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The effect of mechanical extension stimulation combined with epithelial cell sorting on outcomes of implanted tissue-engineered muscular urethras

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ABSTRACT

Urethral defects are common and frequent disorders and are difficult to treat. Simple natural or synthetic materials do not provide a satisfactory curative solution for long urethral defects, and urethroplasty with large areas of autologous tissues is limited and might interfere with wound healing. In this study, adipose-derived stem cells were used. These cells can be derived from a wide range of sources, have extensive expansion capability, and were combined with oral mucosal epithelial cells to solve the problem of finding seeding cell sources for producing the tissue-engineered urethras. We also used the synthetic biodegradable polymer poly-glycolic acid (PGA) as a scaffold material to overcome issues such as potential pathogen infections derived from natural materials (such as de-vascular stents or animal-derived collagen) and differing diameters. Furthermore, we used a bioreactor to construct a tissue-engineered epithelial–muscular lumen with a double-layer structure (the epithelial lining and the muscle layer). Through these steps, we used an epithelial–muscular lumen built *in vitro* to repair defects in a canine urethral defect model (1 cm). Canine urethral reconstruction was successfully achieved based on image analysis and histological techniques at different time points. This study provides a basis for the clinical application of tissue engineering of an epithelial–muscular lumen.

1. Introduction

Urethral defects are a common and disturbing clinical occurrence that usually arises secondary to urinary injury [1,2], tumor resection, infection or malformations [3]. Insufficient urethra rebuilding normally results in stricture and uroschesis that heavily impairs the quality of patients' life.

Traditional urethral reconstruction is limited by the shortage of an ideal repair tissue, but the rapidly expanding tissue-engineering technology provides tremendous opportunities to obtain satisfactory urethral tissue [4–7]. For example, an acellular bladder matrix achieved a high success rate in repairing anterior urethral strictures [8]. Oral epithelial- and muscle-derived cells have been used to construct a two-layered engineered urethra [9], and currently, tissue-engineered autologous urethras in the clinic have been successfully applied [10].

However, some limitations still exist in currently used urethral repair strategies and tissue-engineering techniques [11–13]. The important points of these strategies include avoiding urethral strictures and obtaining enough seed cells [13]. Reconstructive engineered urethras with a well-developed smooth muscle layer represent a good solution for urethral strictures, but smooth muscle cells (SMCs) are difficult to isolate and have lower proliferative ability; therefore, stem cells from different sources need to be differentiated into smooth muscle cells. During the recruitment of these cells, the use of adipose-derived stem cells (ADSCs) is favored because obtaining sufficient numbers of adipose stem cells is easy and minimizes donor site damage [14]. ADSCs have been proven to

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be a promising source of SMCs [15,16], as both chemical and mechanical stimulation can trigger ADSCs' differentiation into SMCs [16,17]. Recently, accumulating evidence has demonstrated that mechanical stimulation is important for maintaining the proper biomechanical function of tissues such as bone, cartilage, muscle and smooth muscle [18–20]. Mechanical stimulation has also been proven to be effective in initiating the differentiation of ADSCs into functional smooth muscle tissue [16,21–23].

A urethral stricture is generated by fibrogenesis; thus, not only a well-developed muscular layer but also a vital urethral epithelium is crucial for the prevention of this disorder. Therefore, it is essential to build a robust epithelium in the engineered urethra. Primary oral mucosal epithelial cells are a promising source of seed cells for the reconstruction of tissue-engineered epithelial-cell sheets [8,24–26], but their proliferation and differentiation potential is impaired by their complex composition [27]. Many cell lineages and cells at different differentiated states are present in primary epithelial cells, and many cells among them have relatively low stemness [27,28]. Therefore, sorting epithelial stem cells from primary oral mucosal epithelial cells using flow cytometry might be a potential strategy to improve the stemness of primary epithelial cells.

To obtain a well-developed muscular tissue-engineered urethra, we combined *in vitro* mechanical stimulation of ADSCs with primary epithelial seed cell sorting. We found that regular mechanical extension can induce ADSCs to differentiate into a muscular layer in a poly-glycolic acid (PGA) scaffold, and this muscular layer is superior to non-mechanical extension-induced control smooth muscle tissue. Furthermore, sorting of primary epithelial cells can increase their proliferation, survival ability and β1 integrin expression.

2. Materials and methods

2.1. Primary cell isolation and culture

Primary oral mucosal epithelial cells were obtained from beagle dogs. After general anesthesia, small pieces of oral mucous membrane were excised from the bucca of the dogs. The harvested oral mucosa was washed three times each with 0.25% chloromycetin solution and PBS, cut into small pieces and digested with Dispase-II (2.5 U/ml) for 15 h at 4 °C. The digestion was terminated by the addition of 10% fetal bovine serum (FBS). The suspended cells were filtered through a cell strainer before cultivation. The primary oral mucosal epithelial cells were cultured in keratinocyte serum-free medium (K-SFM, Gibco company, USA) supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF). Primary cells at passage 1–3 were used in the experiments.

After general anesthesia, primary ADSCs were obtained from fat tissue, which was isolated from the groin. The fat tissue was washed three times with 0.25% chloromycetin solution and PBS, then digested with 0.01% collagenase I for 1 h at 37 °C. The digested cells were collected after filtration through a cell strainer. The primary ADSCs were maintained in Dulbecco's modified Eagle's (DMEM) supplemented with 10% FBS, and primary ADSCs at passage 1 were used in the subsequent experiments.

All cells were cultured at 37 °C at a humidified atmosphere with 5% CO_2.

2.2. Flow cytometer sorting of primary oral mucosal epithelial cells

An RPE-tagged rat anti-dog integrin α6 antibody and a FITC-tagged mouse anti-dog CD71 antibody were added to primary epithelial cells (1×10^6/ml) for 30 min at 4 °C, then washed three times using PBS containing 4% FBS. After centrifugation at 1000 rpm for 5 min, the supernatant was removed, and the precipitate was resuspended in 200 μl cold PBS containing 4% FBS. The fluorescence intensity was detected by a flow cytometer (EPICS® ALTRA™, Beckman Coulter, US). The integrin α6 (positive)/CD71(negative) cells were separated by fluorescence-activated cell sorting, as a minor subpopulation of the oral mucosal epithelial cells.

2.3. Design of a mechanical extension bioreactor

To conduct mechanical extension stimulation, we first designed and manufactured a bioreactor that was composed of a peristaltic pump, a reaction chamber, a fluid collection bottle and connection ducts (Fig. 1A and B). After filling the ducts and chambers with culture medium, the engineered PGA tubes were connected to ducts in the reaction chamber (Fig. 1C). The mechanical extension was rhythmical and was produced by a peristaltic pump at 75 times/min, with a pressure of 0.02 Mpa. Before the engineered muscular urethras were produced, the bioreactor was sterilized and placed in a CO_2 incubator.

2.4. Two-layer epithelial–muscular urethras preparation

The scaffold was tube of fibrous PGA mesh of bulk density 50 mg cm^{-3}, porosity 95%, and fiber diameter 12–15 μm (Albany International Research, Mansfield, USA). The tube diameter was 5 mm, the tube length was 20 mm and the tube thickness was 1.6 mm. PGA scaffold was placed in an incubation chamber containing 20 ml DMEM added 10% FBS. ADSCs (2×10^7) were suspended in DMEM containing 10% FBS and seeded in the PGA tubes. ADSCs and epithelial cells were loaded on scaffold by layered seeding technology. These tubes were rotated 90° every 15 min. 20 ml DMEM

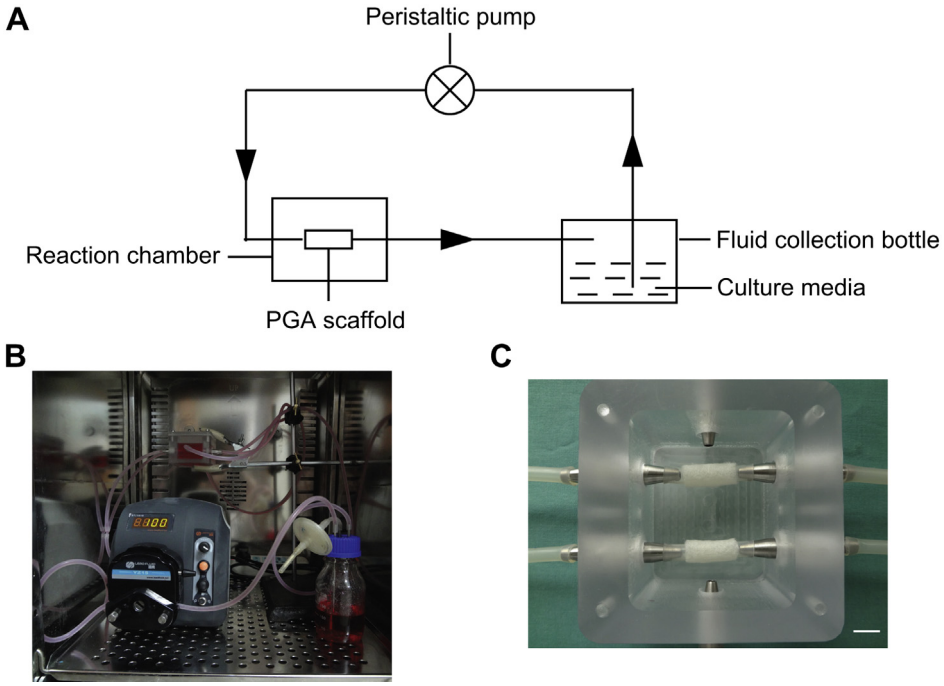

Fig. 1. Illustrations of the mechanical extension bioreactor. (A) Schematic diagram of the mechanical extension bioreactor. (B) An image of the mechanical extension bioreactor in an incubator. (C) An image of the reaction chamber; two PGA scaffolds are connected via ducts. Scale bar = 5 mm.

supplemented with 10% FBS was added 4 h later, and the medium was changed once a day for 1 week, after which the PGA tubes seeded with the cells were attached to the connection ducts of the reaction chamber. Mechanical extension was exerted 75 times/min for 3 weeks, and the medium was changed to DMEM containing 5% equine serum, 5% FBS and 10 μmol/L 5-azacytidine and cultured for 4 weeks. The control non-mechanical, extension-induced muscular urethras were produced using the same procedure but without the mechanical extension stimulation.

2.5. Cell proliferation assay

The sorted or non-sorted primary epithelial cells (3×10^3/well) were seeded in 96-well plates, and their proliferation was monitored by measuring optical density values after adding MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) according to the manufacturer's instructions.

2.6. Colony formation assays

The sorted or non-sorted primary epithelial cells were added in six-well plates at 200/well, and after 1 week, the colony numbers were calculated. The colony formation rate = colony number/200.

2.7. Immunocytofluorescence stain

Primary epithelial cells were fixed in 4% paraformaldehyde at room temperature for 10 min, followed by permeabilization with 0.25% Triton X-100 for 10 min. After blocking with 1% BSA for 30 min, primary mouse anti-canine antibodies against AE1/AE3 or integrin β1 were incubated at 4 °C overnight. Then, corresponding FITC- or PE-conjugated rabbit anti-mouse secondary antibodies were used. Nuclear staining was performed with DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride), and images were captured using a fluorescence microscope (IX70, Olympus, Japan).

2.8. Histochemistry and immunohistochemistry stains

Hematoxylin–eosin (HE) staining was performed on paraformaldehyde-fixed, paraffin-embedded sections. Masson trichrome staining was performed according to the manufacturer's instructions. PFPE sections were incubated with primary antibodies against desmin, α-SMA (Abcam) or AE1/AE3 (Abcam), followed by HRP-conjugated secondary antibodies. After staining development with diaminobenzidine, sections for immunohistochemistry staining were counterstained with hematoxylin. Images were obtained using a microscope

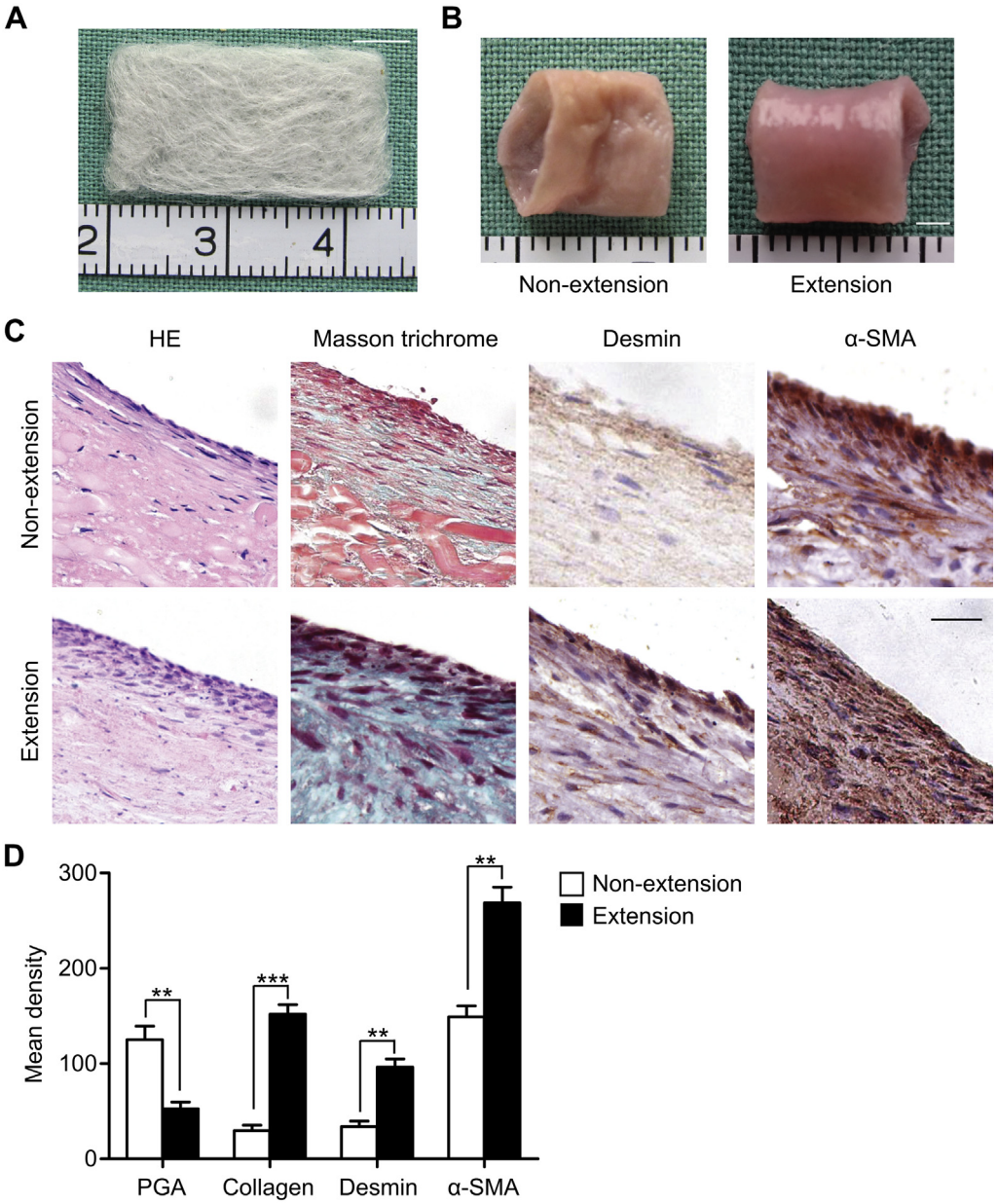

Fig. 2. Mechanical extension improved the viability of ADSC-derived SMCs in the engineered muscular tubes. (A) An image of the PGA scaffold. Scale bar = 5 mm. (B) Images of the extension-stimulated and unstimulated muscular tubes. Scale bar = 2 mm. (C) Images of histological and immunohistochemical staining of sections of extension-stimulated and unstimulated muscular tubes. Scale bar = 20 μm. (D) Quantification of the mean density of histological and immunohistochemical staining of these sections. $N = 3$. Values are means ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$.

(IX70, Olympus, Japan), and quantification was performed using the Image J software.

2.9. Scanning electron microscopy inspection

Scanning electron microscopy inspection was performed as previously reported [29] using a scanning electron microscope (XL30, Philips, Netherlands).

2.10. Canine model of engineered urethral implantation

One-year-old male beagle dogs were obtained from the laboratory animal center of Shanghai Jiao Tong University affiliated Sixth People's Hospital. All procedures were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University affiliated Sixth People's Hospital. All animals were housed under standard conditions according to the institution's approved guidelines.

Eighteen dogs were divided into two groups. The first group was implanted with mechanical extension-stimulated engineered urethras, whereas the second group was implanted with non-mechanical extension-stimulated engineered urethras. After intravenous general anesthesia (napental, 30 mg/kg), the dogs were fixed in a supine position, and the urethras were catheterized using 6F catheters. After sterilization, the urethras were exposed through a ventral longitudinal incision in the penis. Approximately 1 cm of the urethra was removed, and the engineered urethra was implanted to repair the defect. Urinary catheters were removed 7 days post-operation. The outcomes of three dogs in each group were evaluated by retrograde urethrography at 1, 2 and 3 months post-operation. After urethrography, these dogs were sacrificed to observe the survival of the implanted engineered urethras, and the implanted urethras were then removed for further histological observations.

2.11. Retrograde urethrography

The dogs were fasted for 12 h before performing urethrography, and after intravenous general anesthesia, 6F catheters were inserted in their bladders. Diatrizoate meglumine (76%) (Xdhelp, Shanghai, China) was diluted in 0.9% sodium chloride solution (1:1) and slowly injected in the urinary catheters under X-ray fluoroscopy. While the contrast medium was being injected, the catheters were slowly withdrawn until the repaired region was reached.

2.12. Statistical analyses

Significant differences were determined using the two-tailed Student's t test, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Mechanical extension affected ADSC-derived SMCs

We observed the effects of mechanical extension in SMCs induction. After isolating ADSCs, we assembled them into a tubularized bioabsorbable PGA scaffold (Fig. 2A) and cultured it in a mechanical extension-stimulated environment supplemented with chemical induction. After 8 weeks, we found that muscular tubes were formed in both the mechanical extension-stimulated group and the control group without extension stimulation, but the extension-induced muscular tubes were more robust at the macroscopic level (Fig. 2B). Using HE, Masson and immunohistochemical staining analyses, we discovered that the PGA scaffold adsorbed more thoroughly in extension-induced muscular tubes (Fig. 2C and D) and that these tubes exhibited increased expression of collagen fibers (Fig. 2C and D), desmin (Fig. 2C and D) and α-smooth muscle actin (α-SMA) (Fig. 2C and D). Desmin and α-SMA are markers of smooth muscle cells [30], and collagen fibers are the main structural constituents of urethras. Therefore, our results showed that mechanical extension combined with chemical induction can generate SMCs from ADSCs.

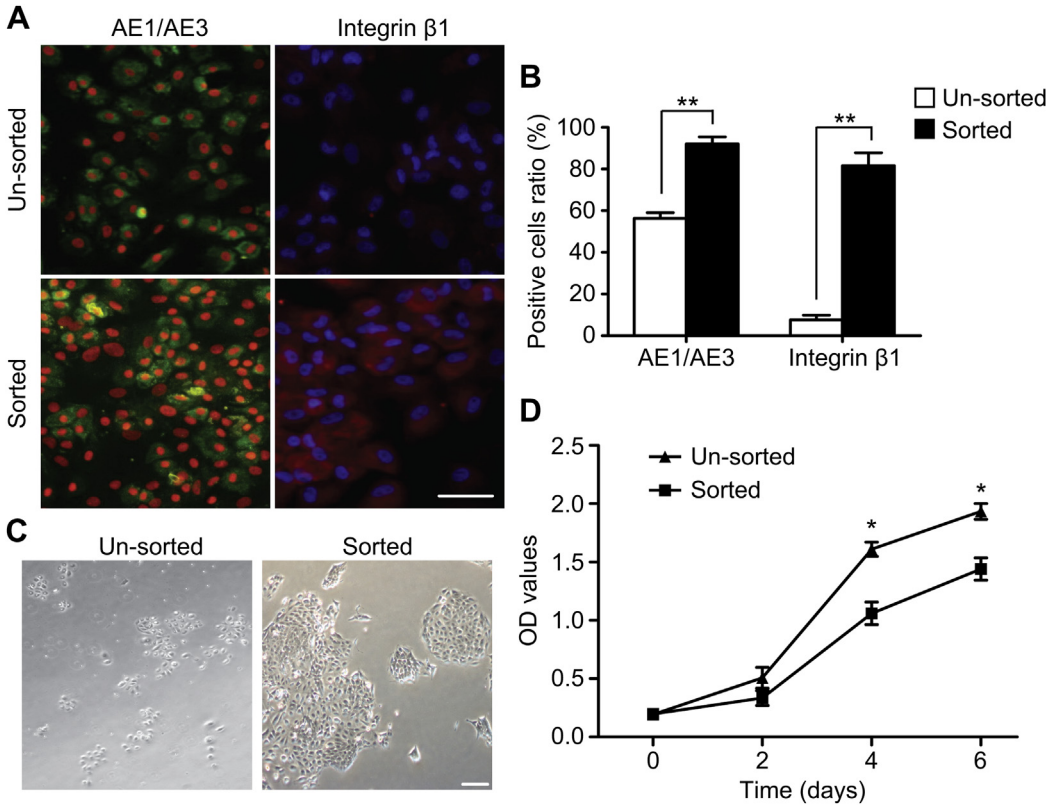

Fig. 3. Flow cytometer sorting ameliorated the stemness of primary oral epithelial cells. (A) Immunocytofluorescence staining images of sorted and un-sorted primary oral epithelial cells. In AE1/AE3 column green color shows AE1/AE3 positive in sorted and un-sorted primary oral epithelial cells; in integrin β1 column red color shows integrin β1 positive in sorted and un-sorted primary oral epithelial cells. Scale bar = 20 μm. (B) Quantification of the positive stained cells from (A). $N = 3$. Values are means ± S.E.M. $^{**}P < 0.01$. (C) Colony formation assays of sorted and un-sorted primary oral epithelial cells showed that larger colonies are formed by sorted epithelial cells. Scale bar = 50 μm. (D) Proliferation curves of sorted and un-sorted primary oral epithelial cells measured by MTT assays. $N = 3$. Values are means ± S.E.M. $^{*}P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Epithelial stem cells enrichment

Oral mucosal epithelial cells are a convenient source of tissue-engineered epithelial seed cells, and they have been successfully applied in mucosal membrane reconstructions [31]. However, the use of primary oral mucosal epithelial cells is limited by their relatively poor proliferation and differentiation potency. To resolve this limitation, we used a flow cytometer sorting technique to select the integrin α6+/CD71− subpopulation of cells. After selection, we cultured these cells in K-SFM supplemented with BPE and EGF and identified them by cytokeratins AE1/AE3 immunocytofluorescence analyses (Fig. 3A and B). Then, we observed the integrin β1 expression (Fig. 3A and B), colony formation ability (Fig. 3C) and proliferation characteristics (Fig. 3D) of the sorted epithelial cells. We found that compared to un-sorted cells, sorted epithelial cells expressed more integrin β1, had a high level of colony formation ability and proliferated more slowly, which is consistent with the characteristics of epithelial stem cells.

These results indicated that flow cytometer sorting can ameliorate the stemness of primary oral mucosal epithelial cells, which might improve the survival of these cells in engineered urethras.

3.3. Two-layer fabricated urethras in vitro

The urethra is a lumen with two layers, an epithelial layer and a muscular layer. Both of these layers are important for the prevention of strictures. The two-layer epithelial–muscular engineered lumen satisfactorily simulates the structure of natural urethras, so it might be a promising tool for urethra repair. To test if our engineered muscular tubes can be fabricated with sorted epithelial cells, we incubated sorted oral mucosal epithelial cells with muscular tubes. After co-culture of the engineered tubes with sorted cells for 1 week, the tubes were histologically examined. HE staining results showed that there was a well-developed stratified epithelium formed in the luminal surface of the engineered tubes (Fig. 4A and B), and AE1/AE3 immunohistofluorescence staining demonstrated that this newly formed epithelium was composed of epithelial-derived cells (Fig. 4C). Scanning electron microscopy inspection showed that an epithelial-cell sheet had formed on the inner side of the tubes (Fig. 4D).

These results showed that fabrication of the mechanical extension-induced muscular tubes is possible with sorted oral mucosal epithelial cells.

3.4. Mechanical extension-induced urethra reconstruction

We further evaluated the clinical application potential of our two-layer epithelial–muscular urethras in a canine model (Fig. 5A). We conducted urethrography, macroscopic anatomic investigation and histological analyses at 1, 2 and 3 months after the engineered urethras were implanted. We found that post-surgery urinary fistulas did not occur. The urethrography (Fig. 5B) and macroscopic anatomic investigation results (Fig. 5C) indicate that there were no severe urethral strictures. Moreover, histological analyses showed that the embedded engineered urethras survived well at all time points, and the grafts had developed nearly normal architecture, compared with nearby autologous urethral tissue, 3 months after implantation (Fig. 5D).

These results demonstrate that our engineered two-layer epithelial–muscular urethras are capable of urethral defect repair, and no obvious adverse consequences occurred after using these urethras.

The dogs implanted with the mechanical extension-induced muscular tubes displayed slight urinary strictures (Fig. 5B and C) and developed into more normal architectures as autologous

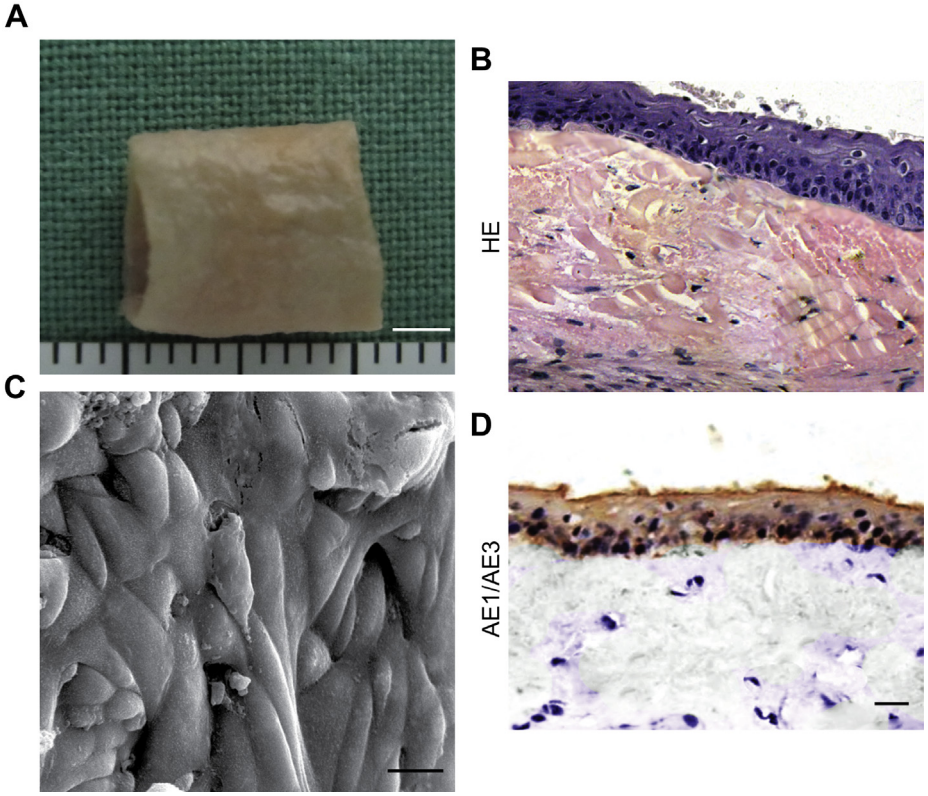

Fig. 4. *In vitro* fabrication of two-layer epithelial–muscular tissue-engineered urethras. (A) An image of two-layer engineered urethras. Scale bar = 2.5 mm (B) HE stained two-layer engineered urethras. Scale bar = 25 μm (C) A scanning electron microscope image of the inner surface of two-layer engineered urethras. Scale bar = 10 μm. (D) AE1/AE3-stained two-layer engineered urethras. Scale bar = 25 μm.

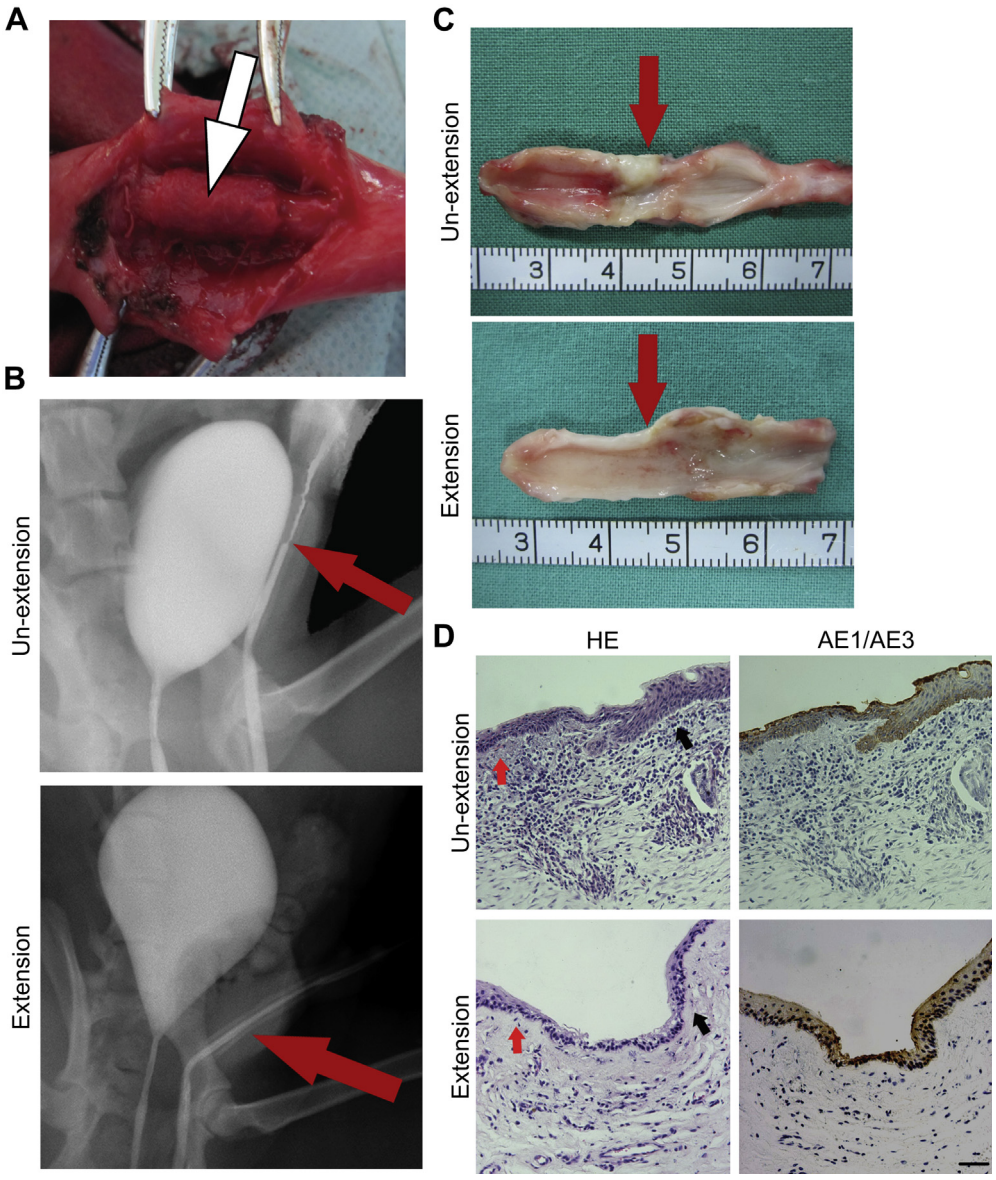

Fig. 5. Mechanical extension-stimulated engineered urethras improved the outcomes of urethra repair. (A) The photo of an implanted engineered urethra. The white arrowhead indicates the engineered urethra. (B) Retrograde urethrography images 3 months post-operation showed that the urethral structure was lighter in dogs that were implanted with the mechanical extension-stimulated engineered urethras. The red arrowheads indicate the engineered urethra. (C) Macroscopic anatomical investigations showed that the mechanical extension-stimulated engineered urethras healed better than control urethras. The red arrowheads indicate the engineered urethra. (D) HE and AE1/AE3 staining of urethral sections revealed that the mechanical extension-stimulated engineered urethras (red arrowhead) developed into more normal architectures resembling nearby autologous urethral tissue (black arrowhead). Scale bar = 50 μm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

urethras (Fig. 5D) compared to the dogs implanted with the non-mechanical extension-induced engineered urethras. These results showed that mechanical extension improved the defect repair outcomes of engineered urethras.

4. Discussion

Urethral defects secondary to diseases such as hypospadias or urethrostenosis are common in urological departments. Additionally, big segmental urethral defects are especially difficult to repair because of the difficulty in obtaining appropriate urethral tissues. Traditionally, autologous tissue from the genital system or non-genital system always results in complications such as hair growth, contraction, stricture, lithogenesis, diverticulum formation and abdominal viscera disturbance, and will injure the donor sites. Tissue-engineering technology is an ideal solution for such problems. Our study proves that rhythmic mechanical extension stimulation can improve the viability of differentiated SMCs in a PGA scaffold, and this approach combined with flow cytometer sorting of primary oral mucosal epithelial cells can help fabricate two-layer epithelial–muscular engineered urethras *in vitro*. Furthermore, this type of engineered urethra is superior in the prevention of secondary urethral strictures after use for urethral defect repair compared to non-mechanical extension-stimulated engineered urethras.

Recent stem cell research progress highlights the development of tissue-engineering technology. CD71 is a human and murine epithelial cell sorting marker [28], but in our primary canine oral epithelial cells isolated from an oral mucosal membrane by trypsin digestion, we found that CD71 expression was sparse. Therefore, we used integrin α6 as an alternative cell sorting marker [28].

Subsequent investigations proved that the phenotypes of integrin α6+/CD71− epithelial cells are similar to those of epithelial stem cells, which include slow proliferation, high colony formation ability and high integrin expression levels. These cells have promise in the construction of epithelial sheets, which is consistent with previous reports [32].

Some researchers have used smooth muscle cells as seed cells for muscular urethral construction [9], but acquiring enough seed smooth muscle cells is difficult because of their low proliferation potential and relatively higher trauma during primary cell harvesting. ADSCs have a higher proliferation potential compared to smooth muscle cells and are relatively easier to obtain; therefore, we used ADSCs to differentiate into SMCs. This approach facilitates the easy procurement of enough seed cells and reduces the injury to donor sites. ADSCs can be isolated from fat tissue using CD90, CD44, CD105, CD34 and CD35 flow cytometer sorting, and these cells have the potential to differentiate into SMCs by 5-azacytidine stimulation. SMCs are capable of generating a contractile force [33] by contraction or relaxation and the extension force generated by a urinary stream can affect each cell [34]. This reciprocal influence is pivotal in modulating the SMC phenotypes and determining the uroflow rate. Additionally, the survival of implanted tissue-engineered urethras is impaired by a limited blood supply and is inadequate for the complex local mechanical microenvironment. We produced muscular urethras by mechanical extension stimulation, which enhanced the viability of differentiated SMCs and the urethra strength; this type of engineered urethra might be more accustomed to the mechanical extension produced by the urinary stream. These reasons might contribute to the lower rate of urethral strictures.

The reaction chamber of the bioreactor used in this study was made of PGA material, which has no cytotoxicity. Our investigation found that this apparatus can transfer mechanical extension to cells and is therefore suitable for the *in vitro* construction of tissue-engineered urethras.

Inducing the formation and maturation of engineered tissues requires suitable inducing factors, stimulations and environments, which is difficult to achieve for complicated tissues. To fabricate two-layer urethras, we optimized our culture media and used 1:1 serum-free K-SFM and DMEM containing 10% FBS, which achieved good results. Examinations revealed that the epithelial cells and smooth muscular cells survived and were healthy *in vitro*.

According to a report in 2011 [10], five male patients who used tissue-engineered autologous urethras had good outcomes after approximately 7 years of follow-up. This research highlights the potential of tissue-engineering technology in urethra reconstruction. However, currently, tissue-engineered urethra research and use are still in the preliminary stages, and further efforts are needed. Our method, which combines mechanical stimulation and sorting of primary epithelial seed cells, produces promising results in a canine model and suggests that mechanical stress stimulation is useful for the fabrication of engineered urethras, as it is in generating engineered cartilage [18], ligament [19] and bone [20].

In our present study, we found that mechanical extension is useful in constructing engineered urethras, but we only used one extension strength, which is not enough to determine the optimal mechanical stimulation mode. Although the epithelium of our two-layer urethras is stratified and resembles that of autologous urethras, the density of SMCs in the muscular layer was relatively low; thus, further investigations are still needed to improve our engineered urethras.

5. Conclusions

We demonstrated that mechanical extension stimulation can improve the viability of ADSCs induced to differentiate into SMCs *in vitro*, and flow cytometer sorting of integrin α6+/CD71− subpopulation primary oral epithelial cells can ameliorate the stemness of these seed cells. The combination of these two technologies can fabricate two-layer epithelial–muscular tissue-engineered urethras, and such engineered urethras can be successfully used in urethra repair, leading to improved outcomes of urethra repair procedures.

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