabbits, bridging a previous surgical urethral defect. The outcome of reconstructive surgery was evaluated histologically at 10 days, 3 weeks, 3 and 12 months. As the time after surgery increased, the neourothelium became less thick, signs of inflammatory response disappeared, and the orientation of collagen fibrils and smooth muscle fascicles resembled that of a normal urethra. The implants displayed abundant vascularization, and the luminal surface started to become irregular. Acellular blood vessels may represent a promising approach to urethral defect therapy for different reasons: (a) unlimited availability, (b) readily obtainable in different lengths and gauges, (c) the potential for being organized as tissue bank, and (d) that just one simple surgical procedure is needed. Nevertheless, before this technique can be applied in humans, it must be tested in more species and animals.

Key words Hypospadias · Acellular matrix · Uroepithelial cells · Rabbits

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Introduction

Urethral reconstruction following failed hypospadias repair or traumatic chronic stricture requires adequate amounts of tissue [4]. While many workers may consider their ideas original, a study of early papers reveals that almost all present-day techniques are based on notions developed by enterprising surgeons of the last century [4]. The principles of reconstruction have involved the introduction of vascularized skin flaps from the prepuce, penile shaft, or scrotum, and for long urethral defects, full-thickness free skin grafts, bladder or buccal mucosa grafts. Over 300 repair techniques for hypospadias are described in the literature, and many different tissues have been used as substitutes; ureter [20], artery [13] vein [23], appendix [18], skin [11], bladder mucosa [5, 14], buccal mucosa [6, 10], and tunica vaginalis [8]. Recently, biodegradable polymers were also used as delivery vehicles for urothelial cell populations in vivo in animals in order to study the possibility of reconstructing the urethra, and keratinocytes derived from the urethral meatus have been involved in urethral grafts for hypospadias repair in humans [1, 7, 9, 16, 17]. However, despite the technical improvements and newer procedures, urethral cripples are still seen. In these patients, the penis is scarred and relatively avascular; local flaps are often no longer available, and fistulas, strictures or diverticulas may be present with or without chronic infection [7, 17].

It was recently demonstrated that the implant of an acellular tissue matrix in the bladder can guide the regeneration of urothelium, blood vessels, smooth muscle

and nerves [21]. Takami et al. [22] reported that acellular dermal matrix may be useful in full thickness defects by providing a vascularized bed for subsequent epidermal coverage.

The aim of this study was to create an experimental model of urethral defect and to repair it using homologous acellular aortic grafts implanted as urethral substitutes. As a first step, acellular matrix was obtained from rabbit thoracic aorta and the growth of urethral epithelium on it was verified in vitro. In the second step, acellular aortic matrices without seeded urothelial cells were implanted in rabbits, and the outcome of reconstructive surgery was evaluated by histological analysis at various intervals of time, up to 1 year post-intervention.

Materials and methods

Preparation of acellular matrix

Thoracic aorta, obtained from male New Zealand White rabbits, were rinsed four times for 15 min in phosphate buffered saline (PBS) containing antibiotics and amphotericin B, and then treated according to Meezan et al. [12] to obtain an acellular matrix. Vessels were processed with distilled water for 18 h at 4°C, 4% sodium deoxycholate (Sigma Chemical Co, St. Louis, MO, USA) for 4 h and 2000 K units deoxyribonuclease I (DNase I) (Sigma) for 2 h. The absence of cellular elements was confirmed histologically and acellular matrices were stored in PBS at 4°C until use.

Fig. 1 **a** The pendulous urethra with the experimental defect and **b** a schematic diagram

Fig. 2 **a** The aortic graft implanted in the urethra defect and **b** a schematic diagram

Cell cultures

Rabbit urethral segments were minced, and incubated overnight with 0.25% trypsin (Sigma). Isolated uroepithelial cells were seeded onto a feeder monolayer of 3T3-J_2 cells previously treated with mitomycin C (4 μg/ml) for 2 h. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (3:1) (Sigma), containing 0.5 ng/ml hydrocortisone, 5 μg/ml insulin, 24 μg/ml adenine, 6 ng/ml cholera toxin, 10 μg/ml transferrin, 10 μg/ml triiodotyronin and 10 ng/ml epidermal growth factor (Sigma) supplemented with 10% Fetal Clone II (HyClone Laboratories, Inc., Logan, Utah, USA), antibiotics and amphoteric B, according to Rheinwald and Green [15]. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

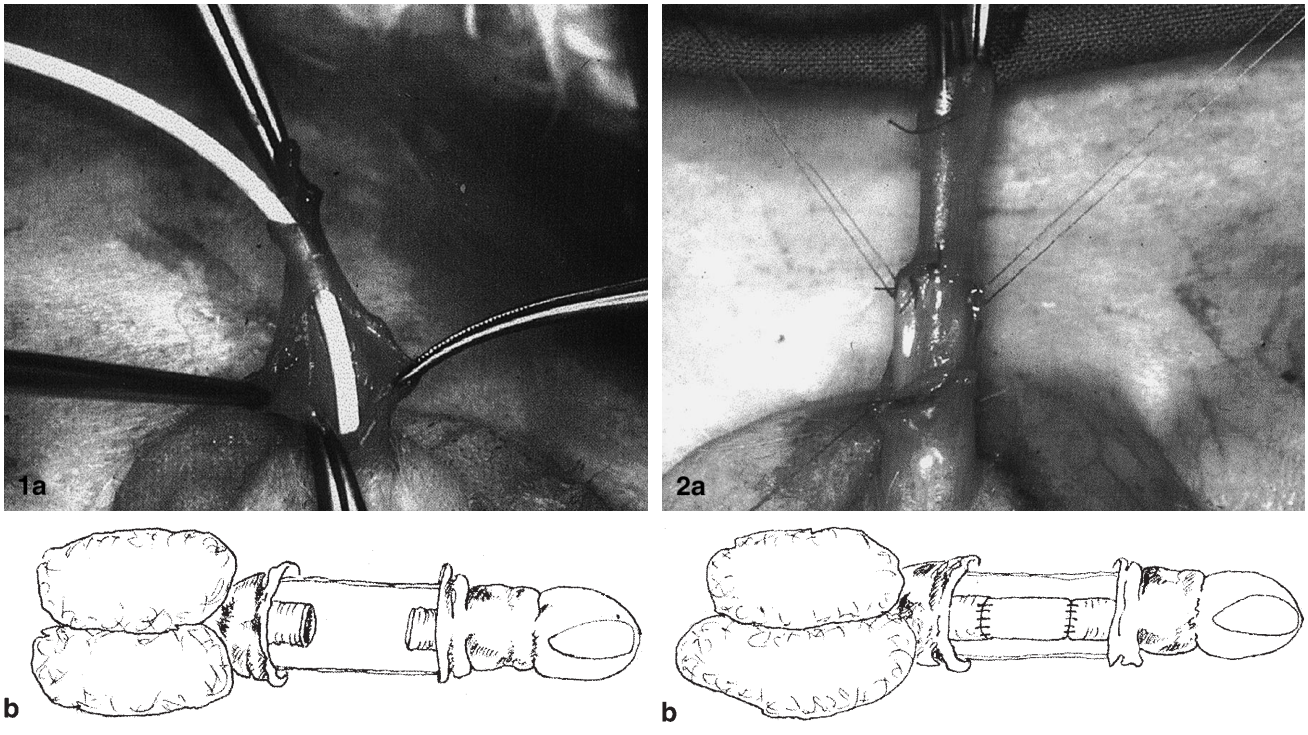
Subconfluent primary cultures were detached by trypsinization, and the cells were seeded on the intimal side of acellular, longitudinally opened vessels, using stainless steel rings that were removed after 24 h. Cells were cultured for 5 days in the above-described medium.

Immunohistochemistry

Cultures grown on acellular vessels were fixed in 10% neutral buffered formalin (Carlo Erba Reagenti, Milano, Italy) and processed for histology. Vertical sections, 5 μm thick, were deparaffinized and immunostained with monoclonal cytokeratin 19 antibody (Bio-Optica, Milano, Italy), and an anti-mouse IgG (Fc specific) peroxidase conjugate (Sigma).

Surgery

Following a 12-h fast, 14 adult male New Zealand White rabbits weighing 3–4 kg each were anesthetized by intramuscular (i.m.) injection of a 50% solution of 10 mg/kg ketamine hydrochloride associated with 12 mg/kg xylazine i.m.; anesthesia was successively maintained by the same solution injected into the ear vein. The perineum and genitals were scrubbed with povidone-iodine solution, and draped with sterile towels. The pendulous urethra was exposed, and a 1 cm segment was totally excised (Fig. 1); simple interrupted 6–0 vicryl (Ethicon, Johnson & Johnson, Pomezia, Italy) sutures were used to anastomose the homologous aortic



acellular graft bridging the urethral defect in an end-to-end fashion (Fig. 2); the wound and skin were then closed in layers with 5–0 catgut (Ethicon) sutures. No urethral stents or catheters were used, nor was cystostomy performed. Gentamicin (5 mg/kg) was administered i.m. preoperatively and for 5 days postoperatively; no immunosuppressive treatment was given. On the second post-operative day, the animals could eat and drink ad lib. Two animals died in the immediate post-operative period; both rabbits were not able to wake up and developed cardiac and pulmonary insufficiency. In the long-term post-operative period one fistula was observed but no stenosis or stones were visible.

Histological studies

To evaluate recovery, groups of three animals were sacrificed at different time intervals, i.e., 10 days, 3 weeks, and 3 and 12 months after surgery. The implants were recovered, fixed in 10% neutral buffered formalin, embedded in paraffin, and 5-μm-thick vertical sections were stained with hematoxylin and eosin.

Results

Acellular matrix

Sections of the matrix obtained following detergent-enzymatic treatment of rabbit thoracic aorta are shown in Fig. 3b. Histological examination revealed the absence of cells; moreover, the acellular matrix maintained the structure of an untreated vessel (Fig. 3a).

Cultures of uroepithelial cells on acellular matrix

Figure 4 shows the histological features of urethral epithelium reconstructed in vitro on the acellular matrix. Five days after seeding, a continuous basal layer, made up of cuboidal cells, and in close contact with the substrate, was visible. The stratification of suprabasal differentiated cells was evident. The superficial layer cells were flattened and lengthened. Moreover, immunohistochemistry revealed the presence of keratin 19 (40 kDa). Negative controls were performed on the acellular matrix and no staining was observed (data not shown). We then verified whether the aortic acellular matrix could be colonized by homologous cultured urothelial cells.

Microscopic examination of the implanted acellular aortic matrix

The pendulous urethra (Fig. 5a) presented an irregular border covered by urothelial cells, beneath which connective tissue with numerous smooth muscle fascicles and vessels could be observed. As expected, none of the rabbits showed signs of rejection.

Ten days after surgery (Fig. 5b), the implant was completely covered by urothelium which was thicker than that of normal urethra. Dense collagenous connective tissue without fiber organization was not apparent in the implanted matrix, but fibroblastic cells were observed. Inflammatory cells and signs of neovascolarization were visible. Three weeks (Fig. 5c) and 3 months (Fig. 5d) after surgery, signs of inflammatory response disappeared, and the collagen fibrils showed an early organization. Moreover, an increased vascularization was observed, and epithelial thickness was equal to that of normal urethra. Twelve months after surgery (Fig. 5e), an almost complete rearrangement of the

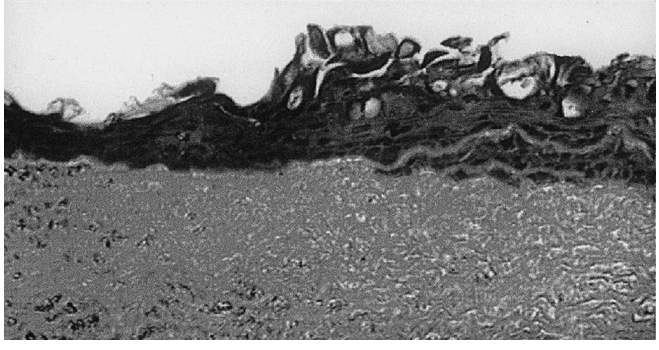


Fig. 4 Immunohistochemistry of urethral epithelium reconstructed in vitro on an acellular matrix. Note the distribution of keratin 19 (magnification ×100)

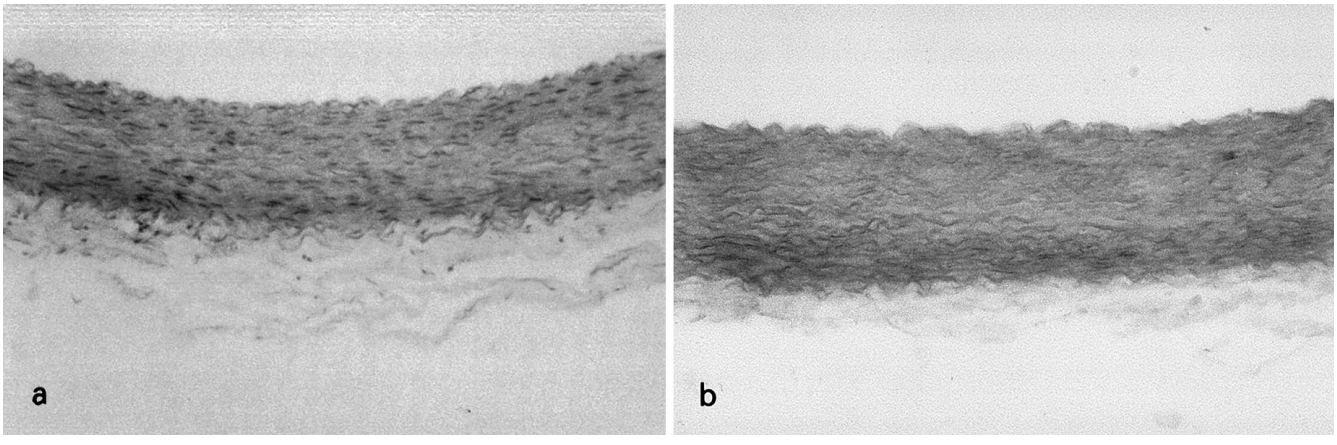


Fig. 3 Transverse sections (5 μm) of rabbit thoracic aorta before (**a**) and after (**b**) treatment. Hematoxylin and eosin (magnification ×160)

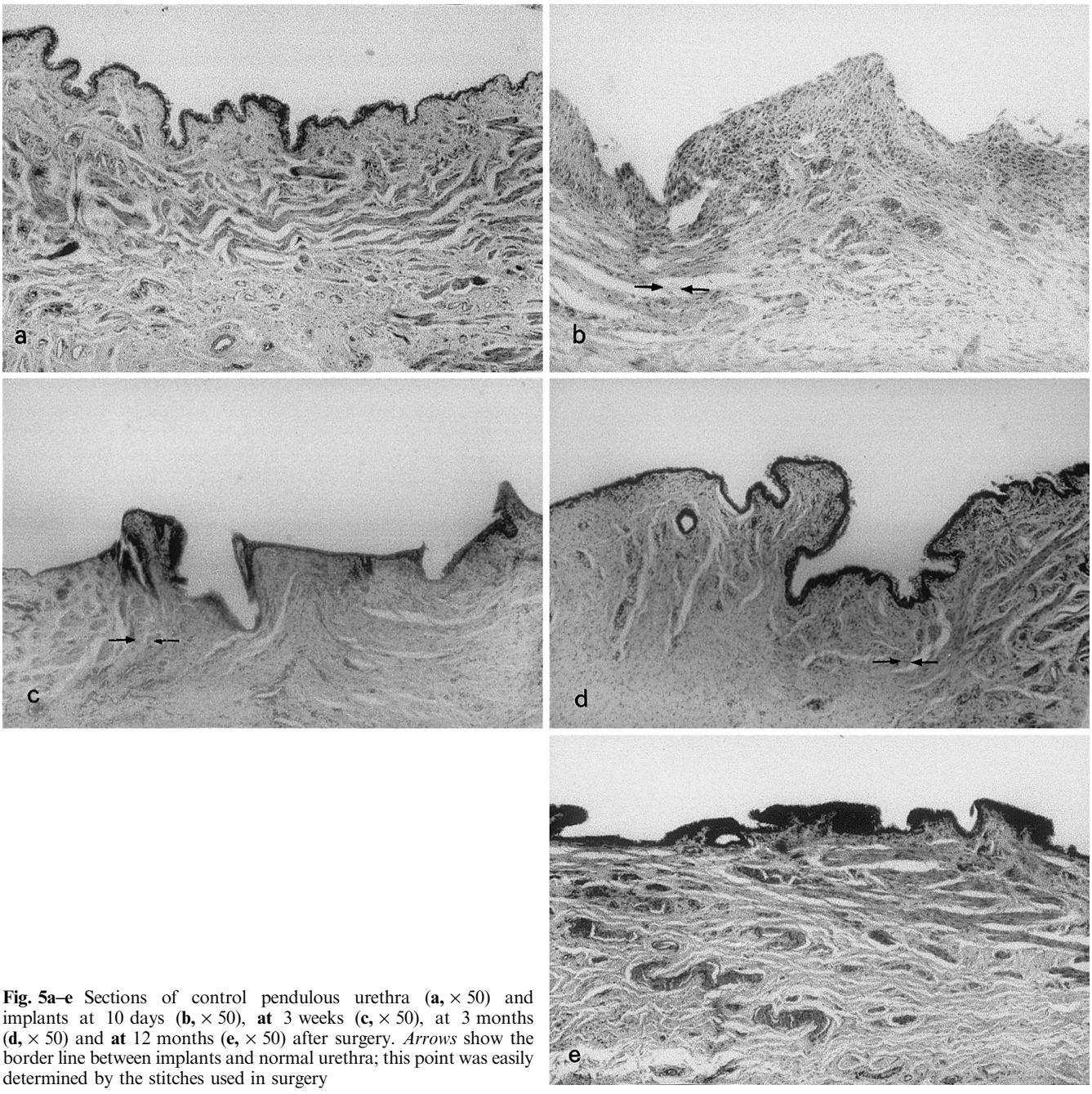


Fig. 5a–e Sections of control pendulous urethra (**a,** × 50) and implants at 10 days (**b,** × 50), **at** 3 weeks (**c,** × 50), at 3 months (**d,** × 50) and **at** 12 months (**e,** × 50) after surgery. *Arrows* show the border line between implants and normal urethra; this point was easily determined by the stitches used in surgery

connective tissue was visible; collagen fibers and smooth muscle fascicles were oriented as observed in normal urethra. The implants presented an abundant vascularization, and their luminal surface started to become irregular.

Discussion

''There is nothing new in surgery not previously described''. This dictum is particularly true in the history of hypospadias. We have certainly advanced from the era in which treatment consisted of amputation beyond the orifice, but almost all present-day techniques are based on ideas developed by enterprising surgeons of the last century [4]. In this study, we employed acellular aortic grafts as urethral substitutes to repair an experimental urethral defect in rabbits.

Like many other workers, we chose the rabbit model for these experiments, because it presents some important advantages: (a) ease of manipulation; (b) ready access to the urethra that is simple to work with; (c) a well-known urethra histology, and (d) not least, it is inexpensive [2, 19]. The choice of thoracic aorta was based on the idea of using a tubular structure of suitable dimensions, already preformed and directly

applicable; this approach has some advantages over others because the surgeon can decide the length of the defect, as well as the size of the urethra to be reconstructed, and with these parameters select the most suitable matrix tube.

To obtain a completely acellular blood vessel, we used the detergent-enzymatic method of Meezan [12] because it is not only simple and quick, but it also maintains the structural characteristics of the matrix in a satisfactory manner; moreover, the basal membrane that plays an important role in epithelial cell attachment is conserved. Furthermore, many investigators have shown that acellular matrices are unable to evoke tissue rejection [21,22]. To verify whether the acellular matrix could provide an adequate surface for urothelial cell attachment and constitute a suitable substrate for cell survival and growth, we seeded primary rabbit urothelial cells on acellular vessels. These cells attached readily to the acellular matrix and after 5 days in vitro formed a continuous epithelium, which expressed a cytokeratin protein associated with rabbit urothelium [1].

Coverage of the matrix with urothelial cells, however, does not seem to be mandatory before the implant, because at 10 days after surgery the implanted area already appeared completely covered by urothelium, formed by epithelial cell migration from the edges of the graft. The matrix was seen to evoke an angiogenic response which promoted cell migration and ingrowth [3]. The grafted area presented a dense structure, probably due to a hyperproduction of fibrillar protein and glycosaminoglycans by fibroblasts. Moreover, the inflammatory response was minimal at 10 days after surgery and disappeared at 3 weeks. After 3 months, the matrix appeared completely integrated in the host's urethra. Indeed after 12 months, the orientation of collagen fibers and muscular fascicles was similar to that present in the host's urethra. Cicatricial retraction and fibrosis of the neo-urethra were minimal in all the animals, and none had any clinical problems. Our results agree with those of Sutherland [21] and Takami [22] who demonstrated that an acellular matrix can guide tissue regeneration in bladder and skin, respectively.

In this study we considered the feasibility of setting up a tissue bank of acellular matrices that could be used when it is impossible to obtain any other tissue. The possibility of always having enough tissue to correct urethral defects, also considered in the experiments of Atala et al. [1] and Italiano et al. [7] utilizing biodegradable polymers, is fascinating but needs further study.

Acellular blood vessels may represent a promising approach to the therapy of urethral defects, because they present numerous advantages: (a) easy and unlimited availability of inexpensive grafts, (b) a choice of different lengths and gauges with already preformed tubular structures, and (c) the opportunity to organize a tissue bank. From a surgical point of view, it is worth stressing that this technique only involves one simple procedure. To date we have not yet used a matrix with implanted autologous urothelium that might further simplify the recovery. However, before utilizing human vascular matrices, it will be necessary to increase the number of species and animals employed in such experiments.

References

1. Atala A, Vacanti JP, Peters CA, Mandell J, Retik AB, Freeman MR (1992) Formation of urothelial structures in vivo from dissociated cells attached to biodegradable polymer scaffolds in vitro. J Urol 148:658
2. Chang KS, Chang JHT (1994) Animal models of pediatric surgical diseases. Pediatr Surg Int 9:307
3. Colville-Nash PR, Willoughby DA (1997) Growth factor in angiogenesis: current interest and therapeutic potential. Mol Med Today 1:14
4. Durham Smith E (1997) The history of hypospadias. Pediatr Surg Int 12:81
5. Fairbanks JL, Sheldon CA, Khoury AE, Gilbert A, Bove KE (1992) Free bladder mucosal graft biology: unique engraftment characteristics in rabbits. J Urol 148:663
6. Humiy GA (1941) A one-stage operation for hypospadias. Br J Urol 29:84
7. Italiano G, Abatangelo Jr G, Calabrò A, Abatangelo Sr G, Zanoni R, O'Regan M, Passerini Glazel G (1997) Reconstructive surgery of the urethra: a pilot study in the rabbit on the use of hyaluronan benzyl ester (Hyaff-11) biodegradable grafts. Urol Res 25:137
8. Khoury AE, Olson ME, McLorie GA, Churchill BM (1989) Urethral replacement with tunica vaginalis: a pilot study. J Urol 142:628
9. Langer R, Vacanti JP (1993) Tissue engineering. Science 260:920
10. Lexer E (1911) On free transplantations. Verh Deutsch Gese Chir 40:386
11. Lorenz C, Maier-Reif K, Back W, Hohl HP, Waag KL (1996) Cultured urothelium in sheep bladder augmentation. Pediatr Surg Int 11:456
12. Meezan E, Hjelle JT, Brendel K (1975) A simple, versatile, nondisruptive method for the isolation of morphologically and chemically pure basement membranes from several tissues. Life Sci 17:1721
13. Morrison WA, Webster HR, Kumta S (1996) Urethral reconstruction using the radial artery forearm free flap: conventional and prefabricated. Plast Reconstruct Surg 97:413
14. Nguyen DH, Mitchell ME, Horowitz M, Bagli DJ, Carr MC (1996) Demucolized augmentation gastrocystoplasty with bladder autoaugmentation in pediatric patients. J Urol 156:206
15. Rheinwald JG, Green H (1977) Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature 265:421
16. Romagnoli G, De Luca M, Faranda F, Bandelloni R, Franzi AT, Cataliotti F, Cancedda R (1990) Treatment of posterior hypospadias by the autologous graft of cultured urethral epithelium. N Engl J Med 323:527
17. Romagnoli G, De Luca M, Faranda F, Franzi AT, Cancedda R (1993) One-step treatment of proximal hypospadias by the autologous graft of cultured urethral epithelium. J Urol 150:1204
18. Shaul DB, Farley Diaz HXJ, Mahnovski V, Hardy BE (1996) Use of tubularized peritoneal free grafts as urethral substitutes in the rabbit. J Pediatr Surg 31:225
19. Scherz HC, Kaplan GW, Boychuk DI, Landa HM, Haghighi P (1992)Urethral healing in rabbits. J Urol 148:708
20. Schmieden V (1909) New method for operation for male hypospadias: free transplant of ureters to form urethra. Arch Kin Chir 90:748

21. Sutherland RS, Baskin LS, Hayward SW, Cunha GR (1996) Regeneration of bladder urothelium, smooth muscle, blood vessels and nerves into an acellular tissue matrix. J Urol 156:571
22. Takami Y, Matsuda T, Yoshitake M, Hanumadass M, Walter RJ (1996) Dispase/detergent treated dermal matrix as a dermal substitute. Burns 22 (3):182
23. Tuffier T (1910) A propos des greffes veineuses uretroplastiques. Bull Mem Soc Chir Paris 36:589
24. Wilson JW, Yeger H, Klement P, Lee M, Courtman DW (1990) Acellular matrix allograft small caliber vascular prostheses. Trans Am Soc Artif Intern Organs 36:340