Repair of urethral defects by an adipose mesenchymal stem cell-porous silk fibroin material

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Abstract. The aim of the present study was to determine whether it was possible to repair urethral defects with a material of adipose mesenchymal stem cells (ADMSCs)-porous silk fibroin (SF). A total of 39 male New Zealand white rabbits were randomly divided into a control group, an SF group and a bromodeoxyuridine (BrdU)-labeled ADMSCs-SF group (SSF group; n=13/group). Defects were made by resecting the posterior urethral wall. The defects in the SF and SSF groups were repaired using SF and BrdU-labeled ADMSCs-SF materials respectively. Then the anterior wall was sutured, and the urethral catheter was retained for 3 weeks following surgery. The catheter was rinsed with nitrofurazone once a day. The cells with positive expressions of factor VIII related antigen (FVIII-RAg), α-smooth muscle actin (α-SMA) and pan-cytokeratin (AE1/AE3) were detected by immunohistochemical assay, and the distributions of BrdU positive cells and macrophages were observed. Urethrography was performed prior to and following surgery. All rabbits had normal urethral morphologies prior to surgery. The incidence rates of postoperative complications in the control, SF and SSF groups were 76.92 (7/13), 23.07 (3/13) and 15.38% (2/13), respectively (P<0.05). The number of positive macrophages in the SSF group was significantly lower than that of the SF group 4 weeks following surgery (P<0.05). In the SSF group, BrdU positive cells were scattered within the SF material following surgery, primarily at the intersection between the SF material and the urethra. The number of FVIII-RAg positive cells in the SSF and SF groups were significantly different (P<0.05), which were also significantly higher than that of control group (P<0.01). The number of α-SMA positive cells in the SSF and SF groups were significantly different (P<0.05), and these values also significantly exceeded those exhibited by the control group (P<0.01). In addition, the SSF and SF groups had positive staining of AE1/AE3. Similar to normal urethral mucosa, the cytoplasm was stained brownish yellow (P<0.05). It is thus feasible to repair urethral defects using ADMSCs-SF material.

Introduction

Urethral defects, which are caused by urethral trauma, congenital malformations and tumors, are common urological diseases. At present, it is still rather difficult to repair a long urethral defect. When the length exceeds 4 cm, additional tissue transplantation is in need (1,2). Researchers have endeavored to repair urethral defects by using various tissue materials, such as the foreskin, bladder mucosa, small intestinal submucosal tissue and tunica vaginalis. However, there is still no ideal substitute material for urethral tissue hitherto, because of high incidence rates of metabolic abnormalities, infection, urethral fistula, urethral stricture and other complications. Besides, repairing tissues with defects by using autologous tissues prolongs the time of urethral surgery. By using tissue-engineered technology, Atala et al (3) reconstructed the bladder by autologous bladder tissues and constructed the urethra.

In traditional urethral tissue engineering, seed cells are mainly derived from autologous urethral tissues. The number of passages of urethral cells is limited, which can be solved by stem cells with strong differentiation and proliferation abilities. For the first time, Zuk et al (4) isolated stem cells with multipotential differentiation from a suspension of adipose tissues that were obtained by suction lipectomy, and referred to them as processed lipoaspirate cells. Afterwards, Li et al (5) reported that bone marrow mesenchymal stem cells (MSCs) had osteogenic and chondrogenic differentiation potentials and secreted proteins (stem cell-derived factor-1 and hepatocyte growth factor). These biological advantages should be considered in the selection of an MSC source for specific clinical application (6).

During the repair of urethral defects, it is also important to find an appropriate biological scaffold material which can relieve inflammation, and benefit the adhesion, growth and proliferation of seed cells, with high tissue compatibility (7-9). As a natural biological material derived from silk, silk fibroin (SF) rarely shows immune response after purification that removes silk sericin. In addition, it can promote cell adhesion, growth and proliferation (10-12). SF carries great promises in
Materials and methods

Ethics. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Animal Care and Use Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai, China).

Materials. Porous SF material was prepared through freeze-drying by Professor Mingzhong Li from College of Chemistry, Chemical Engineering and Material Science, Soochow University (Suzhou, China). This material had a dense lower surface, a lowly porous upper surface and a porous inner structure. The SF membrane consisted of filamentous core protein, SF and sericin. A total of 39 adult New Zealand white rabbits weighing about 3.0-4.5 kg (preoperative rectourethrogram disclosed normal urethrae) were randomly divided into a control group, an SF group and a BrdU-labeled ADMSCs-SF material group (SSF group) (n=13).

Establishment of urethral defect model. The rabbits were anesthetized by intravenous injection with 30 mg/kg pentobarbital sodium in the ear. The surgical area was routinely sterilized three times and covered by a drape. Then an F6 catheter was inserted into the urethra. A longitudinal incision of about 1.5 cm was made on the anterior wall in the middle of the ventral urethra. Finally, the segmental urethral defect model was established after cutting open subcutaneous tissues and the urethra, and longitudinally resecting the posterior urethral wall of about 2.5x1.0 cm.

Culture of ADMSCs. A healthy New Zealand white rabbit (about 2.0 kg) was anesthetized by intravenous injection with 30 mg/kg pentobarbital sodium, and fat near the epididymis was collected under sterile conditions. Discernible small vessels and fascia were resected, and tissues were washed by PBS, cut into pieces, added a double volume of 0.15% type I collagen, digested by shaking at 37°C for 45 min, added an equal amount of DMEM-F12 containing 10% fetal bovine serum (FBS) to stop digestion, filtered through a 200-mesh screen, lysed with 160 mM ammonium chloride at 37°C for 10 min, centrifuged at 2,600 x g for 15 min, washed once with PBS, and centrifuged at 2,600 x g for 15 min. The precipitated ADMSCs were inoculated into 6-wells plate with 3 ml of DMEM-F12 containing 10% FBS in each well, and cultured in a 37°C incubator with 5% CO₂ and saturated humidity. After almost complete confluence was reached, ADMSCs were digested by 0.25% trypsin and centrifuged to discard the culture medium. Then ADMSCs were incubated in DMEM containing 10 µM BrdU and 10% FBS at 37°C in 5% CO₂ and saturated humidity for 3 days. Afterwards, they were diluted by PBS into a final density of 1x10⁶/ml and stored in sterile tubes at 4°C prior to use.

Observation of BrdU-labeled ADMSCs on SF material by inverted microscope. SF material was prewetted with culture medium and placed in a 24-well plate. Then BrdU-labeled ADMSCs were inoculated onto the material at a density of 1x10⁶/ml, incubated for 1 h and further cultured after adding 1.5 ml of culture medium. The growth of these cells was observed by inverted microscope (Fig. 1).

Surgical procedure and postoperative treatment of urethral defects. For the control group, the same width of the urethral mucosa was cut off, and four corners were sutured with 5-0 nylon thread (Cheng-He Microsurgical Instruments Factory, Ningbo, China), as a postoperative marker for tissue collection. An F10 disposable catheter was retained, and the glans was fixed, with about 5 cm retained in vitro. A 6-0 PGA absorbable thread (Suzhou Medical Appliance Factory, Suzhou, China) was used to suture the anterior wall of the urethra continuously as well as the subcutaneous fat and skin interruptedly. The head was fixed with a cervical gear.

For the SF group, the urethral wall defect was repaired by SF material through Inlay surgery, and the edge was sutured interruptedly with 6-0 PGA absorbable thread. Afterwards, the material was longitudinally sutured using 6-0 absorbable thread in an interrupted way, with the penis in the middle, and four corners were sutured with 5-0 nylon thread, as a postoperative marker for tissue collection. The remaining procedures were the same as those of the control group.

For the SSF group, after a suspension of BrdU-labeled ADMSCs at the density of 1x10⁶ was dropped on the posterior wall defect of the urethra into a layer, the defect was covered and repaired with SF material through Inlay surgery, and then the material surface was evenly dropped with the ADMSC suspension. The remaining procedures were the same as those of the SF group.

The urethral catheter, which was washed with nitrofurazone once per day, was retained for about 3 weeks after surgery. The collected urine was diluted by intravenous infusion with 250 ml of glucose-sodium chloride (once per day) for about 3 weeks. To prevent removal of the catheter by the rabbit itself, the cervical gear was retained for about 3 weeks (or 2 weeks for those sacrificed in the postoperative second week), and 800,000 U of penicillin sodium was injected intravenously for about 2 weeks (once per day).

Postoperative observation. All rabbits were sacrificed by air embolization (n=2), 4 (n=9) and 6 weeks (n=2) after surgery respectively. Urethrography was performed after surgery to observe the urethral patency. The repaired urethral segment was taken, fixed in 4% neutral paraformaldehyde, embedded in paraffin and subjected to H&E staining for histological examination. Cells with positive expressions of factor VIII related antigen (FVIII-RAg), α-smooth muscle actin (α-SMA) and AE1/AE3 as well as macrophages were detected by immunohistochemical assay. Two microscopists independently
observed sections without knowing the experimental design. Ten visual fields were randomly selected for each section. The positive cells in each field were counted, and the results were averaged.

Statistical analysis. All experimental data were analyzed by GraphPad software (GraphPad Software, Inc., San Diego, CA, USA). All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. The categorical data were subjected to the χ² test, and the numerical data were subjected to the t test. P<0.05 was considered statistically significant.

Results

Urethrography results. All rabbits had normal urethral morphologies before surgery. Urethral stricture and fistula were observed by retrograde urethrography before the end of the experiment after surgery. The incidence rates of post-operative complications in control, SF and SSF groups were 76.92 (7/13), 23.07 (3/13) and 15.38% (2/13) respectively, with significant differences (P<0.05). The incidence rates of post-operative complications in SSF and SF groups were similar (P>0.05) (Table I).

Histological observation results. In the control group, the urethral defect did not form mucous epithelium 2 weeks after surgery, and many lymphocytes infiltrated, with few blood vessels. A discontinuous urethral mucosa formed 4 weeks after surgery, and the surface was uneven, accompanied by infiltration of lymphocytes and fibrous tissue hyperplasia. The number of layers of mucous epithelial cells in the urethral defect increased 6 weeks after surgery, and the cells were arranged irregularly, with disordered polarity. Many submucosal lymphocytes infiltrated, with fibrous tissue hyperplasia and disordered tissue structures. They were scattered within a small amount of smooth muscle fibers (Fig. 2A and D).

In the SF group, the surface of SF material did not form epithelium 2 weeks after surgery. There were few blood vessels and collagen tissues at the bottom of SF material, with infiltration of a small number of lymphocytes. In addition, the structure of SF was intact, without tissue growth on the top. A stratified epithelial structure formed on the surface of SF material 4 weeks after surgery, and blood vessels, smooth muscle and fibrous tissue grew along SF pores. A part of SF tissues were incomplete and only a few lymphocytes infiltrated. Six weeks after surgery, 3-4 layers of epithelial cells formed on the surface of SF material. Many submucous blood vessels, smooth muscle and fibrous tissue grew along SF pores, with infiltration of a few lymphocytes and complete SF structure. Meanwhile, decomposition hardly occurred (Fig. 2B and E).

In the SSF group, no epithelial cells formed on the surface of SF material 2 weeks after surgery. Many blood vessels and collagen tissues grew at the bottom of SF material, with infiltration of a few lymphocytes and complete SF structure. Tissues did not grow on the top of SF material. Three to four layers of epithelial cells formed on the SF material surface 4 weeks after surgery, which were irregularly arranged. Many submucosal blood vessels, smooth muscles and fibrous tissues grew along SF pores, also with infiltration of a small number of lymphocytes (Fig. 2C). Six weeks after surgery, 6-7 layers of epithelial cells formed on the SF material surface. The cells were arranged in a regular pattern. Considerable blood vessels, smooth muscles and fibrous tissues grew along SF pores. A small number of lymphocytes infiltrated, and SF material decomposed into small pieces (Fig. 2F).

Distribution of macrophages, BrdU, FVIII-RAg, α-SMA and AE1/AE3 positive cells. There were macrophages at the gap and edge of SF material in both SSF and SF groups 2, 4 and 6 weeks after surgery respectively. The number of macrophages with positive expression in the SSF group was (11.66±1.58)/HP, which was significantly lower than that of the SF group ((13.88±2.08)/HP) at the same time point (P<0.05) (Fig. 3; Table II).

In the SSF group, BrdU positive cells were scattered within SF material in the urethral defect 2, 4 and 6 weeks after surgery, which were more obvious at the intersection between this material and the urethra (Fig. 4). There were FVIII-RAg positive cells in the urethral defects of the three groups 2, 4 and 6 weeks after surgery. There were considerable FVIII-RAg positive cells under the mucosae of both SSF and SF groups 4 and 6 weeks after surgery. The numbers of positive cells in SSF and SF groups were (23.44±2.40)/HP and (20.77±2.38)/HP respectively 4 weeks after surgery, both of which were significantly higher than that of the control group ((15.11±1.61)/HP) (P<0.01). The number of the SSF group significantly exceeded that of the SF group (P<0.05) (Fig. 5; Table II).

There were α-SMA positive cells in the urethral defects of the three groups 2, 4 and 6 weeks after surgery. A large number of α-SMA positive cells were observed at the intersections of SF materials with the urethra in SSF and SF groups.
4 and 6 weeks after surgery. The numbers of α-SMA positive cells in SSF and SF groups were (33.00±3.27)/HP and (29.00±3.20)/HP respectively 4 weeks after surgery, which were significantly higher than that of the control group at the same time point ((16.11±1.53)/HP) (P<0.01). The number of the SSF group was significantly higher than that of the SF group (P<0.05) (Fig. 6; Table II). Pan-cytokeratin (AE1/AE3) in both SSF and SF groups was stained positive. The cytoplasm, which was stained brown uniformly, was reticular under the high-magnification microscope, like normal urethral mucosa. It also contained many papillary structures. In contrast, normal urethral mucosa had positive pan-cytokeratin staining, and the cytoplasm was brown (Fig. 7).

Discussion

Autologous substitutes, such as acellular matrix, tunica vaginalis, oral mucosa and small intestinal submucosal tissue, have commonly been used to repair urethral defect. However, they all have disadvantages, such as prolonged surgical time and hospital stay, aggravated trauma, urethral stricture and fistula (13-15).

Shokeir et al (16) repaired a 3-cm defect in the canine urethra by using an acellular matrix material, and found urethral stricture in experimental group by urethrography. Subsequently, Hu et al (17) repaired a rabbit urethral defect of 1.5x1.0 cm by employing a urethral extracellular matrix, and found that the urethral TNF-α level of experimental group exceeded that of control group. Bhargava et al (7) evaluated the clinical outcomes of repair using oral mucosa, and concluded that it was unsuitable for repairing large urethral defect. El-Assmy et al (18) treated a rabbit model with a commercially available small intestinal submucosal tissue. As a result, the experimental group suffered from urethral fistula and obvious hyperplasia of fibrous tissues. In addition, there were no smooth muscle bundles in tissues, so the clinical application of small intestinal submucosal tissue was controversial. The development of tissue engineering has shed a new light on addressing the problems mentioned above (19,20). Dal Pra et al (21) implanted SF material in subcutaneous tissue, and found a large number of vascular reticular connective tissues therein on the 180th day, with a small number of macrophages. There was no lymphocyte infiltration or formation of fibrous capsule. Herein, we found that significantly more new blood vessels formed in SF material of the SSF group than in the SF group. Additionally, Fuchs et al (22) found that after peripheral vascular endothelial progenitor cells and SF material were cultured for 4 weeks in vitro, the vascular structure began to form 1 week later, and the vessel-derived matrix (collagen) was evidently deposited on the surface of SF material. With extended culture time, the vascular area and length as well as the number of blood vessels all increased. SF material was infiltrated with only a few lymphocytes in the urethra, with mild inflammatory reaction. In both SSF and SF groups, a small number of lymphocytes infiltrated in vivo after repair of the urethra, but the control group had considerable lymphocyte infiltration. Panilaitis et al (23) found that SF induced minimal inflammation, but SF particles with diameters of 10-200 µm significantly stimulated macrophages to release TNF-α. Thus, the role of SF material in macrophages was limited by its size, shape and interaction with other molecules. Until now, the inflammatory response to SF materials implanted with stem cells has seldom been studied. In this study, the number of macrophages in the SSF group was significantly lower than that of the SF group. The inflammatory response of the SSF group was significantly milder than that of the SF group, indicating that the response was alleviated due to the interaction between ADMSCs and SF material, the surrounding microenvironment as well as the material surface characteristics, size and chemical composition. Moreover, SF material composites with ADMSCs further mitigated the inflammatory response of tissues.

Large amounts of smooth muscle tissues were observed in the SF material, and the number of urethral smooth muscle cells in the SSF group exceeded that of the SF group. Besides, basic urethral structures formed in both groups. There were many smooth muscles at the periphery of SF material. The interaction between the urethral epithelium and mesenchymal tissue may be conducive to the formation of urethral smooth muscle. Meanwhile, the labeled ADMSCs existed for a long time, which may also play an important role in smooth muscle regeneration. In a specific microenvironment, stem cells may differentiate into smooth muscle cells or promote their proliferation through the paracrine effect. In the SF group, smooth muscles and blood vessels grew markedly on SF.

Table I. Incidence rates of urethral fistula and stricture.

| Group      | Fistula + | Fistula - | Total (n) | Incidence rate (%) |
|------------|-----------|-----------|-----------|--------------------|
| Control    | 10        | 3         | 13        | 76.92              |
| SF         | 3         | 10        | 13        | 23.07*             |
| SSF        | 2         | 11        | 13        | 15.38*             |

*P<0.05 vs. control group; *P<0.05 vs. SF group. SF, silk fibroin; SSF, bromodeoxyuridine-labeled adipose mesenchymal stem cells-SF group.

Table II. Number of FVIII-Rag, α-SMA and macrophage positive cells 4 weeks following surgery.

| Group | FVIII-Rag | α-SMA | Macrophage |
|-------|-----------|-------|------------|
| SSF   | 23.44±2.40 | 33.00±3.27 | 11.66±1.58 |
| SF    | 20.77±2.38 | 29.00±3.20 | 13.88±2.08 |
| Control | 15.11±1.61 | 16.11±1.53 | 0.00       |

*P<0.05 vs. control group; *P<0.05 vs. SF group. SF, silk fibroin; SSF, bromodeoxyuridine-labeled adipose mesenchymal stem cells-SF group; α-SMA, α-smooth muscle actin; FVIII-Rag, factor VIII related antigen.
material in the urethral defect, without obvious decomposition. Contrarily, in the SSF group, SF material decomposed into large pieces 6 weeks after surgery, possibly owing to enzymatic reaction. Accordingly, we postulated that stem
cells may promote the secretion of proteolytic enzymes in vivo, accelerating the decomposition and segmentation of SF material. In both groups, SF material was mostly exposed on the urethra luminal surface 2 weeks after surgery, lacking epithelial coverage. New tissues only grew at the bottom, and SF material was not fixed tightly. Four and six weeks after surgery, a small amount of SF material shed off, with the formation of many new tissues. Urethral epithelial cells were not observed 2 weeks after repair, and SF material was exposed in the urethra. In the fourth week, urethral epithelium formed in both SF and SSF groups, and SF scaffolds were sufficiently immobilized, indicating that tissues began to grow into the gap between SF materials from the second to the fourth week. Therefore, it was necessary to extend the cathetering time to enhance the binding of SF material to the urethra. The urine of normal rabbits contained many phosphate crystals that easily blocked the catheter, so nitrofurazone was used to wash the bladder, with intravenous infusion with an appropriate amount of glucose-sodium chloride solution. As a result, the urine was effectively diluted, preventing possible infection and urethral obstruction. Stratified columnar urethral epithelium structures formed in the urethral defects of both SSF and SF groups 6 weeks after surgery, like normal urethral mucosa. However, the epithelial cells in the SSF group were arranged more orderly, probably because ADMSCs promoted the growth of adjacent urethral mucosal cells along the SF material surface.

In summary, SF material had high biocompatibility with ADMSCs. Repairing urethral defect by ADMSCs-composited SF material induced the formation of a large number of blood vessels, and promoted the growth of smooth muscle tissues,
with mild inflammatory reaction. Furthermore, it restored the basic functions of the urethra by benefiting the formation of urethral epithelial cells. Hence, it is feasible to apply the composite ADMSCs-SF material to repair urethral defect safely.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
BT, LS, TL, ZL, XY and QF performed the study and analyzed experimental data; BT and YL designed the study and prepared the manuscript.

Ethics approval and consent to participate
All animal experiments were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai, China).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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