Curative effect and histocompatibility evaluation of reconstruction of traumatic defect of rabbit urethra using extracellular matrix

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Objective: To investigate the curative effect and histocompatibility of reconstruction of traumatic urethral defect of rabbit using urethral extracellular matrix (ECM).

Methods: Urethral ECM was obtained by excision of the urethra in 20 donor rabbits. In experimental group, 20 rabbits were resected a 1.0 cm-1.5 cm segment of the urethra and artificially made a model of traumatic urethral defect, then reconstructed by the urethral extracellular matrix of the same length. The rabbit immunity response was assessed by lymphocyte transformation test and serum TNF- \( \alpha \) level. The reconstructed urethral segments were stained with hematoxylin-eosin and Van Gieson stain and observed by histological examination postoperatively. The urethrography, urethroscopy and urodynamic examinations were performed.

Results: There was no significant difference in stimulative index of lymphocyte transformation between ECM group and control group. The serum TNF- \( \alpha \) levels of ECM group slightly rose, but the increase was not significant as compared with control group. On postoperative day 10, epithelial cell had migrated from each side and small vessels were found in the extracellular matrix. In the 3rd week, several layers of urothelium covered the whole surface of the matrix tube. In the 6th week, the disorganized arrangements of smooth muscle fibers were firstly observed by Van Gieson staining. In the 24th week, the smooth muscle cells increased and the matrix tube appeared fairly similar to normal urethral wall components. The urethroscopy and urodynamic evaluation revealed that the surface of reconstructed urethra was smooth and emition was unobstructed.

Conclusion: The urethral extracellular matrix might be an ideal and safe biomaterial for the reconstruction of urethral traumatic defect.

Key words: Extracellular matrix; Urethra; Reconstructive surgical procedure

Urethral injury is the most common traumatic disease in urologic surgery. The management of complicated urethral defects caused by urethral injury has been a real challenge to urologists for decades. The reconstruction of urethra has been attempted with various indigenous and allogeneic materials.1,2 However, the ideal materials for urethral replacement have not been found yet.

Extracellular matrix (ECM) was produced by extracting cell components from the cellular matrix through a multistep chemical and enzymatic process. This leaves behind a sheet of homogenous extracellular matrix, which mainly consists of collagen and elastin with the removal of all soluble proteins in the matrix. There have been many reports on regeneration of different tissues in animal model using the ECM scaffold.3-8 Here, we report our experimental results of urethral ECM as a repairing material for urethral reconstruction in rabbit model.

METHODS

Animal groups
A total of 60 New Zealand male rabbits, weighing 2.5-4.0 kg, were provided by the animal experiment center of Wuhan University. The urethral ECM were aseptically obtained from 20 rabbits. The other 40 rabbits were divided into 3 groups: ECM group (n=20), in which homologous urethral ECM graft were used to replace the defected urethra; control group (n=10), in which the animals were given sham operation; homologous urethra group (n=10), in which the animals were transplanted with homologous urethral segment.
Agents

The following reagents and instruments were used: 10 mmol/L phosphate-buffered saline (PBS, pH=7.4), 0.5% ethylenediamine tetraacetic acid (EDTA), Iscoves Modified Dulbeccos Medium (IMDM, Hyclone, USA), methyl thiazolyl tetrazolium (MTT, Sigma, USA), phytohemagglutinin (PHA, Sigma, USA) and rabbit TNF-α ELISA kit (BPB Co, USA).

Urethral ECM preparation

The urethral sections of donor rabbits were immersed in PBS containing 0.1% sodium azide for 12 hours, 0.5% EDTA along with 0.4% trypsin for 5-6 hours, 1.0% formaldehyde together with 0.2% glutaraldehyde for 10 minutes, 1 mol/L sodium chloride containing 40 U/ml DNase for 6-8 hours. Subsequently, the sections were put in 50 ml of 4% sodium deoxycholate containing 0.1% sodium azide and stirred for 5-6 hours. The acellular matrix was washed three times in PBS and stored in 10 % neomycin sulfate at 4°C.

Surgical technique

The rabbits of all groups were anesthetized with isoflurane (0.2% to 3%). In ECM group, 20 rabbits were resected a 1.0 cm-1.5 cm segment of the urethra. Then a model of traumatic urethral defect was artificially established and repaired by the urethral extracellular matrix (ECM) of the same length. The new ECM was sutured to the remaining host urethral proximal and distal ends by 6-0 vicryl sutures in end-to-end anastomosis. An 8-Fr catheter was placed in urethra after operation. In control group, we only exposed the urethral corpora cavernosa and closed the incision immediately. In homologous urethral group, animals were randomly divided into 5 pairs and homologous urethral segments were transplanted from each other. The surgical procedure was the same as ECM group.

Light microscopy and ultrastructural evaluation

The reconstructed urethral segments stained with hematoxylin-eosin (HE) and Van Gieson stain were observed with light microscopy and scanning electron microscopy at the 10th day, 3rd, 6th and 24th week postoperatively. Eight rabbits (4 from ECM group and 4 from control group) underwent the urethrogramic examinations 10 and 24 weeks after operation.

PHA-induced lymphocyte transformation test

In the 3th week, the spleens of rabbits were aseptically resected, ground and homogenized by serum-free IMDM to prepare cell suspension containing approximately 1.0×10⁶ lymphocytes per ml. Then 100 µl cell suspension was added to 96-well flat plate, 100 µl IMDM containing PHA (1000 µg/ml) was inoculated to wells in experiment groups, and IMDM medium without PHA was added in control group. The culture fluid was incubated in a 5% CO₂ atmosphere at 37°C for 48 hours, transferred to a centrifuge tube and centrifuged at 1000 r/min for 10 minutes. Discard the supernatant, drop 10 µl MTT (1 mg/ml) in each tube, then incubate for 5 hours, and centrifuge at 1000 r/min for 5 minutes. Add 200 µl DMSO and vibrate in 37°C water for 10 minutes. Absorbance was determined with an automatic ELISA reader at 570 nm. The stimulative index (SI) of lymphocyte transformation was measured with a formula: SI=OD value in PHA group / OD value in control group.

ELISA for serum TNF-α quantitation

Rabbit blood was collected from the left atrium. The serum was prepared by centrifugation and stored at -70°C. The serum TNF-α concentration was determined using a rabbit TNF-α ELISA kit according to the manufacturer’s instructions. The TNF-α levels were detected at 4 time points: before operation, at postoperative 12 hours, 24 hours and 48 hours.

Urethroscopy and urodynamic examination

The urodynamic examination was performed for 4 rabbits each in experimental groups and control group. Four rabbits in ECM group were examined by urethroscopy at postoperative 24th week.

RESULTS

Characterization of the urethral ECM

After being de-cellularized, the urethral tissue appeared as a white, semitransparent and wider caliber. Histologically, the implants were confirmed to be acellular before implantation. The structure of the extracellular matrix was regularly composed of many eosinophilic reticular collagen fibrils by HE staining, which were closely connected with each other. The fragment of cell was not noticed. Under a scanning electron microscope, the matrix fibers were fabricated as network and there was no cell fragments in the interstices (Fig.1). Scanning electron microscopy showed the intact nature of the urethral matrix surface and confirmed the scaffold-like structure of the graft without evidence of cellular elements.
Observation of reconstructed urethra

In ECM group, the surface of the matrix tube was covered with urothelium 1 week after surgery (Fig.2). A minimal infiltration of erythrocytes and mononuclear cells were seen on day 10, indicating an acute inflammatory reaction. Three weeks after operation, several layers of urothelium covered the whole surface of the matrix tube more or less uniformly, showing no difference from the urethra of the host. Six weeks after operation, the matrix tube was composed of several layers of urothelium and some capillaries. The disorganized arrangements of smooth muscle fibers were firstly observed by Van Gieson staining and the ingrowth of muscle fibers occurred from adjacent edges of the host urethra. The matrix specimens showed a lower density of myofilaments than the normal rabbit urethra. The complete disappearance of the mononuclear cells was observed and there was no evidence of fibrosis or scar in the urethra. At the 24th week, neo-muscularization was well developed. The smooth muscle cells were arranged in parallelled rows in the longitudinal direction. The thickness of smooth muscle bundles had increased in the central part of the matrix. The number of myofilaments was significantly increased (Fig.3). The degenerative changes such as fibrosis, calcification or necrosis of the smooth muscle layer were not observed. The urothelial lining and muscularization of the matrix tube appeared fairly similar to normal urethral wall components. In control group, there was no urethral histological abnormality. In homologous urethral group, 6 rabbits died 10-12 days after operation. The carcass dissection revealed urethral stenosis and extravasation of urine or stone formation. Urodynamic examination showed that differences in bladder capability, maximum bladder pressure, volume of remnant urine and urethral lateral pressure between experimental groups and control group were not significant confirmed by the Student’s t test (P>0.05).

**Table 1.** The changes of serum TNF-α levels in 3 groups (pg/ml)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum TNF-α levels (pg/ml)</th>
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<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Control</td>
<td>19.9±7.8</td>
</tr>
<tr>
<td>ECM</td>
<td>18.5±8.8</td>
</tr>
<tr>
<td>Homologous urethra</td>
<td>19.2±9.3</td>
</tr>
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For comparison:

- $P_1$: control group vs ECM group
- $P_2$: control group vs homologous urethra group

$P_1$, $t_1$: control group vs ECM group; $P_2$, $t_2$: control group vs homologous urethra group.

Serum TNF-α levels assay

The results showed that the serum TNF-α levels of ECM group increased slightly as compared with control group, but the differences were not significant ($P>0.05$). The TNF-α levels of homologous urethra group had elevated significantly as compared with control group ($P<0.05$ or $P<0.01$, Table 1).

Lymphocyte transformation test

Our experiment demonstrated that the stimulation index (SI) of lymphocyte transformation had rised slightly in ECM group ($2.432 ± 0.287$) as compared with control group ($2.136 ± 0.325$), but the difference had no significance ($P>0.05$). The SI in homologous urethra group ($3.315 ± 0.317$) had increased significantly ($P<0.05$).

Urethroscopy and urodynamic examination

Urethroscopy showed that the urathral wall of rabbit was smooth and integral and the inside diameter and appearance of the urethral lumina was normal in ECM group. There was no stenosis, extravasation of urine or stone formation. Urodynamic examination showed that differences in bladder capability, maximum bladder pressure, volume of remnant urine and urethral lateral pressure between experimental groups and control group were not significant confirmed by the Student’s t test (P>0.05).

Fig. 1. The construction of ECM under scanning electron microscope (SEM, original magnification×900). Fig.2. The surface of the matrix tube was covered with urothelium 1 week after surgery (HE staining, original magnification×40). Fig.3. The normal-appearing muscle bundles were seen 24 weeks after implantation (Van Gieson staining, original magnification×40).
DISCUSSION

For many years, the management of urethral defects or strictures caused by urethral injury is a formidable problem for urologists. Although traditional end-to-end anastomosis is used for short urethral defects or strictures, the defect is too extensive to be repaired by direct end-to-end anastomosis in some cases.

Due to the shortage of urethral tissue for urethral reconstruction, various non-degradable synthetic materials have been previously tested for urethral replacement, including silicone, polytetrafluoroethylene, synthetic polyester, textile fabric and collagen tubes. Vozzi et al. had used microfabricated PLGA as a scaffold to reconstruct urethra in animal experiments. These materials have failed because of generated infection, bio-incompatibility, crystal deposition.

Biodegradable synthetic polymers and naturally derived collagen-based materials have been introduced as materials that may guide urothelial tissue regeneration in experiment and in clinic. The main goal of urethral replacement is to find a material that allows reliable regeneration of a functionally normal urethra. The major problem is how to identify a biomaterial as a suitable scaffold for this remodeling process. Atala et al. reported their experience in the animal model with the formation of urothelial structures in vivo from dissociated cells attached to biodegradable polymer scaffolds in vivo. They showed that urothelial cells may be harvested, cultured and then bonded to artificial biodegradable polymers as scaffold materials for urethral regeneration. Nevertheless, although the idea is appealing, further studies must be done to investigate their initial results.

Autologous tissue from various sources has been clinically used for urethral reconstruction, including bladder mucosa free grafts, scrotum skin, prepuce mucosa, buccal, labial, testis tunica vaginalis and rectal grafts. Although each tissue type has specific advantages and disadvantages, it is evident that the application is associated with additional procedures for graft retrieval, mucosal glandular protrusion, prolonged hospitalization and donor-site morbidity. Moreover, there are many complications such as skin flapor, graft shrink, stricture, and stone formation. Researchers are looking for more ideal material for urethral reconstruction.

Our laboratory has made an acellular collagen matrix derived from urethral tissue, which can be experimentally used for adequate urothelium cell epithelialization and urethral tissue regeneration. We have demonstrated that the urethral extracellular matrix is suitable for urethral repair in an animal model. All animals in the experimental groups showed a patent and functioning urethra, as evidenced by radiographs, histological examinations of biopsies and urethroscopic examinations. There was no evidence of infection, graft rejection, fistula, or stone formation in any animal in the experimental groups. Tabularized repair for urethral defects with extracellular matrix was achieved successfully.

The urethral acellular matrix used in this study was obtained from homologous urethral tissue. The cellular components were removed, leaving only the collagen-based matrix, composed of a complex mixture of structural and functional proteins, glycoproteins, and proteoglycans, arranged in a unique, tissue-specific, three-dimensional ultrastructure. These proteins have many functions, including the provision of structural support and tensile strength, attachment sites for cell surface receptors, and reservoir for signaling factors that modulate such diverse host processes as angiogenesis and vasculogenesis, cell migration, cell proliferation and orientation, inflammation, immune response, and wound healing. In this study, after being grafted successfully, the matrix in the experimental groups became covered with urothelium that had migrated from host, then neovascularization occurred, followed by the formation of smooth muscle cells. The normal and functional muscle lining was observable and no signs of antigenicity were evident. On postoperative day 10, epithelization and vascularization were observed in the urethral acellular matrix. In the 6th week after operation, Van Gieson staining showed the regeneration of smooth muscle cells and capillaries and the inflammatory reaction had disappeared. In the 24th week, few differences were observed between graft and host tissue. Histological, urodynamics and radiographic studies demonstrated desirable tissue regeneration and functional reconstruction of defected urethra. There were no obvious postoperative complications.

The lymphocyte transformation test is an available way to evaluate the immune function of lymphocytes. Some unspecific stimulants such as PHA, ConA and specific antigens can stimulate small lymphocytes to
transform into lymphocytoblasts. The SI of lymphocyte transformation reflects the level of cell-mediated immunity. Our study revealed that the SI of lymphocyte transformation increased slightly in ECM group as compared with control group, but the increase was not significant. Nevertheless, the SI in homologous urethra group had elevated significantly. The results showed that the urethral extracellular matrix had finer histocompatibility.

The level of serum TNF-α reflects the status of immune response. TNF-α is a pleiotropic cytokine produced by macrophages and T lymphocytes in response to a variety of stimuli such as bacterial or viral infection, in which it plays a protective role. Meanwhile, TNF-α is a primary mediator of the inflammatory response with many potent proinflammatory actions and hence has been involved in the initiation and development of the rejection response. The rejection response is initiated by activation of CD4+ T helper cells by alloantigens, either through direct stimulation by donor antigen presenting cells, or indirectly by recipient antigen presenting cells. Activated T helper cells will release initiator cytokines such as IL-1β, IL-2, and IFN-γ, which in turn activates macrophages to release TNF-α. TNF-α participates in initiating the response through upregulation of MHC molecule expression required for specific T cell activation and increased cellular infiltration through endothelial cell activation and adhesion molecule expression. TNF-α will further maintain the inflammatory response within the rejection infiltration through upregulation of adhesion molecules, increased vascular permeability, and activation of inflammatory cells. The rising of serum TNF-α levels indicates that the immune response is active. Our experiment showed that the serum TNF-α levels in ECM group increased slightly as compared with control group, but the differences were not significant. The results showed that the urethral extracellular matrix did not cause rabbits’ inflammatory response.

In this study, we have demonstrated that the urethral acellular matrix is an ideal biocompatible biomaterial for the reconstruction of the urethral injured defect in rabbit. The results may indicate that the graft has the potential for functional neo-muscularization that will result in its maintenance as a physiologic urethral wall. The detailed functional and molecular biologic experiments are needed to evaluate the mechanism of angiogenesis in the urethral acellular matrix graft.

REFERENCES


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