Tissue-engineered autologous urethras for patients who need reconstruction: an observational study

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Summary

Background Complex urethral problems can occur as a result of injury, disease, or congenital defects and treatment options are often limited. Urethras, similar to other long tubularised tissues, can stricture after reconstruction. We aimed to assess the effectiveness of tissue-engineered urethras using patients' own cells in patients who needed urethral reconstruction.

Methods Five boys who had urethral defects were included in the study. A tissue biopsy was taken from each patient, and the muscle and epithelial cells were expanded and seeded onto tubularised polyglycolic acid:poly(lactide-co-glycolide acid) scaffolds. Patients then underwent urethral reconstruction with the tissue-engineered tubularised urethras. We took patient history, asked patients to complete questionnaires from the International Continence Society (ICS), and did urine analyses, cystourethroscopy, cystourethrography, and flow measurements at 3, 6, 12, 24, 36, 48, 60, and 72 months after surgery. We did serial endoscopic cup biopsies at 3, 12, and 36 months, each time in a different area of the engineered urethras.

Findings Patients had surgery between March 19, 2004, and July 20, 2007. Follow-up was completed by July 31, 2010. Median age was 11 years (range 10–14) at time of surgery and median follow-up was 71 months (range 36–76 months). AE1/AE3, α actin, desmin, and myosin antibodies confirmed the presence of cells of epithelial and muscle lineages on all cultures. The median end maximum urinary flow rate was 27·1 mL/s (range 16–28), and serial radiographic and endoscopic studies showed the maintenance of wide urethral calibres without strictures. Urethral biopsies showed that the engineered grafts had developed a normal appearing architecture by 3 months after implantation.

Interpretation Tubularised urethras can be engineered and remain functional in a clinical setting for up to 6 years. These engineered urethras can be used in patients who need complex urethral reconstruction.

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Introduction

Complex urethral problems can be caused by injury, which can lead to an inability to void. Catheters might need to be inserted below the abdomen in the suprapubic region for adequate bladder emptying, because often the effects of the trauma on the involved tissues need to be minimised. Some patients with urethral strictures repeatedly have infections, experience straining and major discomfort, and have blood in their urine. Urethral functional inadequacy can occur in patients with pelvic fractures or straddle trauma; previous manipulation with indwelling catheters, endoscopy, or surgery; and congenital or acquired disease.

Reconstructive techniques depend on the urethral defect location, length, and the surgeon's preference and previous experience. Short, non-complex urethral defects can be repaired with an end-to-end anastomosis by aligning and joining the normal urethral ends.[1] For long defects, surgeons might need to do a pubectomy, to gain better access to the damaged tissue and to help shorten the urethral gap.[2] An onlay repair, in which about half the strictured circumference portion of the tubular urethra is replaced with a tissue graft (eg, skin or buccal mucosa), is often used for damaged urethras that have a healthy urethral bed.[3,4] Tubularised tissue grafts might be needed for complex or long urethral defects, but have a high proportion of failures (sometimes over 50%).[5]

Regenerative medicine might help to overcome some of the drawbacks associated with the native tissues that are used for urologic reconstruction.[6,7] However, engineered tubularised constructs (eg, urethras or small blood vessels) tend to stricture over time.[5,8–10] We aimed to assess whether engineered autologous tubularised urethral tissue could be used as an alternative method for the treatment of complex posterior urethral defects.

Methods

Patients

Five boys with urethral defects were invited to participate in this study of engineered urethras at the Federico Gomez Children's Hospital in Mexico City, Mexico. Three patients presented with a complete posterior urethral disruption caused by pelvic trauma and had substantial widespread injury and two had previous failed posterior urethral repairs, one with a buccal mucosa graft and one with a skin graft, both of which were tubularised.

The study protocol was approved by the hospital's investigational review board (protocol number 2002055)

and ethics board. Written informed parental consent was obtained before acquirement of the tissue biopsy and 4–7 weeks before surgery.

Procedures

All five boys had an engineered urethra created from autologous cells and underwent urethral tubularised posterior urethroplasty by the same surgical method and procedure. Before surgery, the urethral defect length and characteristics such as location and associated pelvic pathology were measured by retrograde urethrography, voiding cystourethrograms, and urethroscopy. We took a detailed history from all patients and completed symptom questionnaires from the International Continence Society (ICS).

We took tissue samples from every child and used these to engineer the urethras. All tissue samples were taken by the same person (A Raya-Rivera). A 3 cm suprapubic transverse incision was made and a bladder biopsy (1×1 cm) was taken and sent to the approved manufacturing facility at the Metropolitan Autonomous University, Mexico City, Mexico, where the urethras were engineered under regulatory guidelines (HIM87120BSO) from the Federal Commission on Safety and Health Protection; Investigation, Ethics, and Biosafety Division (COFEPRIS). Primary cultures of smooth muscle and urothelial cells were collected as described previously.[6] Briefly, cells were collected by explanting pieces of muscle tissue and scraping the uroepithelial tissue onto 10 cm culture plates. The muscle tissue was placed in Dulbecco's modified Eagle's medium (Invitrogen; Carlsbad, CA, USA) and the epithelial tissue was placed in keratinocyte growth medium, both with epidermal growth factor. The cells were expanded to a density of $1·0–3·0×10^7$ per cm^2 for 3–6 weeks before seeding.

Muscle and urothelial cells were characterised by histology and immunohistochemistry before seeding onto the scaffolds. 5 μm sections of formalin-fixed, paraffin-embedded tissues were processed and stained with haematoxylin and eosin. We used representative sections for immunohistochemistry. Uroepithelial cell layers were identified with broadly reacting monoclonal mouse anti-human cytokeratins AE1/AE3, desmin, and myosin (Dako, CA, USA; product codes M3515, D33, and M3558) antibodies at 1:100, 1:50, and 1:50 dilutions, respectively, for 24 h at 4°C. Smooth muscle fibres were labelled with monoclonal α smooth muscle anti-actin antibodies (Novocastra, Newcastle, UK) at 1:100 dilution for 24 h at 4°C. Sections were incubated with the Dako Real EnVision Peroxidase/DAB detection system (Dako). All sections were counterstained with hematoxylin. Images were taken with a light microscope.

We seeded cells onto scaffolds as described previously.[7,11,12] Epithelial cells were seeded onto the luminal surface and muscle cells onto the outer surface of the tubular scaffolds. Briefly, the cells were removed from the culture plate with a 0·05% trypsin solution. Cells were centrifuged, rinsed with medium with no additives, and the pellet was re-suspended to a concentration of $1×10^7$ cells per mL. A biodegradable mesh made of polyglycolic acid (PGA; Sherwood Medical, St Louis, MO, USA) was tubularised with running 5-0 PGA sutures (Ethicon; Piscataway, NJ, USA) and sized for every individual patient, according to measurements of urethral defect from radiography, urethral calibration, and endoscopy. The size of the engineered urethras ranged from 4 to 6 cm (median 5 cm), with a 16 French diameter. The scaffolds were constructed in the same manner as used previously,[7,12] and the biomaterials were processed with the same techniques as used previously for engineered bladders implanted in people.[12,13] Briefly, the mesh was sprayed with a 50:50 liquid PGA:poly(lactide-co-glycolide acid) (Sigma; St Louis, MO, USA). The scaffolds were placed in a vacuum desiccator; samples confirmed scaffold purity.[13] The scaffolds were further sterilised with ultraviolet light and sequentially seeded with epithelial cells within the lumen and smooth muscle cells on the outer surface. The seeded scaffolds were incubated in culture for 7 days with Dulbecco's modified Eagle's medium. Overall, construction of the neo-urethras took 4–7 weeks.

We measured cell viability on the seeded grafts with an MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) assay.[14] After cells were incubated with 1 mg/mL MTT reagent for about 4 h, an isopropanol:hydrochloric acid (ratio 1:1) solution was added. The samples were read with a GENios (Tecan, Durham, NC, USA) ELISA plate reader (630 nm wavelength). Measurements from non-seeded grafts were used as controls.

We did scanning electron microscopy for seeded and non-seeded scaffolds to assess whether cells had grown on the scaffolds. Random samples were taken on days 3 and 6 after seeding. Fragments were washed with a 0·1 M sodium cacodylate buffer and fixed for 2 h in a glutaraldehyde (2·5%) solution. Samples were post-fixed in a 1:100 sodium cacodylate osmium tetroxide solution and dehydrated in a series of ethanol concentrations. We dried the preparations, covered

	Age (years)	Primary diagnosis	Previous urethroplasty	Defect site	Defect length (cm)	Follow-up (months)
1	10	Motor vehicle accident	No	Membranous urethra	5	76
2	14	Straddle trauma	Buccal mucosa	Membranous urethra	6	73
3	11	Motor vehicle accident	No	Membranous urethra	4	71
4	11	Motor vehicle accident	Foreskin	Membranous urethra	4	65
5	12	Straddle trauma	No	Membranous urethra	5	36*

*Patient followed up for 36 months because he was the last patient to enter the study.

Table: Characteristics of patients

them with ionised gold, and examined them with a JEOL JSM-5300 microscope.

Once the engineered urethras had been made, the patients underwent surgery. The urethras were catheterised and exposed through a perineal inverted semicircular incision. Defects were identified, the stricture and scar tissue was removed, and the urethral ends were dissected free from surrounding scar tissue. The tubularised engineered urethral constructs were surgically implanted with absorbable sutures. All engineered urethras were trimmed in the overlap region with the native urethras. A urethral catheter was left in place for 2 weeks in the first patient, and for 4 weeks in the other patients. The catheter size ranged from 12 to 14 French. We took patient history, asked patients to complete the ICS questionnaires, and did urine analyses, cystourethroscopy, cystourethrography, and flow measurements at 3, 6, 12, 24, 36, 48, 60, and

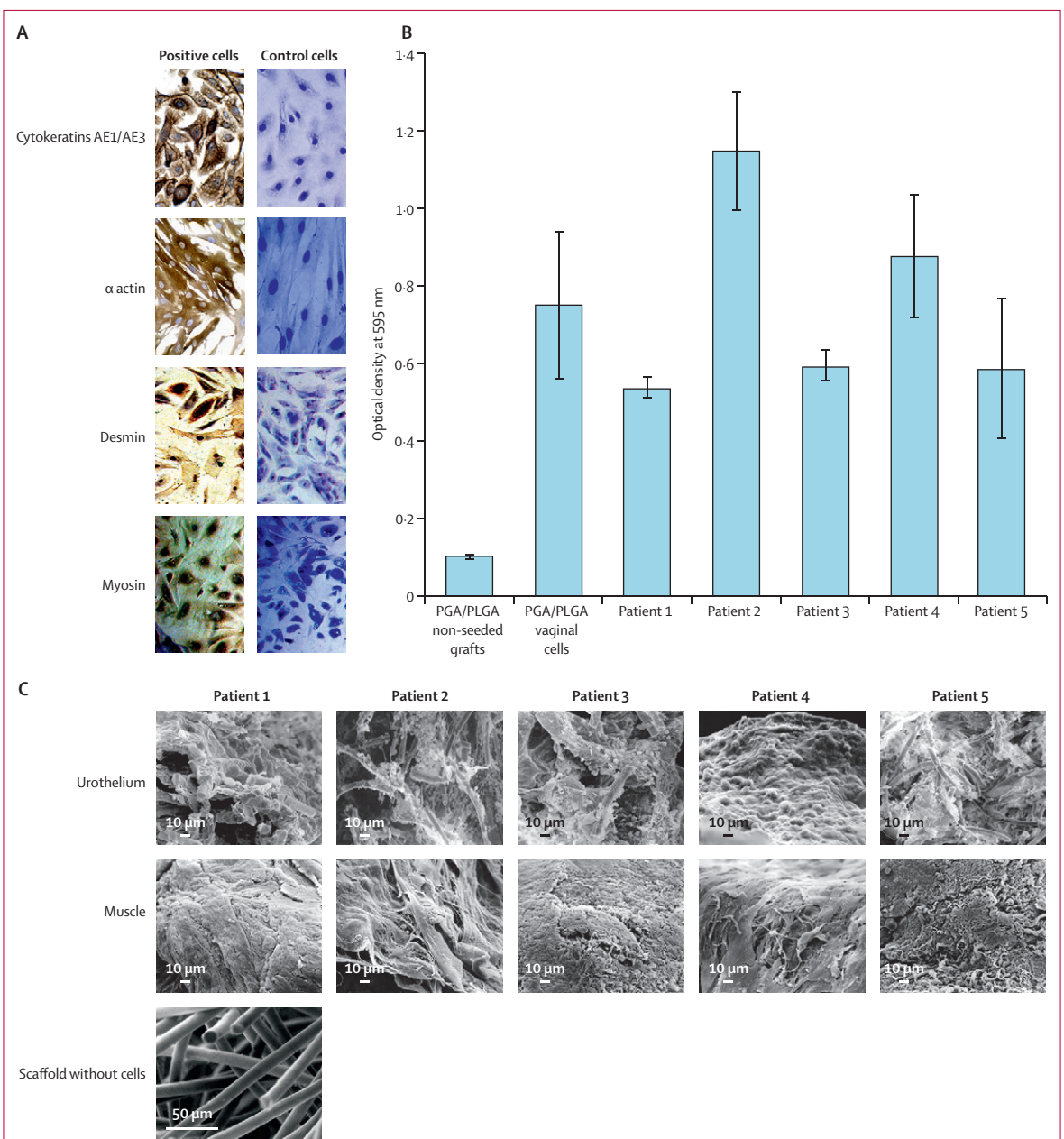

Figure 1: **Cellular expression of epithelial and muscular markers, and viability assay and scanning electron microscopy assessments**
(A) Labelling of smooth muscle and uroepithelial cells in cultures incubated with primary antibodies (positive cells) and without (control cells). All these cell cultures came from patient 3. (B) Assessment of cell viability on the seeded grafts from every patient. Vaginal cells were assessed as positive controls. Bars represent SD. (C) Scanning electron microscopy (15 Ku) of seeded scaffolds 6 days after seeding.

72 months after surgery. We did serial endoscopic cup biopsies at 3, 12, and 36 months, each time in a different area of the engineered urethras.

Role of the funding source

The sponsor funded the basic research and its analysis but had no role in the study design (preparing the

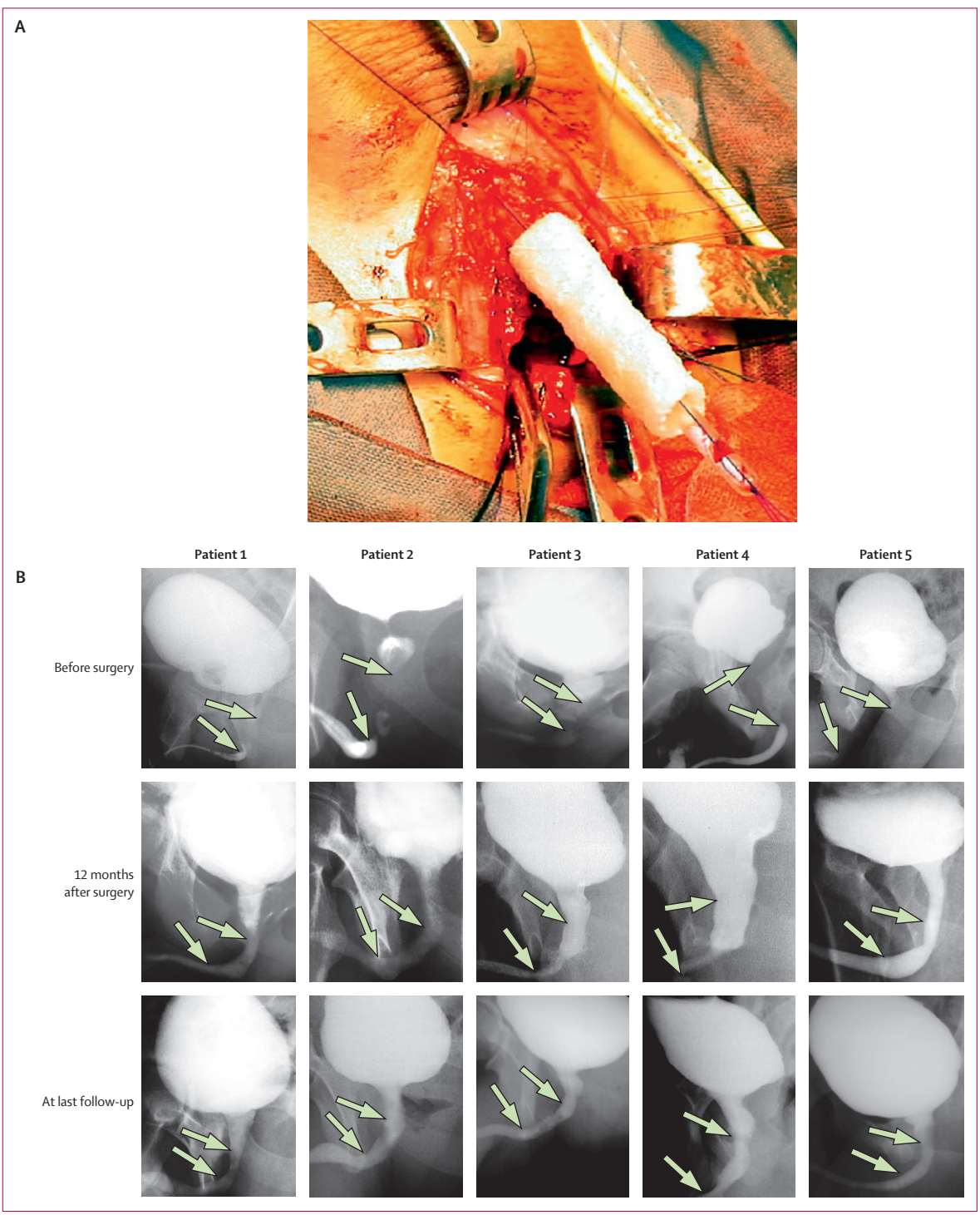

Figure 2: **Neo-urethra implantation and clinical outcome**
(A) A cell-seeded graft sutured to the normal urethral margins from the first patient. (B) Voiding cystourethrograms of all five patients before surgery (arrows show the abnormal margins), 12 months after surgery (arrows show margins of tissue engineered urethras), and at last follow-up (arrows show margins of tissue engineered urethras).

protocol), data collection, data analyses, data interpretation, or writing of the report. All authors had full access to all the data and had final responsibility for the decision to submit for publication.

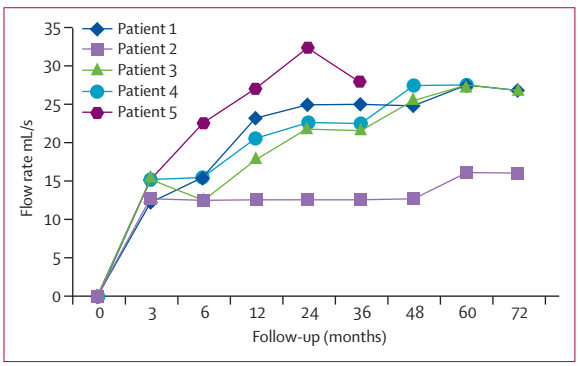

Figure 3: **Uroflow analyses**

Results

Patients had surgery between March 19, 2004, and July 20, 2007. Follow-up was completed by July 31, 2010. The table shows the characteristics of each patient. Median age was 11 years (range 10–14 years) at the time of surgery and patients were followed up for a median of 71 months (range 36–76 months) after surgery.

AE1/AE3, α actin, desmin, and myosin antibodies confirmed the presence of cells of epithelial and muscle lineages on all cultures (figure 1). There was a substantial increase in cell growth on the seeded scaffolds after 7 days in standard culture conditions. Scanning electron microscopy on day 6 of culture showed all scaffold surfaces were covered with cells.

The engineered urethras were implanted without intraoperative complications (figure 2). None of the patients had fistulae or urinary tract infections during follow-up. Patient history and serial symptom questionnaires completed by the patients with their

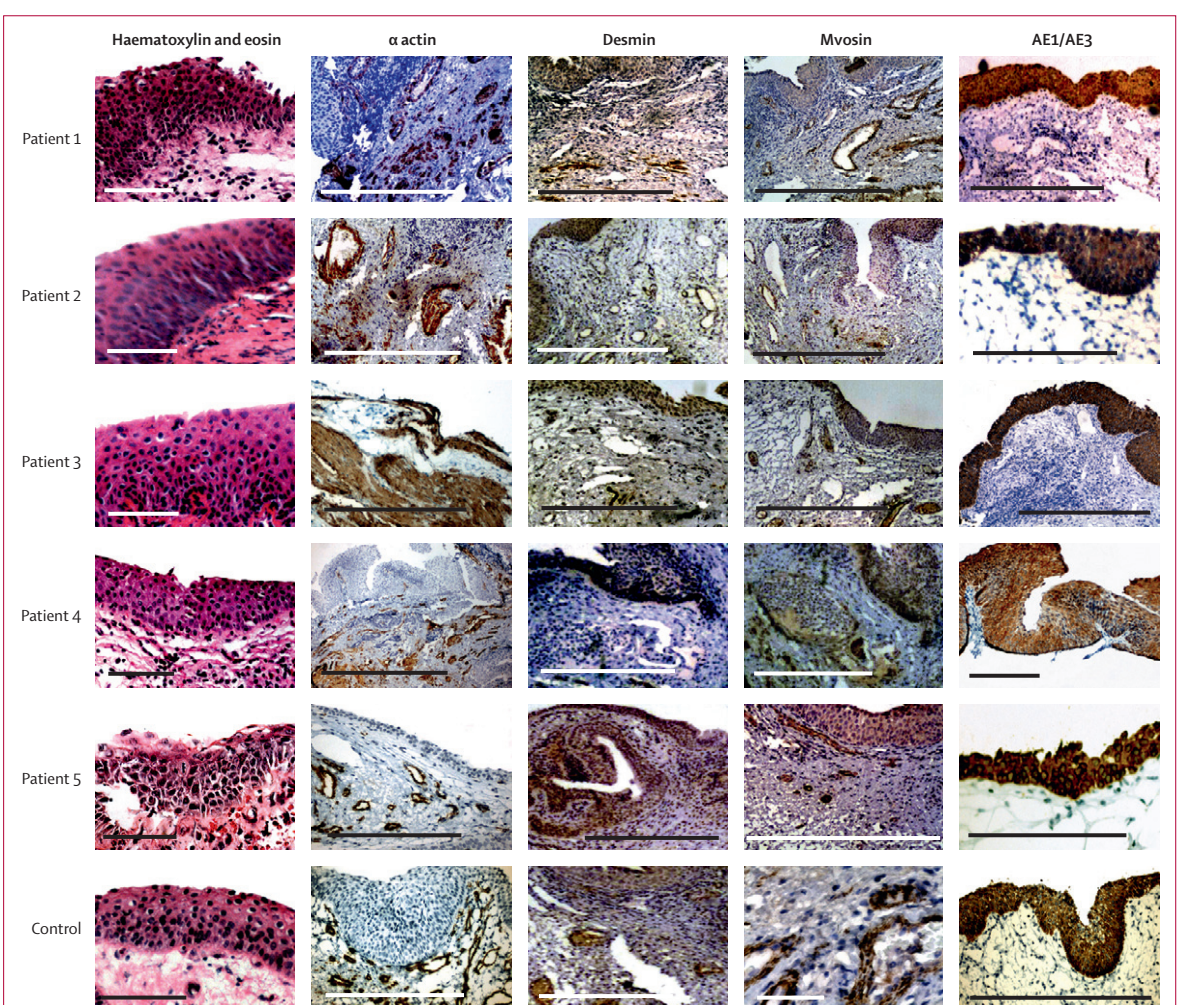

Figure 4: **Histological and immunocytochemical analyses**
Results of urethral biopsies of engineered segments from every patient at 1 year after surgery and from a control sample of healthy urethral tissue. All scale bars indicate 25 μm.

parents confirmed patients' satisfaction with the results of the operation, as measured by different parameters including the absence of dysuria, voiding frequency, straining, or dribbling.

The first patient, who had their catheter removed 2 weeks after surgery, presented with a decreased urinary voiding stream 2 weeks later. Cystourethroscopy showed a narrowing at the proximal superior graft anastomotic site that needed a transurethral incision 4 weeks after surgery. The patient was able to void well thereafter, and no further interventions were needed. Cystourethroscopy confirmed the radiographic findings of a patent urethra in all patients (figure 2). The fourth patient had pelvic disruption that involved the sphincter, and he needed a pubovesical sling after surgery for his daytime stress incontinence. All patients are now continent.

Voiding cystourethrograms, which showed the extent of the defects before surgery, showed the maintenance of wide urethral calibres without diverticula 12 months after surgery (figure 2). The absence of strictures and diverticula was confirmed with serial cystourethroscopy.

***Panel:* Research in context**

Systematic review

We did a Medline search for original articles on tissue engineering or regenerative medicine and urethras with stricture disease. We used the search terms "tissue engineering", "regenerative medicine", and "urethras" from January, 1991, to August, 2010. We found no studies on reconstructive surgery with a constructed tubularised engineered urethra. We did identify three studies that described urethral reconstruction in patients who needed an on-lay, non-tubularised repair.[19–21] The first two studies used a collagen-based extracellular matrix, derived from bladder submucosa, for urethral repair.[19,20] In the first study, 24 of the 28 patients had a successful outcome at 48 months.[19] The second study, a randomised comparative study, assessed the outcome of the extracellular matrix compared with buccal mucosa.[20] This study showed that the collagen-based acellular matrix is a viable option for urethral repair in an on-lay, non-tubularised manner, but only in patients with a healthy urethral bed, where the normal cells from the native urethra can regenerate over the extracellular matrix.[20] In the third study, five patients with an unhealthy urethral bed secondary to Lichen sclerosus underwent urethroplasty using buccal mucosa cell seeded grafts.[21] The grafts were used for urethroplasty in a one-stage (n=2) or a two-stage procedure (n=3). By 37 months, one patient needed a complete excision of the grafted urethra, one needed partial graft excision, and three had a patent urethra with the graft in situ, although all three needed some form of postoperative treatment.[21]

Interpretation

Present methods of urethral reconstruction, with tissue grafts such as buccal mucosa or skin, have shown that on-lay repairs often have better outcomes than tubularised repairs. Tubularised tissue grafts might be needed for complex or long urethral defects, but have a high proportion of failures.[5] There has been no previous work done in terms of creating tubularised engineered urethras for patients. Previous studies have shown that whenever a healthy urethral bed is present, an on-lay repair can be done with an off-the-shelf extracellular matrix, and no cells are needed. If the urethral bed is not healthy, and it is preserved as part of the repair, the matrices, either with or without cells, might not lead to optimum outcomes. The present study shows that tubularised engineered urethras can be created using patients' own cells and can lead to adequate long-term outcomes.

Uroflow studies showed adequate flow rates, with a mean end maximum flow rate of 25·1 mL/s, and a median of 27·1 mL/s (range 16–28) at the last follow-up (figure 3).

Histology of the serial biopsies, confirmed with immunohistochemistry, showed that the engineered urethras appeared to have a normal architecture by 3 months after implantation, and consisted of distinguishable layers of epithelia and smooth muscle (figure 4). There were no aberrant histological changes over time.

Discussion

We have successfully constructed engineered urethras with autologous cells and implanted them into patients with urethral defects. All five boys were continent at last follow-up.

The results of this study are consistent with the results from experimental studies we did in the early 1990s, when we seeded biodegradable matrices with autologous cells.[15] In an experimental model in rabbits, autologous bladder epithelial and smooth muscle cells were grown and seeded onto preconfigured tubular matrices.[11] Entire urethral segments were resected and anterior urethroplasties were done with tubularised matrices, either seeded with cells or without cells. The tubularised matrices that were seeded with autologous cells formed new tissue that was histologically similar to native urethra. There was poor tissue development, fibrosis, and stricture formation from those matrices that were not seeded. The study showed that the use of engineered urethras with matrices of cells was crucial to the success of tubularised repairs in the long term. Although the scaffold alone was not sufficient for a tubularised repair and there was fibrosis and scarring present, we noted that there was limited tissue regeneration at the edges of the construct. In animal experiments, we noted that the maximum distance at which native cells from the normal urethra migrated onto the scaffold and contributed to adequate tissue regeneration was 0·5 cm.[16] This finding led us to assess the use of matrices without cells to replace only the top part of the tubular urethra, in an on-lay manner, instead of replacing the entire tubular urethral circumference.[17] Thereafter, we showed that if a portion of the circumferential urethral tissue is healthy and can be preserved, off-the-shelf matrices without cells can be used as on-lay grafts with adequate long-term outcomes.[18–20] Buccal mucosal cell-seeded matrices have been used as on-lay grafts for urethral disease in patients with lichen sclerosus.[21] Lichen sclerosus is a hard condition to treat because of the propensity of the tissue to scar over time.[21] Patients with extensive lichen sclerosus stricture disease might need excision of the entire urethra to the proximal normal area and replacement by graft. Overall, previous regenerative studies showed that for on-lay repairs, matrices alone might be sufficient for reconstruction, but for complex urethral strictures, both

matrices and cells might be needed, especially for tubularised repairs (panel).

We experimented extensively with many different biomaterials, and PGA-based biomaterials were used both experimentally for the creation of urethral tissue and clinically for bladder replacement.[6,7,12,21] The biomaterials we used in this study have already been used safely in patients for multiple indications,[13] including bioengineered bladders in patients who were followed up for up to 12 years.[7,12] The biomaterials we used were deemed to work best for this patient series because additional strength and anatomical support was needed to adequately mould the engineered structures for the posterior urethral location. As shown previously, the cells survive on the scaffolds in culture and when initially implanted in vivo through nutrient diffusion.[22,23] Angiogenesis and neovascularisation starts when the construct is implanted and occurs in concert with further cell expansion and tissue formation.[22,23]

Primary anastomosis remains the procedure of choice for short urethral defects or for most primary posterior injuries. Urethral defects, such as prostatomembranous urethral rupture, often differ in children and adults, and, as a result, the presentation and surgical methods for repair might differ. The entire posterior urethra is delicate in boys, whereas in men it is supported by the mature prostate. As a result, posterior urethral injuries in men are usually limited to a short area of the membranous urethra distal to the prostate, and those of boys can occur throughout the posterior urethra, including the bladder neck. In boys, there is marked upward displacement of the prostate; in adults this displacement is usually minimal.[24] The unique features associated with urethral defects in children might need more complex repairs. Complex urethral strictures or defects might need tubularised grafts, usually with the use of native tissue such as buccal mucosa or skin, and are associated with a high proportion of complications[5] Therefore, alternative materials and repair procedures are needed, and tissue engineering techniques might be a viable option for urethral reconstruction.

Although physicians agree on many details of the urethroplasty procedure, some controversy exists about the earliest feasible time to remove the catheter after urethroplasty. The time to remove the catheter after anastomotic buccal mucosal urethroplasty ranges from 7 to 28 days.[25] The time of catheter removal remains largely a matter of physician opinion. The occurrence of a narrowing at the proximal superior graft anastomotic site in the first patient of this series shows the importance of leaving the indwelling catheter for 4 weeks in patients undergoing repair with engineered urethras, to maximise the success of the procedure.

The engineered urethral implants had a similar phenotypic and histological make-up to native urethral tissue, with normal epithelium surrounded by connective tissue and muscle, normal patency of the neo-urethras, and bladders that emptied adequately. These functional characteristics were confirmed by a median end maximum flow rate of over 27 mL/s. Although normal values for maximum flow rates are hard to calculate, in the paediatric population values greater than 10 mL/s are usually not associated with any adverse symptoms.[24] In a study of 335 children with no urological disease, the normal mean maximum flow was 19·95 mL/s (range 9·95–29·95).[26]

We have shown that urethral structures can be engineered and remain functional in a clinical setting in the long term. The study was limited in that only pediatric patients were treated. The results might differ in the adult population. The study aimed to treat patients with posterior urethral defects. Additional studies will be needed to determine if anterior urethral defects can be repaired in a similar way.Tissue engineered urethras, created with patients' own cells, can be used to successfully treat complex urethral defects. The tubularised engineered urethras showed histological and functional characteristics similar to native urethras and maintained an adequate outflow for up to 6 years. Tissue engineered urethras could be a new alternative source for reconstruction.

Contributors

AR-R, JJY, and AA obtained funding and wrote the manuscript. All authors designed the study, analysed the data, interpreted the results, revised the manuscript, and approved the final version.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

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