

In Vivo Evaluation of a Multilayered, Fibrin-Agarose Urethral Substitute Generated by Tissue Engineering

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Abstract

Background: Tissue engineered urethral substitutes could offer alternative approaches for urethral regeneration. In this work, we generated and evaluated *in vivo* a multilayered urethral substitute generated with nanostructured fibrin-agarose biomaterials. **Methods:** A three-layered urethral substitute was generated and grafted to repair surgically induced penile urethra defects in rabbits (acellular multilayered urethral substitute graft (UREG)). Native urethras served as positive controls (positive control (P-CTR)), unrepared defects as negative controls (negative control (N-CTR)), and oral mucosa autograft (AUTO), as the gold-standard treatment. Results were analyzed after 3 months for surgical feasibility, complications, urine stain patterns and histological, histochemical and immunohistochemical analyses. **Results:** All procedures were feasible, with no severe complications. Both in N-CTR and AUTO groups, 2/5 animals developed urethral fistulas, with none in UREG. Altered urine stains appeared in 3/5 of N-CTR and AUTO animals, and in 1/5 of UREG. Histologically, the UREG implants resembled native urethra, with high expression of pancytokeratin, cytokeratins 7, 8 and 19, involucrin and uroplakin, and stromal expression of versican, biglycan and decorin, with similar collagen and glycosaminoglycan contents. However, UREG grafts showed lower alcian blue staining intensity, higher number of blood vessels and a discontinuous muscular layer at the grafting site. In contrast, N-CTR and AUTO groups failed to reproduce the structure and molecular composition of the native urethra. **Conclusions:** A multilayered fibrin-agarose urethral substitute generated by tissue engineering could support urethral regeneration in an animal model of urethral repair. These findings support its potential as a promising alternative for urethral reconstruction.

Keywords: Urethra, multilayered graft, fibrin-agarose biomaterials, tissue engineering.

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Introduction

Severe conditions affecting the human urethra, including congenital diseases such as hypospadias and epispadias [1], and acquired conditions such as urethral strictures and fistulae remain challenging to manage, and current treatments are associated with high rates of complications and side effects [2,3]. Because of its tubular structure, the human urethra is particularly susceptible to stenosis and urethral stricture formation following trauma or surgical in-

jury, which could ultimately lead to obstructive urinary symptoms [4]. Despite their prevalence, conditions affecting the human urethra remain a therapeutic challenge, and results are typically suboptimal, with a high tendency for recurrence [5].

Although endoscopic approaches can be applied in certain cases, urethral defects are normally treated by urethroplasty, a method consisting in a surgical repair and reconstruction of the damaged tissues [6]. When a segment of the urethra is partially or completely altered, urethroplasty

is typically performed using tissues harvested from peripheral donor sites, which are used as flaps allowing reconstruction of the urethral defect [7,8]. However, large defects and recurrent urethral alterations are increasingly treated using grafts of non-urolological tissues, especially the oral mucosa (OM), which can be obtained from the patient's oral cavity and grafted at the urethral defect [7]. Although OM grafting can be considered as the gold-standard technique, particularly in complex cases, it is considered to be technically complex [9], and the rate of complications and adverse effects, including stenosis, fistula formation and graft detachment is comparable to that observed with flap techniques [10]. These results underscore the need for continued research in the field.

One potential alternative is the use of bioartificial grafts and scaffolds generated by tissue engineering. Tissue engineering is an emerging field of research that allows the generation and clinical application of different types of bioartificial tissues and organs, such as the human skin [11], cornea [12] and, within the urological system, the bladder [13,14], ureter [15] and urethra [16]. However, reproduction of the multilayered tubular structure of the human urethra, composed by a urethral mucosa, a spongy layer and a tunica albuginea, is very complex, resulting in highly heterogeneous results in urethral repair, and varying degrees of biocompatibility of biomaterials grafted on this organ [17]. In this context, nanostructured fibrin-agarose biomaterials emerged as promising biomaterials demonstrating excellent *ex vivo* and *in vivo* biocompatibility, allowing the generation of multilayered structures [18]. This biomaterial is obtained by combining human plasma fibrin with marine agarose and showed to be well tolerated when implanted in patients with skin [11], cornea [12] and palate defects [19]. Although this biomaterial allowed the successful generation of a bioartificial, multilayered human urethra, its *in vivo* behavior after urethral implantation has not been evaluated.

In the present study, we have analyzed *in vivo* a multilayered urethral substitute generated by tissue engineering using fibrin-agarose biomaterials, assessing its biosafety and efficacy in an animal model of urethral defect, as compared to controls and animals receiving an oral mucosa autograft (AUTO).

Materials and Methods

Animal Experimentation and Groups of Study

In vivo experiments were carried out on wild-type, adult, male New Zealand rabbits of 6 months of age, weighing approximately 4 kg. Animals were kept at the Experimental Unit of University Hospital, Spain, where animal welfare was guaranteed at all times. Animal studies fulfilled European Union (EU) and Spanish guidelines for ethical animal care (EU Directive 63/2010; RD 53/2013). Details were previously published following the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) Guidelines 2.0 for reporting animal research

[20]. This study was approved by the Ethics Committee for Animal Research (Junta de Andalucía), protocol code 17/12/2024/178; date of approval: 20 December 2024 and by the regional ethics committee CCEIBA (Comité Coordinador de Ética de la Investigación Biomédica de Andalucía), ref. 0268-N-23 (date of approval 13th April 2023). Four groups of study were established, with 5 animals randomly assigned to each experimental group (20 in total). Randomization was carried out by assignment numbers, and group assignment was not blinded to the researchers. As a preliminary exploratory study, sample size was not calculated. All animals were analyzed individually (the experimental unit was each single animal). No exclusion criteria were established, and all animals in each experimental group were analyzed with no exclusions.

- Positive control (P-CTR): native animals in which the urethra was not subjected to any surgical intervention.
- Negative control (N-CTR): animals in which the ventral side of the penis urethra was surgically sectioned to create a longitudinal defect of approximately 1 cm of length. This defect was left unrepaired, and the soft tissues and the skin surrounding the urethra were repaired and replaced to their original sites, using reabsorbable PDS suture material (ref. Z303H, Ethicon, Raritan, NJ, USA).
- AUTO: animals in which the ventral side of the penis urethra was surgically sectioned to create a longitudinal defect of approximately 1 cm of length. This defect was repaired by grafting a fragment of autologous OM obtained from the same animal, in the same intervention, using PDS suture material. Once the urethra was repaired, the soft tissues and the skin surrounding the urethra were repaired and replaced to their original sites, using the same suture material.
- Acellular multilayered urethral substitute graft (UREG): animals in which the ventral side of the penis urethra was surgically sectioned to create a longitudinal defect of approximately 1 cm of length. This defect was repaired by grafting an acellular urethral substitute generated by tissue engineering as described below, using PDS suture material. Once the urethra was repaired, the soft tissues and the skin surrounding the urethra were repaired and replaced to their original sites, using the same suture material.

All surgical procedures were performed on animals subjected to deep general anesthesia using ketamine (25 mg/kg) (Imalgene®, Boehringer Ingelheim, Ingelheim am Rhein, Germany) and xylazine (5 mg/kg) (Xilagesic®, Calier, Barcelona, Spain), and analgesia with magnesium metamizole (1.6 mg/mL) (Nolotil®, Boehringer Ingelheim) was applied for 48 h after surgery. Details on the surgical procedures were previously published. Animals were kept in the animal facility for a follow-up time of 3 months. Then, animals were deeply anesthetized and euthanatized with a euthanasia solution (Eutanax 200®, Fatro Ibérica, Barcelona, Spain).

Generation of an Acellular Multilayered Urethral Substitute by Tissue Engineering

An acellular multilayered urethral substitute was generated by tissue engineering, as previously reported [21]. In brief, we first generated fibrin biomaterials by mixing 760 μL of human plasma obtained from healthy donors with 125 μL of Dulbecco's Modified Eagle Medium (DMEM) (D6429, Merck, Darmstadt, Germany), 15 μL of tranexamic acid (Amchafibrin™, Fides-Ecofarma, Spain), 50 μL of 2% calcium chloride in water (211221.1210, Panreac Química S.L.U., Barcelona, Spain) and 50 μL of phosphate-buffered saline (PBS) (D8662, Merck). Fibrin-agarose biomaterials were generated by replacing the 50 μL of PBS by 50 μL of melted 2% type-VII agarose (A0701, Merck) in PBS. In both cases, the mixture was carefully aliquoted in culture plates (83.3920.300, Sarstedt, Nümbrecht, Germany) and allowed to jelly for 24 h in a cell incubator at 37 °C with 5% CO_2 . Then, a multilayered structure was generated by plastic compression nanostructuration, as described elsewhere [21]. In short, a fibrin-agarose hydrogel was placed on a polyamide membrane with a pore diameter of 0.2 μm (WHA10414012, Merck) sited on top of several layers of sterile filter paper (84783, Thermo Fisher Scientific, Waltham, MA, USA). Then, a fibrin hydrogel was placed on top of the previous fibrin-agarose biomaterial, and another fibrin-agarose hydrogel was set on the fibrin hydrogel. Another nylon membrane and several layers of sterile filter paper were carefully placed on top the hydrogels, and 500 g of weight were applied for 3 min to induce the physical fusion of the three hydrogels to form a multilayered structure.

Variables of Study

The following parameters were quantified in each animal ($n = 5$ for each analysis method). In order to reduce potential confounders, all animals were randomly assigned to an experimental group before these variables were analyzed:

(A) Surgical and functional parameters: feasibility of the intervention, determined using a previously published Likert-like scale ranging from 1 (unfeasible) to 5 (easily performed); animal tolerance to the procedure, evaluated in terms of mortality and complications (infections, dehiscence, hemorrhage, necrosis); anatomic inspection of the urethra to identify its macroscopic appearance (normal, presence of fistulae, stenosis or dilatations); and quality of urine voiding, evaluated by inspecting the urinary stream after filling the bladder with commercial povidone-iodine solution (Betadine®, Cooper Consumer Health, Paris, France) and the presence of abnormal urination, as determined by inspecting the urine stain patterns left by the animals in the absorbent paper deposited under each cage [22]. In this case, the stains were classified as: 1 (normal), defined as large stains concentrated in one corner of the paper, 2 (partially abnormal), defined as large stains scattered

across the paper, and 3 (abnormal), defined as small stains dispersed throughout the paper.

(B) Histological parameters. The bladder and urethra were excised from each animal and fixed in neutral formalin. Transversal sections corresponding to the region of the penile urethra where the surgical procedure was performed were obtained and immersed in paraffin. Thin sections (2 μm -thick) were then obtained using a microtome, mounted on glass slides (MAD-15-188-55/100, Vitro MD, Granada, Spain), dewaxed in xylene (251769.2714, Panreac), and rehydrated using decreasing concentrations of ethanol (141086.1214, Panreac). For a global analysis of tissue structure, samples were stained with hematoxylin and eosin (H&E) and Masson's trichrome method, following standard protocols. For scanning electron microscopy (SEM), transversal penile sections were obtained at the graft region, fixed in 2.5% glutaraldehyde (253857.1611, Panreac), washed in cacodylate buffer (C-0250, Merck), dehydrated with acetone (131007.1611, Panreac), dried using the critical point method, and sputter-coated with gold and palladium. Samples were analyzed using a desktop scanning electron microscope (NANOS®, Sempior, Nuenen, Netherlands). The surface of the epithelial cells was analyzed to determine the specific differentiation pattern found on these cells, according to a previous classification described for human mucosae [23].

(C) Histochemical parameters. To stain the main fibrillar and non-fibrillar components of the tissue extracellular matrix (ECM), samples were subjected to histochemical methods, as previously described [24–26]. Briefly, collagen fibers were identified using picosirius red staining (PSR), elastic fibers were stained with the histochemical method of Verhoeff (VER), reticular fibers were identified with the metal reduction method of Gomori (RET), proteoglycans were stained with alcian blue (AB), and the periodic acid-Schiff (PAS) method was used to identify basement membrane glycoproteins.

(D) Immunohistochemical parameters. To identify specific components of each tissue, samples were subjected to indirect immunohistochemistry using specific primary antibodies. In brief, tissue sections were subjected to antigen retrieval with ethylenediaminetetraacetic acid (EDTA) (AGEL3554, Bionova, Madrid, Spain), citrate (MAD-004071R, Vitro) or Tris-EDTA (MAD-004070R, Vitro) buffer at 98 °C (for 25 min) or with chondroitinase ABC (C3667, Merck), endogenous peroxidase was quenched for 10 min in 1:9 hydrogen peroxide (131077.1211, Panreac) in PBS, and samples were preincubated for 30 min in normal horse serum (S-2012, Vector Labs, Newark, CA, USA) and casein (SP-5020, Vector Labs). Primary antibodies were applied overnight at 4 °C, and a ready-to-use secondary antibody mixture conjugated with peroxidase (MP-7401-50, Vector Labs) was used for 1 h at room temperature. Signal was revealed using a diaminobenzidine (DAB) substrate kit (SK-4105, Vector Labs), and tissues were slightly

counterstained with Harris hematoxylin (6765002, Epredia, Portsmouth, NH, USA). Primary antibodies and specific conditions used are shown in **Supplementary Table 1**.

In all cases, slides were dehydrated in ethanol, cleared in xylene and coverslipped using histological mounting medium (A20100, Deltalab, Barcelona, Spain), and images were obtained using a Panoramic DESK II DW slide scanner (3D Histotech, Budapest, Hungary).

Quantification and Statistical Analysis

Results corresponding to the immunohistochemical analyses were semiquantitatively assessed as strong positive signal (+++), positive signal (++), mildly positive signal (+), or negative (–), as previously reported [15,24,27]. Quantification analyses were performed by three independent histologists. For the histochemical analyses using PAS, PSR and AB staining methods, we quantified the staining signal in each study group using ImageJ software (National Institutes of Health, Bethesda, MD, USA), as previously reported [24]. In brief, microscopic images were obtained using the same conditions (exposure, light intensity, etc.) for each study group, and the area of interest was selected using the multi-point tool of the software. Then, we used the automatic quantification tool of the software to determine the mean staining intensity in each image. A total of 20 measurements were obtained from each study group.

Quantitative results were analyzed for normality using the Shapiro-Wilk and D'Agostino-Pearson tests. As most variables demonstrated to be non-normally distributed, non-parametric statistics was used. For the feasibility results and histochemical analyses, statistical differences between two specific groups were assessed using Mann-Whitney *U* tests. For the comparison of results expressed as percentages (fistulas and urine stain patterns), we used the Exact Test of Fisher. In order to mitigate the risk of type I error arising from multiple comparisons, we applied Bonferroni adjusted *p* values, and values below 0.01 were considered as statistically significant for the double-tailed tests. Statistical analyses were carried out using the Real Statistics Resource Pack software (Release 7.2) available at <https://www.real-statistics.com/> (Purdue University, West Lafayette, IN, USA).

Results

Surgical and Functional Analyses

Results of the surgical and functional analyses are summarized in **Supplementary Table 2**. Analysis of the feasibility of the surgical procedure revealed that the surgical intervention was feasible in all groups, with an average score of 4.80 ± 0.45 in the Likert-like scale used in the present work in the N-CTR group, 4.20 ± 0.45 in the AUTO group and 3.80 ± 0.84 in the UREG group. Differences among the three groups were not statistically significant ($p > 0.01$ for the Mann-Whitney test). All animals tolerated very well the procedure, and mortality was zero in all

groups. No infections, dehiscence, hemorrhage, necrosis or other complications were detected in any of the animals.

When the urethra of the animals was macroscopically inspected at the end of the follow-up time, we found that none of the animals showed urethral stenosis or dilations, and all urethras could be easily catheterized. However, two animals (40%) in the N-CTR group and two animals (40%) in the AUTO group showed urethral fistulas, whilst none of the animals in the P-CTR and UREG groups showed fistulas. Differences between UREG and N-CTR or AUTO groups were statistically significant ($p < 0.0001$ for the exact test of Fisher), but not between UREG and P-CTR. Macroscopic images corresponding to each group are shown in Fig. 1.

In addition, analysis of the urinary stream confirmed that all animals had a normal stream morphology (**Supplementary Video 1**), except for 2 animals in the N-CTR group and 2 animals in the AUTO group that showed a normal main stream, associated with a secondary stream arising from the urethral fistula (**Supplementary Video 2**). Assessment of the urine stains left in the absorbent paper (Fig. 1) revealed that 3 animals (60%) in the N-CTR and 3 animals (60%) in the AUTO group had abnormal or partially abnormal urine stains (1 abnormal and 2 partially abnormal in each group), whereas 4 animals (80%) in the UREG group left normal stains, and 1 (20%) had partially abnormal stains. The statistical analysis showed that the UREG group was significantly different to the N-CTR and AUTO groups ($p = 0.0032$ for the exact test of Fisher).

Histological Evaluation of the Urethral Structure in Each Study Group

The morphostructural analysis of the urethra of each group of animals at the implant site first revealed that the native, normal urethra corresponding to the P-CTR group consisted of an epithelial layer of columnar cells organized in 1 to 4 cell layers, and a subjacent lamina propria or stromal layer with abundant cells scattered throughout a dense ECM and blood vessels (Fig. 2). In turn, the urethra of the N-CTR group was very thin, with only 1–2 cell strata of cubic or flattened cells, and the lamina propria apparently contained lower amounts of cells. For the AUTO group, we found a very thick epithelial cell layer containing around 20 cell strata, and cells were organized in a basal layer, stratum spinosum, and stratum granulosum, and a lamina propria with few scattered cells. In contrast, the UREG group displayed an epithelial layer with 1–4 layers of columnar cells and abundant stromal cells within a dense ECM with numerous vessels, similar to the N-CTR group. When the acellular multilayered urethral substitute kept *ex vivo* was analyzed, we found a dense acellular biomaterial in which all layers were firmly joined and attached to each other. Finally, the OM graft obtained from the oral cavity of each AUTO animal displayed the typical histological structure of the native OM, with a stratified epithelium with around

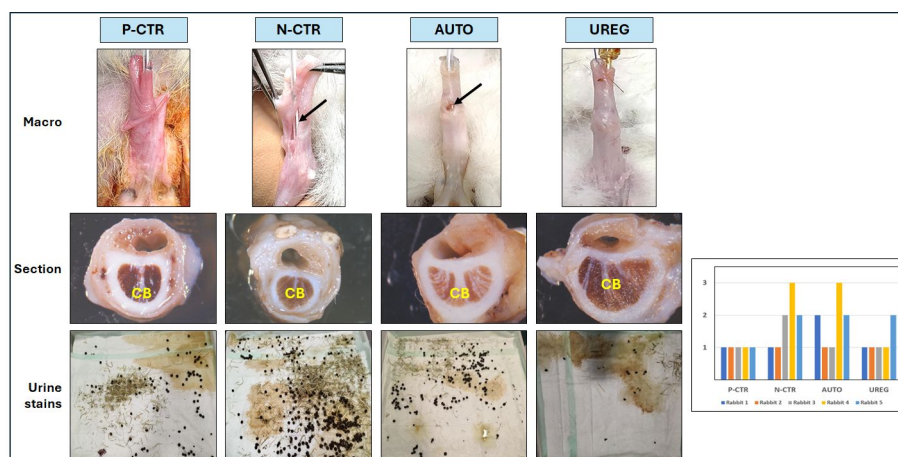


Fig. 1. Assessment of the urethra and the urine staining patterns of animals included in each study group. Macro: macroscopic appearance of the penis of each animal, after placing a urinary catheter into the urethra. Black arrows indicate the presence of urethral fistulas; Section: transverse section of the penis at the level of the urethral graft. CB: cavernous bodies; Urine stains: representative urine staining patterns for each study group. Stains corresponding to the P-CTR and UREG groups are large and concentrated in one corner of the paper (considered normal), whereas the image shown in the N-CTR group had large and dispersed stains (partially abnormal), and the image shown in the AUTO group shows small, scattered stains (abnormal). P-CTR: positive control group of native animals not subjected to any surgical intervention; N-CTR: negative control group of animals with an unrepaired longitudinal urethral defect; AUTO: animals in which the urethral defect was repaired with an autologous oral mucosa graft; UREG: animals in which the urethral defect was repaired with an acellular urethral substitute generated by tissue engineering. The histogram on the right shows the raw urinary stain scores for each animal in each study group, classified as follows: 1 (normal), characterized by large stains concentrated in a single corner of the paper; 2 (partially abnormal), characterized by large stains scattered across the paper; and 3 (abnormal), characterized by small stains dispersed throughout the paper. This figure was generated using Microsoft Office 365 PowerPoint version 2605 (Microsoft Corp., Redmond, WA, USA).

40 cell strata differentiated into the basal layer, stratum spinosum, stratum granulosum, and stratum corneum, and a dense lamina propria with abundant cells and blood vessels (Fig. 2).

SEM analysis of the urethral surface at the implant site (Fig. 3 and **Supplementary Table 3**) showed that the urethra was covered by epithelial cells in all groups, although some differences were detected among groups. Specifically, we found that cells in the P-CTR group were elongated and well organized, and their surface was covered with abundant smooth linear ridges and microplicae and microridges organized in a fairly uniform, parallel orientation, resembling a type-II surface pattern. The surface of cells in the N-CTR group was rather flat and less defined, and was mostly devoid of any surface patterns, with some cells showing poorly-defined parallel microridges. In the AUTO group, cells were wider and thicker than those in the control groups, exhibiting a flat, squamous-like morphology, and were covered with abundant small pits, arranged in an irregular, granular pattern, compatible with a pattern type V. Finally, UREG urethral cells were elongated and well organized, thicker than those in the P-CTR group, but thinner than cells in the AUTO group, and their surface was rough and granulated, with few microplicae and microridges that tended to form parallel structure, and was consistent with a mixed pattern between type I and type II. On the other hand,

the urethral stroma contained abundant collagen bundles, without differences among groups, and the cavernous bodies contained high amounts of red blood cells within wide cavities in all groups.

Analysis of Relevant Markers of the Urethral Epithelium in Each Study Group

Analysis of the epithelial layer of the urethral mucosa in each group of study and control OM tissues using immunohistochemistry revealed several differences among groups (Fig. 4 and Table 1). First, the analysis of cytokeratins (CKs) using PANCK antibodies revealed that all samples, including the OM control, were strongly positive (+++) for this marker, with few differences among groups. When CK1 was analyzed, we found that all groups were mildly positive (+), except for the OM control tissues, which were positive (++). In turn, the expression patterns found for CK7 and CK8 were very similar, with strongly positive signal found in P-CTR, N-CTR and UREG, and negative signal in OM samples, although the AUTO group was positive for CK7 and mildly positive for CK8. When CK15 was evaluated, we found a positive signal for P-CTR, AUTO and UREG, and a mildly positive signal for N-CTR and OM. In turn, CK19 was strongly positive in the P-CTR, N-CTR and UREG groups, and positive in the AUTO group and in control OM tissues. Assessment of involucrin (IVL)

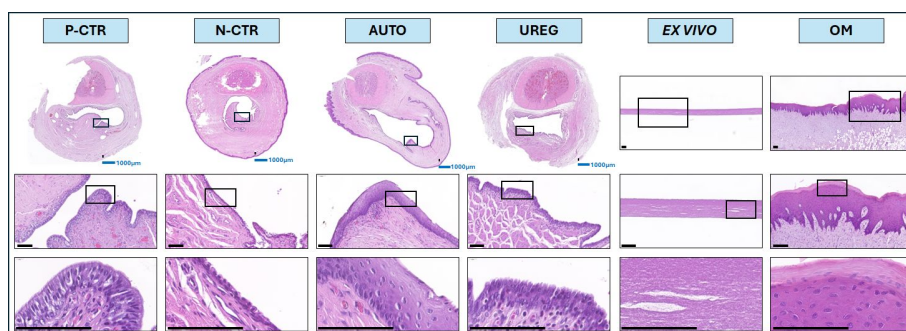


Fig. 2. Histological analysis of the penile urethra of animals included in each study group, and the acellular multilayered urethral substitute and OM grafts using hematoxylin and eosin (H&E) staining. Images of the urethra at the graft site are shown at increasing magnifications. Black squares correspond to the higher-magnification images displayed below each image. P-CTR: positive control group of native animals not subjected to any surgical intervention; N-CTR: negative control group of animals with an unrepaired longitudinal urethral defect; AUTO: animals in which the urethral defect was repaired with an autologous oral mucosa graft; UREG: animals in which the urethral defect was repaired with an acellular urethral substitute generated by tissue engineering; *EX VIVO*: acellular multilayered urethral substitute generated by tissue engineering and kept *ex vivo*; OM: oral mucosa grafts. Scale bars: 100 μm . For the lower magnification *in vivo* images, additional scale bars corresponding to 1000 μm are shown in blue. This figure was generated using Microsoft Office 365 PowerPoint version 2605.

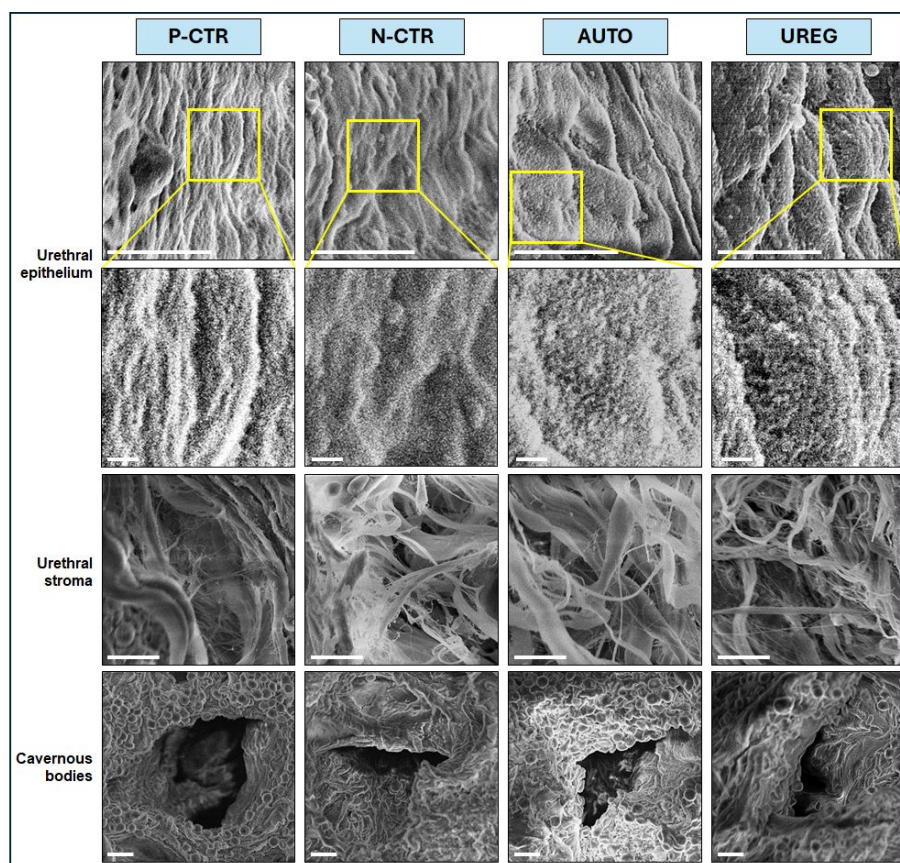


Fig. 3. Scanning electron microscopy analysis of animals in each group of study. Images are shown for the surface of the urethral epithelium, fibers in the urethral stroma, and cavernous bodies. For the urethral epithelium, yellow squares correspond to the areas shown at higher magnification in the images below. P-CTR: positive control group of native animals not subjected to any surgical intervention; N-CTR: negative control group of animals with an unrepaired longitudinal urethral defect; AUTO: animals in which the urethral defect was repaired with an autologous oral mucosa graft; UREG: animals in which the urethral defect was repaired with an acellular urethral substitute generated by tissue engineering. Scale bars: 10 μm for the top urethral epithelium images and for the stroma and cavernous bodies, and 1 μm for the higher magnification images of the urethral epithelium. This figure was generated using Microsoft Office 365 PowerPoint version 2605.

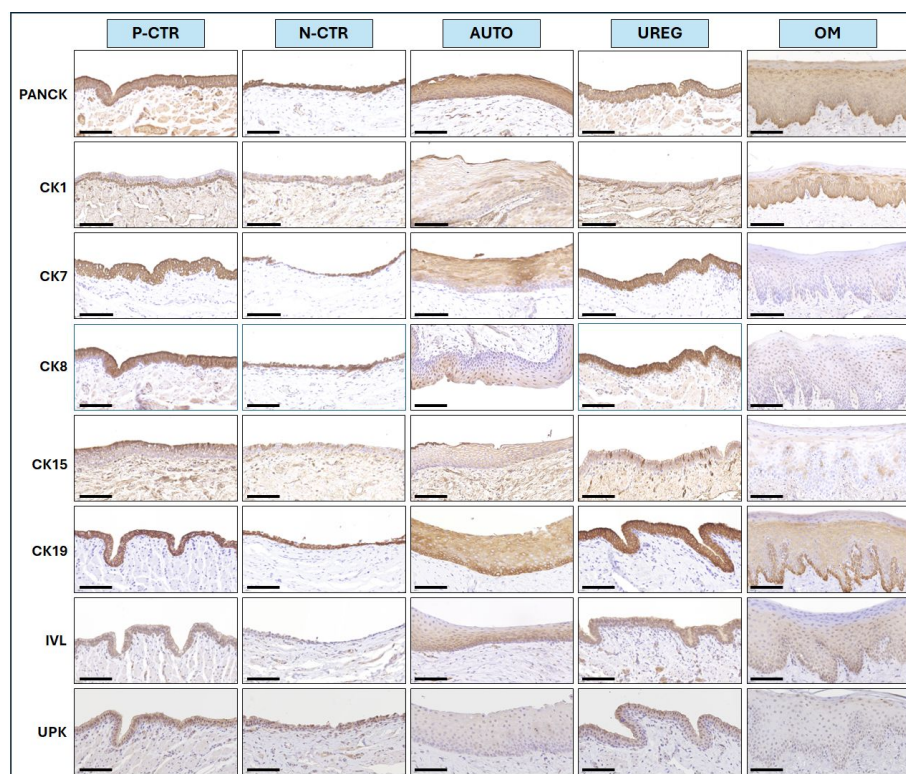


Fig. 4. Immunohistochemical analysis of several epithelial markers in the urethral epithelium of each group of animals and native OM. Images are shown for the analysis of pancytokeratin (PANCK), CKs 1, 7, 8, 15 and 19, involucrin (IVL) and uroplakin (UPK). P-CTR: positive control group of native animals not subjected to any surgical intervention; N-CTR: negative control group of animals with an unrepaired longitudinal urethral defect; AUTO: animals in which the urethral defect was repaired with an autologous oral mucosa graft; UREG: animals in which the urethral defect was repaired with an acellular urethral substitute generated by tissue engineering; OM: oral mucosa grafts. Scale bars: 100 μ m. This figure was generated using Microsoft Office 365 PowerPoint version 2605.

revealed a strongly positive signal in P-CTR and UREG, a positive signal in AUTO and OM, and a mildly positive staining signal in N-CTR. Finally, we found strongly positive uroplakin (UPK) expression in the P-CTR, N-CTR, and UREG groups, whereas the AUTO and OM samples were mildly positive.

Evaluation of the Basement Membrane in Each Study Group

Evaluation of the basement membrane of the different samples analyzed in the present work using PAS histochemistry (Fig. 5 and Table 2) confirmed the presence of this structure at the stomal-epithelial interface of each sample. When the PAS staining intensity was quantified, we found that the native OM had significantly higher PAS intensity than P-CTR and AUTO, whereas differences among the other groups of study were not significant ($p > 0.01$).

Analysis of Relevant Components of the ECM at the Stromal Layer in Each Study Group

In order to characterize the ECM of the stromal layer, we first quantified the presence of collagen fibers using PSR (Fig. 5 and Table 2). Results showed that the PSR histochemical signal was similar in P-CTR and UREG, whilst

N-CTR showed significantly higher amounts of collagen. The analysis of proteoglycans as determined by AB histochemistry revealed that P-CTR samples contained similar amounts of these components than AUTO, and N-CTR was comparable to OM. However, the AB signal was significantly higher in N-CTR and OM than in P-CTR, and UREG was significantly lower than P-CTR. Then, we analyzed the presence of relevant proteins of the stroma ECM using immunohistochemistry (Fig. 6 and Table 1). Results of these analyses showed that P-CTR was positive for versican (VCAN), biglycan (BGN), and decorin (DCN), whereas N-CTR was mildly positive for VCAN and positive for BGN and DCN, AUTO was positive for VCAN and mildly positive for BGN and DCN, and UREG samples were positive for these three markers. When control OM was analyzed, we found a mildly positive signal for VCAN and DCN and a strong signal for BGN.

In addition, our analysis of blood vessels using anti-CD31 primary antibodies revealed that all urethra samples contained abundant amounts of vessels, although some differences were detected among groups (Fig. 6). First, we found that P-CTR and control OM samples contained 3.20 ± 0.95 and 2.85 ± 1.23 vessels per area of $35 \mu\text{m}^2$, respectively, with non-significant differences between these sam-

Table 1. Semiquantitative analysis of the immunohistochemical signal for pancytokeratin (PANCK), cytokeratin (CK) 1, 7, 8, 15 and 19, IVL and UPK in the urethral epithelium, and versican (VCAN), biglycan (BGN) and decorin (DCN) in the stromal layer of each group of animals and native OM.

Markers		P-CTR	N-CTR	AUTO	UREG	OM
Epithelial markers	PANCK	+++	+++	+++	+++	+++
	CK1	+	+	+	+	++
	CK7	+++	+++	++	+++	–
	CK8	+++	+++	+	+++	–
	CK15	++	+	++	++	+
	CK19	+++	+++	++	+++	++
	IVL	+++	+	++	+++	++
	UPK	+++	+++	+	+++	+
Stromal components	VCAN	++	+	++	++	+
	BGN	++	++	+	++	+++
	DCN	++	++	+	++	+

P-CTR: positive control group of native animals not subjected to any surgical intervention; N-CTR: negative control group of animals with an unrepaired longitudinal urethral defect; AUTO: animals in which the urethral defect was repaired with an autologous oral mucosa graft; UREG: animals in which the urethral defect was repaired with an acellular urethral substitute generated by tissue engineering; OM: oral mucosa grafts. The staining intensity was assessed as strong positive signal (+++), positive signal (++), mildly positive signal (+), or negative (–).

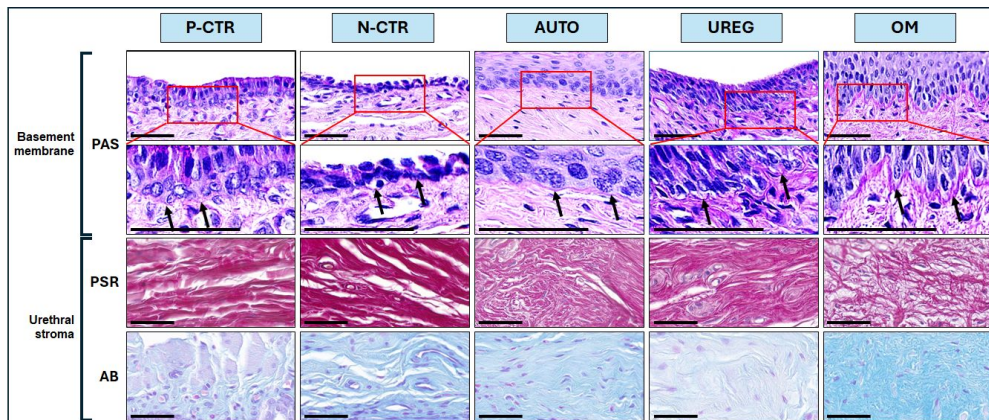


Fig. 5. Histochemical analysis of the basement membrane and urethral stroma as determined by PAS staining, picrosirius red (PSR) and AB in each group of animals and native OM. For PAS, the higher magnification images correspond to the red inserts in the lower magnification images. Arrows highlight illustrative areas of positive PAS signal. P-CTR: positive control group of native animals not subjected to any surgical intervention; N-CTR: negative control group of animals with an unrepaired longitudinal urethral defect; AUTO: animals in which the urethral defect was repaired with an autologous oral mucosa graft; UREG: animals in which the urethral defect was repaired with an acellular urethral substitute generated by tissue engineering; OM: oral mucosa grafts. Scale bars: 50 μ m. This figure was generated using Microsoft Office 365 PowerPoint version 2605.

ples. Then, our analysis revealed that the N-CTR, AUTO and UREG groups showed significantly higher amounts of blood vessels than P-CTR and OM (4.90 ± 1.21 , 4.70 ± 1.66 and 4.85 ± 1.35 , respectively), and differences among N-CTR, AUTO and UREG were non-significant.

Analysis of the Muscular Layer in Each Group of Animals

Analysis of the different samples using anti-smooth muscle actin (SMA) antibodies showed the presence of a muscular layer surrounding the urethral mucosa in all study

groups, but not in the OM control tissues (Fig. 6). Whereas the muscular layer was continuous in the P-CTR group, we found that the N-CTR and AUTO urethras had lost continuity and showed very few smooth muscle fibers at the site of the surgical procedure. In the case of the UREG group, some scattered bundles of muscle fibers were found at the site of the surgery, although these bundles seemed not to be connected to each other, and continuity was also lost in this group.

Table 2. Quantitative analysis of the histochemical signal obtained for PAS, at the basement membrane, and picrosirius red (PSR) and AB, at the stromal layer of each group of animals.

Groups	PAS	PSR	AB
P-CTR	60 ± 22.5	109.5 ± 40.68	32.7 ± 14.13
N-CTR	71.65 ± 32.14	155.3 ± 22.8	49.75 ± 14.77
AUTO	55.85 ± 9.58	86.35 ± 17.77	36.05 ± 9.76
UREG	71.15 ± 31.07	109.9 ± 17.39	22.95 ± 6.59
OM	89.5 ± 25.73	77.5 ± 31.03	57.35 ± 12.41
P-CTR vs. N-CTR	0.2012	<0.0001*	0.0001*
P-CTR vs. AUTO	0.4777	0.0304	0.1918
P-CTR vs. UREG	0.1274	0.7381	0.0039*
P-CTR vs. OM	0.0002*	0.0103	<0.0001*
N-CTR vs. AUTO	0.0810	<0.0001*	0.0026
N-CTR vs. UREG	0.9042	<0.0001*	<0.0001*
N-CTR vs. OM	0.0596	<0.0001*	0.0763
AUTO vs. UREG	0.0263	0.0001*	<0.0001*
AUTO vs. OM	<0.0001*	0.2534	<0.0001*
UREG vs. OM	0.0634	0.0004*	<0.0001*

P-CTR: positive control group of native animals not subjected to any surgical intervention; N-CTR: negative control group of animals with an unrepaired longitudinal urethral defect; AUTO: animals in which the urethral defect was repaired with an autologous oral mucosa graft; UREG: animals in which the urethral defect was repaired with an acellular urethral substitute generated by tissue engineering; OM: oral mucosa grafts. *p* values for the statistical comparison of the results obtained in two specific groups are shown in the lower rows, and statistically significant results are highlighted with asterisks (*).

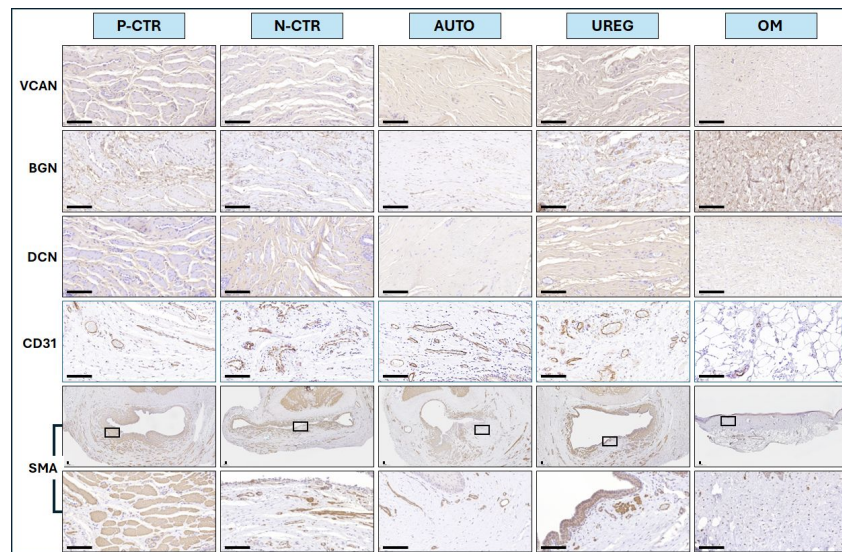


Fig. 6. Immunohistochemical analysis of relevant components of the ECM and blood vessels in the stromal layer and muscle fibers in each study group. Images are shown for the analysis of VCAN, BGN, and DCN, and for blood vessels showing positive signal for CD31. The lower rows correspond to the immunohistochemical identification of smooth muscle fibers of the urethra using smooth muscle actin (SMA) immunohistochemistry. For SMA, images are shown at two magnifications, and black squares correspond to the higher-magnification images displayed below. P-CTR: positive control group of native animals not subjected to any surgical intervention; N-CTR: negative control group of animals with an unrepaired longitudinal urethral defect; AUTO: animals in which the urethral defect was repaired with an autologous oral mucosa graft; UREG: animals in which the urethral defect was repaired with an acellular urethral substitute generated by tissue engineering; OM: oral mucosa grafts. Scale bars: 100 μ m. This figure was generated using Microsoft Office 365 PowerPoint version 2605.

Discussion

Development of novel regenerative strategies allowing a regenerative treatment of severe urethral diseases is one of the main areas of research in human urology. Since the preliminary description of these methods applied to the field of urology [28], several researchers attempted to generate a human urethra by tissue engineering using different approaches [29–31]. However, generation of an urethral substitute consisting of several layers has been very seldom achieved, and full-thickness multilayered urethral substitutes have been only tested *ex vivo* [21].

In the present work, we have generated an acellular multilayered urethral substitute based on fibrin-agarose biomaterials subjected to nanostructuration. Based on plastic compression methods, nanostructuration has been demonstrated to be useful for the generation of multilayered structures, as previously demonstrated by our group for the hard palate [18] and the human urethra [21]. Although the multilayered human urethra generated with these methods showed potential usefulness, *in vivo* characterization in laboratory animals is one of the requirements of the European Medicines Agency (EMA) for the clinical translation of advanced therapies medicinal products (ATMP).

In this regard, the analysis of the UREG grafted on the urethra of animals subjected to severe urethral damage allowed us to demonstrate that the procedure was feasible, and the implant of this product was safe for the host animals, and no severe side effects were detected in any of the animals. Biosafety is probably the main requirement for novel ATMP, and novel tissue-engineered products should be demonstrated to be safe in laboratory animals before clinical translation [32]. In addition to biosafety, ATMP need to prove to be functional, and they should be able to potentially improve clinical outcomes, as compared to traditional treatments. Therefore, we analyzed the functional results of the novel UREG generated in this study by quantifying urethral fistulas and analyzing urine stains. Our results showed that UREG significantly reduced the incidence of fistulas and the proportion of abnormal urine patterns, as compared not only with N-CTR, but also with the gold-standard AUTO procedure. Although these results should be confirmed in a larger cohort of animals, our findings support the use of UREG bioartificial tissues for urethral repair, suggesting a better performance than autografts obtained from the animal OM.

Histologically, the native urethra had a stratified epithelium with columnar cells showing a surface pattern of epithelial differentiation. However, the urethral epithelium of N-CTR had important structural alterations, with few epithelial cell layers at the site of the intervention, whose surface was compatible with an immature tissue devoid of the typical differentiation patterns found in control tissues. These results suggest that the epithelium formed at the area of urethral damage was not comparable to a normal tissue and had significant alterations. When we analyzed the ep-

ithelium of the AUTO group, we found that this cell layer was more comparable to the native OM than to the urethra, with numerous strata showing different keratinocyte types, as it is the case in the OM, with a basal layer, stratum spinosum, and stratum granulosum, and highly differentiated surface patterns [23,33]. In contrast, the epithelial tissue found at the regeneration site of the UREG group was partially similar to that of the P-CTR group, although the surface pattern corresponded to a lower differentiation level than the native urethra. In general, these results support the use of a urethral substitute, and the results were more similar to the native urethral epithelium than the gold-standard AUTOs.

In addition, the analysis of typical epithelial markers using immunohistochemistry confirmed that the native urethra is characterized by the high expression of most of these markers, especially PANCK, CK7, CK8, CK19, IVL and UPK, as previously suggested [34,35]. On the contrary, the epithelial layer of N-CTR had lower expression of CK15 and IVL. Interestingly, the AUTO group showed several similarities with the native OM, with lower expression of CK7 and CK8 than P-CTR, and the expression pattern for CK19, IVL and UPK was similar between the AUTO and OM groups.

Strikingly, we previously demonstrated that OM grafts implanted in the urethra of laboratory rats tended to experience a progressive process of tissue transdifferentiation, and grafts partially lose their original OM phenotype and acquire an urethral-like phenotype after 60–90 days of follow-up [36]. However, the results of the current study suggest that the OM grafted in the rabbit urethra remains more similar to their original profile than to the urethral phenotype, although some changes were detectable, such as an increase in the expression of CK7, CK8 and CK15, and a decrease in CK1, suggesting that a progressive differentiation process could be ongoing. These results are in agreement with previous works carried out in laboratory rabbits demonstrating that the histological structure of OM grafts implanted in the urethra tends to retain its original histological structure, although the results were evaluated only after 6 weeks of the grafting procedure [37]. Additionally, the analysis of OM implanted for 12–36 months in the urethra of patients with severe urethral damage confirmed that the grafts retained their original phenotype and histological structure, and were more similar to an oral tissue than to the urethral epithelium [38]. Most likely, the intrinsic differences among species could explain these functional differences, and confirm that results obtained in a specific animal species cannot be directly translated to the human setting [39]. In this regard, we could hypothesize that the transdifferentiation process could take different periods of time in each animal species. Future studies should be carried out using longer follow-up times to determine if the OM grafted in the urethra of laboratory rabbits could eventually result in a tissue fully resembling the native urethra.

Regarding the animals in which the artificial tissue was grafted, our results confirmed that this tissue generated by tissue engineering resulted in a urethral epithelium that was very similar to the native tissue and had a similar expression pattern for the epithelial markers analyzed here. The fact that the expression profile of epithelial markers resembled that of the native urethra confirms the possibility of achieving urethral regeneration at the functional epithelial level by using the UREG technology. Although future studies are in need, these results suggest that the native urethral cells from the host animal could migrate and repopulate the grafted artificial tissue, resulting in a functional epithelial layer in this group of animals.

On the other hand, we evaluated both the basement membrane and the stromal tissue in each study group to determine the potential of each technique to promote urethral regeneration. A well-structured basement membrane showing a similar PAS staining signal to control urethras was found in the N-CTR, AUTO and UREG groups. Given that the basement membrane plays a crucial role in epithelial attachment [40], and the development of an adequate basement membrane is a key objective in tissue engineering [41], these findings suggest that epithelial attachment was likely adequate across all the study groups. Analysis of the stromal layer revealed that the staining pattern of P-CTR was comparable to that of the UREG group for PSR, indicating the presence of collagen fibers and the stromal proteins VCAN, BGN, and DCN, which typically associate with collagen fibers to form a mature ECM [42], although the amount of proteoglycans identified by AB histochemistry was significantly reduced in UREG compared to P-CTR. In contrast, N-CTR urethras showed significantly higher PSR and AB signal, along with reduced VCAN expression, whereas AUTO showed lower PSR staining, higher AB signal, and lower immunohistochemical expression of BGN and DCN. In general, these findings may imply that N-CTR may reflect a fibrotic response, characterized by an increased synthesis of collagen and proteoglycans, whereas AUTO may indicate insufficient collagen synthesis, as compared to control urethras. Conversely, the use of a bioartificial graft in the UREG group resulted in a more biomimetic regenerative tissue with appropriate amounts of most ECM markers analyzed here, with the exception of proteoglycans detected by AB. Interestingly, the presence of adequate amounts of collagen associated with VCAN, BGN and DCN suggests that these tissues may efficiently reproduce the biomechanical properties of the native urethra, which largely depend on these specific components [43]. Again, future work should determine whether the proteoglycan content of the UREG group normalizes after longer follow-up times.

One of the main requirements for a regenerative tissue to be functional is angiogenesis, as formation of new vessels in the grafted tissue directly impacts the integration and function of tissues implanted *in vivo* [44]. In this sense,

assessment of blood vessels in the tissues analyzed in this work demonstrated the presence of abundant vessels in all groups subjected to surgical procedures, with few differences among groups. It has been demonstrated that capillary sprouts from the host tissue can invade grafts within a few days, organizing a rich microvascular network in the implanted tissue [45]. Although we found that this vascular network was more abundant in the graft than in native tissues, it is expected that development and maturation of the grafted tissue will result in a progressive reduction of the density of vessels [45].

Another relevant question is whether the regenerative urethra was able to properly reproduce the structure of the muscular layer of this organ. The muscular layer surrounding the human urethra plays important roles as structural support for this organ, and in urinary control in both males and females [46]. Therefore, formation of a proper muscular layer should be one of the objectives of urethral tissue engineering. In this regard, our results suggest that the implant of an artificial tissue could improve formation of a muscular layer, as compared with N-CTR and AUTO, although none of the study groups showed the well-structured, continuous layer found in P-CTR. Although UREG animals included in the present study showed proper functional results, it may be possible that the lack of a well-structured muscular layer in this group could limit the future applications of this technology in patients with damaged urethral structure.

In summary, in the present study, we have generated and evaluated *in vivo* one of the first models of tri-laminar urethral substitutes described in the literature. Although these bioartificial tissues may have clinical potential, it will be necessary to carry out additional studies able to determine their usefulness in a real clinical setting. In addition, future studies should be carried out using a larger cohort of animals and longer follow-up times.

Conclusions

In this study, we generated and evaluated *in vivo* one of the first multilayered urethral substitutes generated by tissue engineering using nanostructured fibrin-agarose biomaterials, and we found that these novel substitutes -being easy and rapid to generate- can be used as efficient grafts for urethral repair. Once implanted, these artificial tissues were capable of promoting functional urethral regeneration in a penile urethral defect model. Compared with negative controls and gold-standard AUTOs, the multilayered urethral substitute showed superior functional outcomes, a more biomimetic epithelial phenotype, and a stromal architecture that more closely resembled native urethral tissue, although some components of the ECM and the muscular layer remain incompletely regenerated. These results support the use of this novel approach for future clinical translation in human urethral reconstruction.

List of Abbreviations

AB, alcian blue; AUTO, oral mucosa autograft; BGN, biglycan; CK, cytokeratin; DAB, diaminobenzidine; DCN, decorin; DMEM, Dulbecco's Modified Eagle Medium; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; IVL, involucrin; N-CTR, negative control; OM, oral mucosa; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; P-CTR, positive control; PSR, picrosirius red staining; RET, metal reduction method of Gomori; SMA, smooth muscle actin; UPK, uroplakin; UREG, acellular multilayered urethral substitute graft; VCAN, versican; VER, histochemical method of Verhoeff; EMA, European Medicines Agency; ARRIVE, Animal Research: Reporting of *in Vivo* Experiments; ATMP, advanced therapies medicinal products; SEM, scanning electron microscopy; H&E, hematoxylin and eosin; EU, European Union.

Availability of Data and Materials

The data supporting the findings of this study are openly available in the public repository Zenodo at <https://doi.org/10.5281/zenodo.19241543>, with the DOI number 10.5281/zenodo.19241543 with the license Creative Commons Attribution 4.0 International.

Author Contributions

OOA, JAMD, ELL, EDM, ODGG, MCSQ, MA, DSP and RFV planned and designed the research study; OOA, JAMD, FC, JCA, ELL, EDM and RFV performed the research and collected data; OOA, FC, JCA, ODGG, MCSQ, MA, DSP and RFV contributed to the analysis of the data. All authors contributed to manuscript preparation and editing. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee for Animal Research (Junta de Andalucía), protocol code 17/12/2024/178; date of approval: 20 December 2024) and by the regional ethics committee CCEIBA (Comité Coordinador de Ética de la Investigación Biomédica de Andalucía), ref. 0268-N-23 (date of approval 13th, April, 2023).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.22203/eCM.v059a10>.

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