Continued sustained release of VEGF by PLGA nanospheres modified BAMG stent for the anterior urethral reconstruction of rabbit

Ji-Hong Wang, Yue-Min Xu*, Qiang Fu, Lu-Jie Song, Chao Li, Qin Zhang, Ming-Kai Xie

Department of Urology, Affiliated Sixth People’s Hospital, Shanghai Jiaotong University, Shanghai 200233, China

Objective: To study the biocompatibility and neovascularization of the PLGA nanospheres wrapped with vascular endothelial growth factor (VEGF), which can improve bladder acellular matrix graft (BAMG) with local continuous release of VEGF.

Methods: A total of 18 rabbit model (length of stenosis: 3cm) with anterior urethral stricture were used as experimental animals and divided into three groups. Group A as the control group: Simple BAMG scaffold materials for urethral reconstruction. Group B as the blank group: PLGA microspheres modified BAMG for urethral reconstruction. Group C: PLGA conjugated with VEGF and modified BAMG for the urethral reconstruction. All rabbits underwent urethral angiography after 7 days, 15 days, 1 month and 3 months after the operation, and one rabbit in each group was sacrificed to be prepared for the organization histologic examination, HE staining, Masson staining, CD31, 34 and α-SAM immunohistochemical detection in the repaired sites.

Results: In group A, significant urethral restenosis occurred in two rabbits after 15 days of the operation, HE and Masson staining showed a lot of collagen arranged in the repaired sites, and there were a large number of inflammatory cell infiltration, and there were also CD31, 34 in the repaired sites. α-SAM microvascular tag count showed a small amount of microvascular; Group B showed anastomotic restenosis, HE and Masson staining showed inflammatory cell infiltration and collagen deposition; Group C: urethrography showed lumen patency. There were a small amount of inflammatory cell infiltration after 7 and 15 days after the operation, and there were also CD31, 34 in the repaired sites. The α-SAM microvascular tag count showed many microvascular. And the difference was significant.

Conclusions: Anterior urethral reconstruction with sustained-release of VEGF by PLGA nanospheres modified BAMG stents can reduce postoperative restenosis. It can also reduce collagen deposition and scar formation, promote angiogenesis of the repair tissue; therefore it is valuable in the tissue-engineered urethral reconstruction.
rabbit urethral defect model.

2. Materials and methods

2.1. Materials and main reagents

A total of 18 healthy New Zealand white male rabbits aged 3–5 months were selected, and the animals were provided by the Experimental Animal Center of our hospital. These animals were then randomly divided into three groups, with six white rabbits in each group. Group A, the control group had simple BAMG scaffold materials to repair the urethra; Group B: Blank PLGA microspheres modified BAMG for urethral reconstruction; Group C: PLGA conjugated with VEGF and modified BAMG for the urethral reconstruction. The vascular endothelial growth factor (VEGF) reagents, CD31, 34 and α-SAM immunohistochemistry kit were purchased from Wuhan Boster Biological Engineering Co., Ltd.; The Masson trichrome kits were purchased from Shanghai Rong Bo Biotechnology Co., Ltd.; HE staining kit was provided by our laboratory. Polylactic acid–glycolic acid (PLGA) were purchased from Jinan DaiGang Biotechnology Co., Ltd.; Dimethyl sulfoxide, polyvinyl alcohol: Sigma Company; Methylene chloride, sodium chloride, analytical grade: Zhengzhou Honglida Chemical Co., Ltd.

2.2. Methods

Preoperative preparation and anesthesia: After fasting for 12 h and water deprivation for 4 h before the operation, 100,000 U penicillin were given to the white rabbit 30 min preoperative by intramuscular injection (2–3 times a day). The pentobarbital sodium was formulated as 3% saline solution, 2 mL sodium pentobarbital solution were injected slowly through ear vein for anesthesia. After effective anesthesia, animals were fixed to the operating table at prone position, and animals’ heads were fixed with the head frame. The damaged urethra was dissected and fully exposed. BAMG scaffold material which prepared by different methods of each group were placed in the repaired sites. Its ends were continuously sutured to the urethral stump with the 0/6 DG thread and then formed the urethra. The muscle, deep fascia and skin were closed layer by layer after hemostasis. All rabbits underwent urethral angiography after 7 d, 15 d, 1 month and 3 months after the operation, and one rabbit in each group was sacrificed to be prepared for the histological examination, HE staining, masson staining, CD31, 34 and α-SAM immunohistochemical detection in the repaired sites. It was analyzed by (Image Pro plus 4.02) multimedia color pathological image software. Positive staining and the background were automatic measured by gray level transformation respectively. For different regions, five non–overlapping high–power fields in this experiment was selected to analyze the positively stained area and the vision of the total area by percentage and an average was applied.

2.3. Effect evaluation

If diameter of the repaired urethral was ≤80% diameter of the original urethral, it was regarded as urethral stricture. The immunohistochemistry artificial count analysis of pathological diagnosis was performed. The CD31, CD34 and α–SAM–positive reactants revealed from light brown to dark brown particles under the light microscope.

2.4. Statistical methods

Data in every group were expressed with Mean±SD. The differences between the groups were analyzed by one–way ANOVA.SNK test was used to compare among groups. P<0.05 was regarded as statistical significance.

3. Results

3.1. Pathological morphology observation

Group A: No obvious urethral restenosis was observed in the animals 7 d after the operation, HE and masson stain showed slight fibrosis in the repaired sites, and there was a large number of inflammatory cell infiltration. Reexamination after 15 after of the operation showed there was significant urethral restenosis in two rabbits, lot of arranged collagen and there were obvious infiltration in inflammatory cells. Reexamination one month after operation showed slight fibrosis and a small amount of inflammatory cell infiltration. Reexamination three months after operation showed no obvious urethral restenosis among the animals.

Group B: Blank PLGA microspheres modified BAMG for urethral reconstruction. Reexamination after 15 after of the operation showed there was significant urethral restenosis in two rabbits, lot of arranged collagen and there were obvious infiltration in inflammatory cells. Reexamination one month after operation showed no obvious urethral restenosis among the animals.

Group C: PLGA conjugated with VEGF and modified BAMG for the urethral reconstruction. Reexamination after 15 after of the operation showed there was significant urethral restenosis in two rabbits, lot of arranged collagen and there were obvious infiltration in inflammatory cells. Reexamination three months after operation showed no obvious urethral restenosis among the animals.

3.3. Statistical analysis

Data in every group were expressed with Mean±SD. The differences between the groups were analyzed by one–way ANOVA. SNK test was used to compare among groups. P<0.05 was regarded as statistical significance.
increased compared with group A and group B (mean ± sd) (%).

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Degree of restenosis for the urethra of the white rabbit (%)</th>
<th>n=6</th>
<th>n=5</th>
<th>n=4</th>
<th>n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>96.5±2.4</td>
<td>83.2±5.3</td>
<td>81.6±4.6</td>
<td>78.6±3.6</td>
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<tr>
<td>Group B</td>
<td>96.8±2.2</td>
<td>81.5±4.2</td>
<td>80.1±4.0</td>
<td>79.3±3.8</td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td>99.3±0.5</td>
<td>99.0±0.4*</td>
<td>98.6±0.6*</td>
<td>98.6±0.6*</td>
<td></td>
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</tbody>
</table>

*Compared with group A, P<0.05.

### 3.2. CD31, 34, a–SAM expression in urethral tissue

Positive staining of CD31 protein were expressed in all microvascular endothelial cells of the granulation tissue, which was located in the cytoplasm. The expression of it in group C was significantly increased compared with group A and group B (P<0.05). Positive staining of CD34 protein were expressed in vascular endothelial cells and located in the cytoplasm. The expression in group C was significantly increased compared with group A and group B (P<0.05).

### Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Immunohistochemical expression of CD31, 34 a–SAM of white rabbits (mean±sd) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>CD31 3.1±0.7, CD34 0.4±0.1, a–SAM 6.5±0.5</td>
</tr>
<tr>
<td>Group B</td>
<td>CD31 3.0±0.7, CD34 0.5±0.1, a–SAM 6.1±0.6</td>
</tr>
<tr>
<td>Group C</td>
<td>CD31 11.5±2.2*, CD34 5.5±0.9*, a–SAM 0.4±0.1*</td>
</tr>
</tbody>
</table>

*Compared with group A, P<0.05.

### 4. Discussion

The most important factors of tissue engineering includ seed cells and scaffold. Currently the urethral acellular matrix, BAMG and small intestinal submucosa are the main sources of material for urinary reconstruction. Because BAMG comes from the urinary system, it has good histocompatibility, and as the membrane materials, BAMG can also repair defects according to the shape of the urethra. Therefore, the study on BAMG stent receives much concern. However, urethral stricture is the main factor for the poor treatment. The systemic therapy fails to achieve a satisfactory effect for the urethral stricture, and is also with systemic complications. A large number of studies suggest that reducing the local inflammatory response and increasing the local blood supply are the main direction to solve this problem, therefore the nanocarrier material PLGA which has a good bio–security has gained much attention[3]. This material is a synthetic biodegradable polymer, which causes no side effect in humans. Drugs can be released more slowly by it, which is more conducive to maintain drug concentration and guarantee the treatment effect.

In the study of angiogenesis, the commonly used endothelial cell markers are CD31 and CD34. Previous studies have found that CD31 can be expressed on vascular endothelial cells, platelets, macrophages and neutrophils, and plays an important role in inflammatory angiogenesis[4]. CD34 is often expressed in hematopoietic stem cells, endothelial cells, and embryonic mesenchymal cells, which can accelerate the vascular buds endothelial cell migration in the process of capillary formation[5]. In this study, the expression of CD31 and CD34 were significantly increased in white rabbit which underwent persistent release VEGF treatment. There were apparent edema and a large number of capillary proliferations at the two junctions in the urethral repaired sites. Considering the vascular endothelial growth factor belong to the family of platelet–derived growth factor, and as a specific mitosis of the arteriovenous and lymphatic endothelial cells, it can not only promote the 3D angiogenesis in vitro, but also promote the division of capillary endothelial cell and plasminogen glial enzymes and vasoactive substances[4].

Although the mechanism of VEGF and CD31, CD34 is still not clear, we believe that the VEGF still has no species specificity. It can stimulate the release of vasoactive substances of vascular endothelial cell, thus can facilitate the growth and repair of tissue. That is consistent with the related literatures[6,7]. a–SAM is the most important actin microfilaments in the smooth muscle cell, which is mainly related to the degree of tissue damage and played an important role in the mechanism of tissue fibrosis[8,9]. This study showed that after VEGF treatment, a–SAM protein expression of the repair urethral tissue was significantly reduced, thus can effectively avoid the urethral stricture which is caused by fibrosis in the repaired sites. Studies have shown that a–SAM can be used as early predictors of the chronic fibrosis[10]. We think that the mechanism of therapeutic effect of VEGF may be that it effectively inhibit fibrosis by improving tissue ischemia and hypoxia, thereby preventing the stricture in the repaired sites of the urethra. In summary, we use BAMG as the scaffold material in this study, and performed the anterior urethral reconstruction by increasing the PLGA nanospheres which can sustained–release VEGF, thus can also reduce restenosis after the operation. We initially established a construction method of a nice tissue–engineered urinary tract epithelial tissue in
vivo. Because this method has the multiple effects including promote angiogenesis and reduce scarring, we think there will be broad prospects for the applications of tissue-engineered urethra reconstruction.

Conflict of interest statement

We declare that we have no conflict of interest.

References


