Histologic Changes After Urethroplasty Using Small Intestinal Submucosa Unseeded With Cells in Rabbits With Injured Urethra

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OBJECTIVE To determine whether small intestine submucosa has the same regenerative capacity when urethroplasty is performed in injured urethras.

METHODS Our experiment was conducted in 30 New Zealand male rabbits, all of which had urethral injury. One month after the injury, the animals were randomized into a control group or a group with onlay urethroplasty with small intestine submucosa. The animals were euthanized at 2, 4, 12, 24, and 36 weeks after urethroplasty, and their urethras were removed for histologic and immunohistochemical examination. Before the scheduled euthanasia, urethrography and cystoscopy were performed.

RESULTS After 2 weeks, there was evidence of a continuous monolayer of stratified epithelial cells and absence of smooth muscle fibers. One month later, the epithelium showed no changes from the previously observed features, but some smooth muscle fibers (representing newly formed vessels) became apparent. After 3 months, the graft showed increased concentration of smooth muscle fibers. After 6 and 9 months, the density of smooth muscle cells remained unchanged. Fiber arrangement was irregular, particularly at the anastomosis site. Epithelial and smooth muscle phenotypes were confirmed by immunohistochemistry using anti-pan-citokeratin (AE1/AE3) antibodies and anti-α-smooth muscle actin, respectively.

CONCLUSION Small intestine submucosa promotes regeneration in traumatized urethras, with slightly delayed epithelialization and abnormal distribution of smooth muscle. Urethral damage caused by trauma interferes with the normal healing process. UROLOGY ■: ■—■, 2013. © 2013 Elsevier Inc.

Reconstruction of the male pendulous urethra for congenital or acquired pathology remains a challenge for urologists. More than 200 different techniques have been developed for the treatment of hypospadia and urethral stricture, most of which use penile skin or foreskin.[1,2] Several reports provide evidence of complications associated with these surgical techniques, such as hair growth, graft scarring, diverticula, and stenosis.[3-7] Buccal mucosa, bladder mucosa, tunica vaginalis, and skin patches, among other tissues, have also been used in research and in clinical practice.[8,9] However, the use of nongenital tissue can increase morbidity rates at the donor site.

Recent reports have described use of porcine small intestinal submucosa (SIS), a xenogenic, nonimmunogenic collagen matrix obtained from pig small intestine. SIS induces the regeneration of multiple tissues and has been shown to work at multiple locations.[10] The exact mechanism by which regeneration occurs remains unknown and is currently under investigation. SIS has been found to induce regeneration of the 3 urinary bladder tissue layers in rats and dogs.[11,12] Histologically, the host reacts to SIS with fibrovascular scarring and neovascularization, inflammatory infiltration with fibroblasts, and remodeling. Because the results of urinary bladder regeneration in animals were encouraging, SIS was tested with urethras of rabbits, where epithelial and smooth muscle regeneration was obtained. However, these studies used healthy animals whose urethras had not suffered previous damage, whereas patients who are eligible for a SIS graft urethroplasty do not have normal urethras. Therefore, the purpose of this study in rabbits is to establish whether SIS has the same regenerative capacity when urethroplasty is performed in diseased or injured urethras.

MATERIALS AND METHODS

The study protocol was reviewed and approved by the Italian Hospital of Buenos Aires Ethics Committee.

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Animals

For the experiment, we used 30 New Zealand rabbits, weighing between 2.5 and 3.5 kg, sleeping in individual cages at a constant temperature of 17° to 18°C, and having free access to food and water. All the animals were kept in a fasting condition the night before surgery. The first surgery performed was urethrotomy with nonabsorbable suture closure for future identification. At 1 month after the procedure, the animals were randomized to a control group that did not undergo further surgery and a urethroplasty group that underwent onlay urethroplasty with unseeded SIS.

Procurement of SIS

SIS was obtained, with some modifications, following a previously published protocol by Lai et al.[13] Briefly, the procedure involves removing the small intestine of a pig within 10 minutes of euthanasia and placing it in a saline solution. The tissue is cut into 10- to 20-cm-long segments and stripped off the mesentery. It is subsequently cut along the mesenteric edge, thereby removing the outer seromuscular layer. The intestine is everted and the mucous layer removed with wet gauze, exposing a 0.1-mm-thick whitish membrane. This membrane is subsequently decellularized using 0.5% Triton X-100 plus 0.1% sodium azide for 48 hours under constant agitation on an orbital shaker (Thermo Scientific) at 200 rpm and 37°C. The decellularized membranes are cut into 3- × 4-cm rectangles, sterilized in ethylene oxide, and stored until use.

Urethral Injury (First Surgery)

Anesthetic induction was performed with an intramuscular injections of ketamine (35 mg/kg) and 20% xylazine (5 mg/kg). The rabbits were placed supine on the operating table. Endotracheal intubation was placed, and anesthesia was maintained with 2% isoflurane under spontaneous ventilation. The animals were shaved before their skin was prepared with povidone-iodine, and sterile surgical drapes were placed around the genital area and lower abdomen. The urethra was catheterized with a K31 catheter lubricated with xylocaine jelly, and urethrotomy was performed with scissors up to 1.5 cm of the urethral meatus and closed with X-shaped sutures using 4-0 Prolene (Ethicon) for future identification (Fig. 1).

Onlay Urethroplasty

Rabbits were anesthetized and prepared for the first surgery as described above. With the K31 catheter still in the urethra, the Prolene suture was identified, and a patch 1 cm in diameter around this suture was resected. The SIS was oriented with the mucosal side facing the luminal side of the urethra and sutured to the edges of the urethral defect using continuous running 5-0 Vicryl (Ethicon) suture. The subcutaneous tissue was subsequently reapproximated, and the skin was sutured with 4-0 Vicryl. Sutures of 4-0 Prolene were used proximally and distally for future reference at the time of euthanasia (Fig. 2).

Urethrography and Cystoscopy

All animals underwent urethrography and cystoscopy before euthanasia. After sedation with phenobarbital, a K31 catheter was placed in their urethral meatus and iodine contrast was injected during a fluoroscopy. Cystoscopy was performed to visualize the urethra with the use of a pediatric cystoscope (Wolf, Germany).

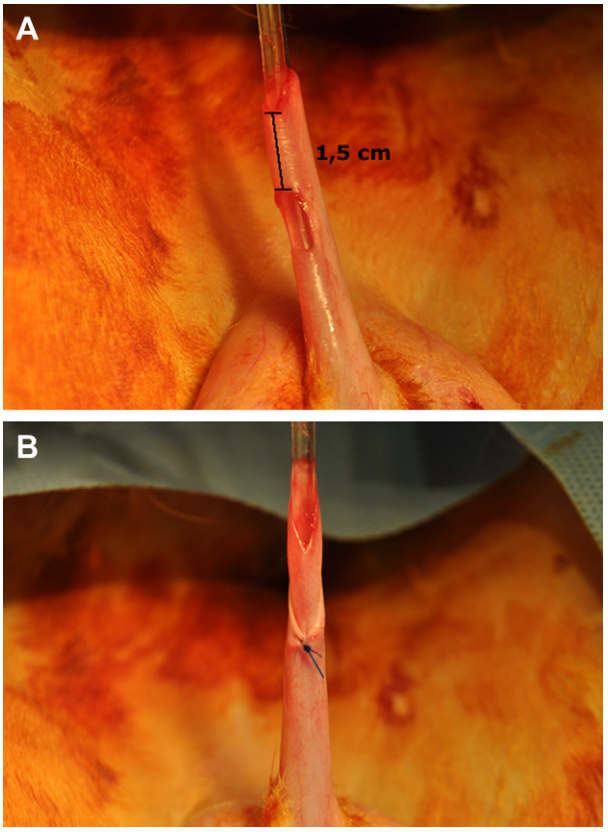

Figure 1. (A) 1.5-cm injury to the urethral meatus in all rabbits. **(B)** The injury is marked with nonabsorbable suture for future reference.

Euthanasia Scheme

All the animals were killed at 2, 4, 12, 24, and 36 weeks after the last surgery with an overdose of pentobarbital once urethrographies and cystoscopies were performed. The rabbits were shaved and their penises sectioned between the nonabsorbable sutures in the urethroplasty group, and around the Prolene suture in the control group. The specimens were fixed in a 4% formaldehyde solution.

Histology

A macroscopic and microscopic examination was carried out. The specimens were embedded in paraffin, cut with a microtome, stained with hematoxylin and eosin and with Masson trichrome staining, and examined under a light microscope. Immunohistochemistry was also performed using anti—pan-citokeratin (AE1/AE3) antibodies (Chemicon International Inc) for epithelial cells and anti-α-smooth muscle actin antibodies (Novocastra Laboratories Ltd) for smooth muscle fibers.

Statistical Analysis

ImageJ 1.44p software (National Institutes of Health) was used to count the number of cells in the samples, and a 2-tailed Student *t*-test calculated using STATA 8 software (StataCorp LP) was used to assess the distribution of smooth muscle fibers in the samples.

RESULTS

All animals survived both surgeries, with no evidence of infection or urinary disorders. Stenosis was found in 2 of the control group animals and in 1 animal in the

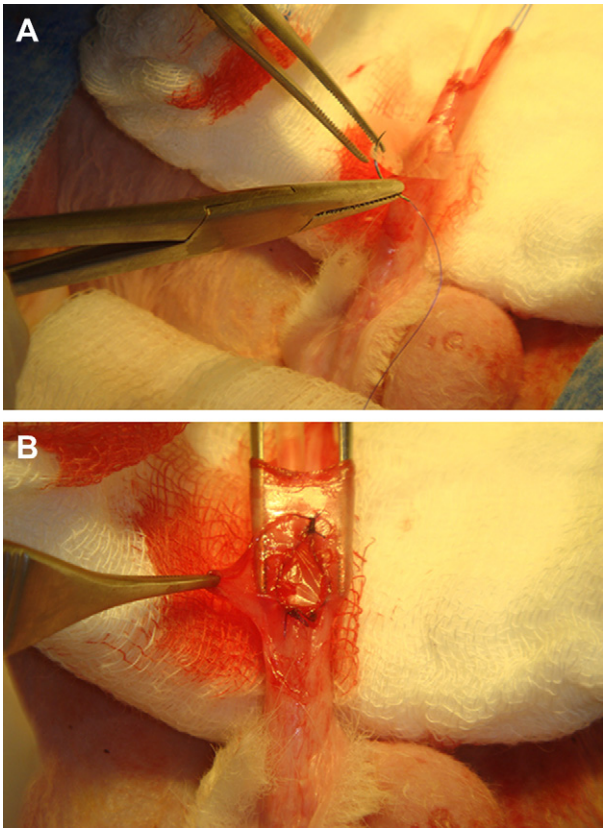

Figure 2. (A) Suturing unseeded small intestinal submucosa. **(B)** Onlay urethroplasty.

urethroplasty group. Three fistulae developed in the latter group. None of the animals were catheterized in the postoperative period for either surgery, so there were no catheter-related problems, as opposed to those described by the authors who used urethral catheters. Urethrographies performed before the scheduled euthanisia showed stricture of the pendulous urethra in 2 control animals (1 was killed after 4 weeks and the other after 12 weeks) and in 1 of the animals in the urethroplasty group (killed after 24 weeks). Three pendulous urethra fistulae were found in the urethroplasty group (1 animal after 4, 12, and 24 weeks, respectively) and none in the control group. Cystoscopy was done after urethrography. Where no stenosis or fistulae were found, a normal epithelium could be seen even 15 days after the urethroplasty, which demonstrates that the SIS patch undergoes a normal process of epithelialization in urethral cells.

Urethrotomy Group

Regardless of when the euthanisia was performed, columnar epitheliums with 3 to 4 layers of cells were observed in the urethral lumen. Muscle fibers were found around the area of the urethrotomy, where there was evidence of inflammatory cells with areas of hemorrhage and fibrosis.

Urethroplasty Group

There was no evidence of diverticula formation or retraction of the SIS patch. A microscopic examination 15 days afterward showed a complete epithelium, with 3 to 4 layers of epithelial cells and some areas containing a monolayer of epithelial cells (Fig. 3). At this stage, a collagen matrix was found, showing complete infiltration by inflammatory cells but absence of smooth muscle cells. The urethra of the animals that were killed 1 month after urethroplasty showed normal columnar epithelium of 3 to 4 layers and no areas containing monolayers of epithelial cells. Remnants of the collagenous matrix with a lower density of lymphoid cells and absence of smooth muscle fibers were observed. Only a few smooth muscle cells with newly formed small vessels were found. After 12 weeks, the epithelium remained unchanged, with no remnants of SIS or disorganized smooth muscle fibers with any specific pattern, or greater density at the anastomosis sites. After 24 weeks, complete replacement of the collagen matrix and increased concentration of smooth muscle cells at the sites of anastomosis were observed, with lower density at the center of the patch ($P < .05$). At 36 weeks after the procedure, practically no difference with the urethras evaluated after 24 weeks was observed (Fig. 4).

COMMENT

Biocompatible materials are being widely used in regenerative medicine. Porcine SIS is one of the most frequently used biomaterials in urology and was originally used to expand bladders in rats and dogs.[14] SIS has been found to promote cell migration[15] and 3-dimensional cellular organization within the biological scaffold[16] in animals and in humans. The reasons SIS promotes regenerative processes are related to growth factors, neovascularization, re-epithelialization, and tissue differentiation.[14] Some studies have documented that SIS is rapidly degraded by the host within 3 weeks of implantation.[17] Although SIS is a nonimmunogenic membrane, inflammatory reaction around the graft was observed,[18] which could contribute to the degradation process of the material or promote neovascularization.[16]

In their first article, Badylak et al[19] reported the use of non-decellularized SIS, but after some time, several researchers began to use decellularized SIS. They used different decellularization methods (chemical, physical, enzymatic, and detergent-based) or combined them to remove cells from collagen matrices, with no serious effect on the matrices. Triton X-100 was widely used. This detergent requires 5 to 21 days to decellularize collagen matrices[20] and can alter the components of extracellular matrices without detriment to the regenerative capacity of SIS. In this respect, only one difference exists between other authors' SIS and our SIS, and this lies basically in the 2-day processing time in our work, which was much shorter than theirs.

Given the growing acceptance of (decellularized) SIS among researchers, it began to be used for the replacement of hollow organs, such as the urethra. The urethra is an external organ and thus is easily accessible for

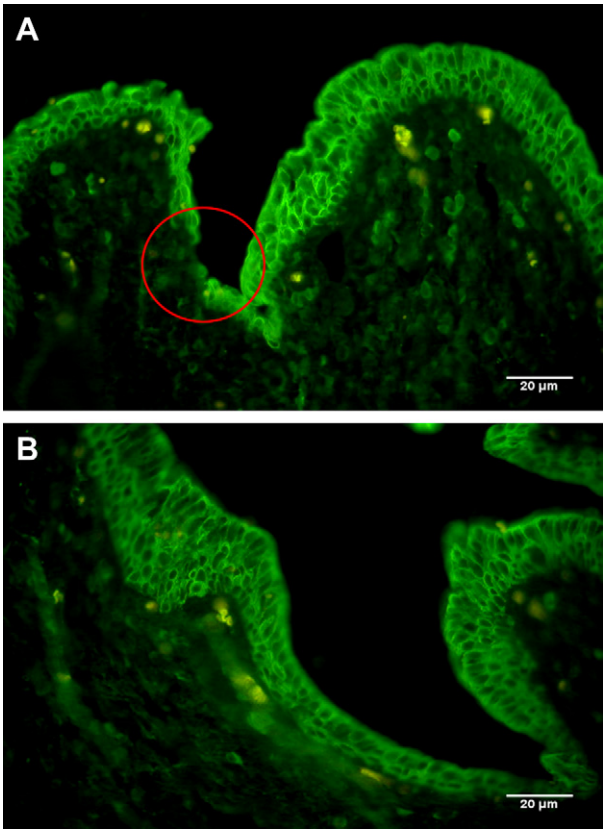

Figure 3. Staining with anti–pan-citokeratin antibodies (green). **(A)** At 15 days after urethroplasty, stratified epithelium is seen with some monolayer areas. **(B)** At 9 months after urethroplasty, normal stratified epithelium is seen.

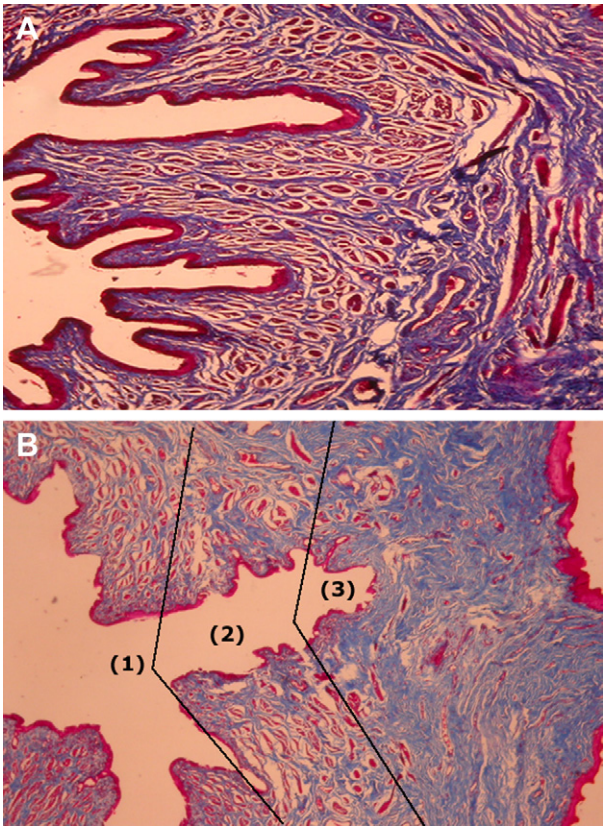

Figure 4. Staining with Masson trichrome shows **(A)** normal urethra and **(B)** at 9 months after the urethroplasty shows (1) normal urethra, (2) peripheral zone, and (3) central zone.

treatment; also, it can be regarded as a simple organ, because its function is basically to carry off urine from the urinary bladder. Different techniques for repairing the urethra using SIS in animals have been reported, the most widely used procedure being onlay (graft) urethroplasty, although tubular urethroplasty has also been described. Both techniques can be performed with seeded or unseeded SIS.

Several studies have reported urethral regeneration in animals; for example, the 1998 study by Badylak et al[16] compared 3 different treatment groups of rabbits. In their study, 1 group was treated with urethrotomy alone; another with urethroplasty with a preputial flap, and a third with an onlay unseeded SIS graft. At the 3-month follow-up, the SIS group showed complete epithelialization of the mucosa with a stratified epithelium of 3 to 4 layers of cells. The smooth muscle fibers were irregular and discontinuous, with a markedly abnormal distribution.

In 1999, Atala et al[21] used porcine bladder submucosa as collagen matrix in rabbits with a longer follow-up and reported complete regeneration of the epithelial layer, as Badylak et al[16] had done before. Smooth muscle fibers were discontinuous and irregular within 2 months; 6 months later, however, the organization of muscle fibers was normal. Hence, they concluded that 6 months is enough time to develop a urethra that is almost indistinguishable from a normal one.

In 2002, Atala et al[22] compared the outcomes of tubular urethroplasties with and without cells. A small bladder biopsy was performed in one of their study groups, and the urothelial cells obtained were cultured in vitro and then placed in a collagen matrix to develop a tube, which was later used as a tubular graft; the same procedure was repeated without cells for a comparison between the 2 types of tubular urethroplasty. At the 6-month follow-up, the unseeded segments showed irregular epithelium and disarranged smooth muscle fibers, and all had stenosis or collapsed; however, the tubular grafts with cells showed no stenosis, had a normal macroscopic appearance, were composed of normal epithelium, and showed arranged smooth muscle fibers comparable to those of a normal urethra.

In 2004, El-Assmy et al[23] compared different techniques for urethral repair in animals, which included onlay urethroplasty using commercial unseeded SIS and cell-free tubular grafts. Three months postoperatively, the onlay SIS group had complete epithelialization and absence of smooth muscle regeneration; conversely, all tubular grafts developed fistulae and stenosis.

It is currently known that when a collagen matrix patch of less than 1 cm in diameter is used, cells need not

be placed on biological scaffolds. In contrast, for patches larger than 1 cm in diameter or tubular grafts, successful results will depend on the use of cells.[22]

All the studies we have reviewed were conducted in healthy animals with normal urethras. Patients who are eligible for urethroplasty, however, do not have healthy urethras; on the contrary, they usually have some kind of birth defect or urethral trauma or have undergone a failed surgery. For this reason, our purpose was to study different ways to improve our animal model to obtain more accurate and better information for use in the treatment of our patients. This is why we developed an animal model with previous urethral injury to assess whether the regenerative power of SIS is maintained in an environment of tissue damage and fibrosis.

To this purpose, we damaged the urethra with a small dissection, performed urethrotomy, and closed the organ. One month later, we repaired the ventral urethra by means of an onlay urethroplasty with unseeded SIS and assessed histologic changes over time. With regard to the epithelium, 15 days after the urethroplasty procedure, complete epithelialization of SIS with columnar epithelium was observed, except for some areas, which were covered with a monolayer of epithelial cells. One month later and thereafter, the epithelium appeared to be normal. No smooth muscle cells appeared in any of the euthanized animals, neither at 15 nor at 30 days after urethroplasty. After 3 months, irregular and discontinuous smooth muscle fibers were apparent. Within 6 months, a higher density of muscle fibers, predominantly at the anastomosis sites, was observed. After 9 months, the distribution remained unchanged and almost identical to that observed after 6 months.

That the urethra thus developed is not histologically normal raises the question of whether SIS should be used. When other tissue types, namely the dermis, buccal mucosa, and so on, are used, acceptable results are achieved despite the absence of resemblance between these tissues and the normal urethra. Consequently, further research should be conducted to demonstrate whether the urethra obtained with tissue engineering, although not morphologically equal to a normal urethra, could be used as a graft with comparable results.

CONCLUSIONS

Our study suggests that SIS can promote regeneration in traumatized urethras. The histologic examination of the studied urethras, however, showed an abnormal pattern, with slightly delayed epithelialization and abnormal distribution of smooth muscle. Hence, our urethral damage model may resemble our patients more closely than previously undamaged models because damage caused by trauma interferes with the normal healing process. In this respect, we believe our urethral damage model could be used in future research to develop new biomaterials for the clinical setting.

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