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Original Article

A preliminary study of a rabbit model of bladder mucosa reconstruction

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Abstract: Objectives: To investigate the practicality of bladder mucosa reconstruction using an autologous peritoneal free graft in a rabbit model. Materials and methods: The bladder mucosa were reconstructed using autologous free peritoneal grafts after mucosa denudation in the rabbit models. Blood analyses, cystography, and urodynamic studies were performed at 1, 3, and 6 months postoperatively. Then the bladder specimens were harvested for histological analyses and immunohistochemical analyses. Results: Our urodynamic study suggested that the maximum bladder capacity and compliance in the experimental group were significantly lower than they were in the control group at 3 and 6 months postoperatively ($P < 0.05$), but there were no significant differences in the bladder leak point pressures at each time point ($P > 0.05$). Cystography suggested the bladder function was good, and no bladder contraction was seen in the experimental group. By 1 month after the bladder mucosa reconstruction, a histological analysis demonstrated that no peritoneum mesothelial cells were observed, and the reconstructed bladder mucosa was covered with 1-2 epithelial layers, which stained positively for CK 7 and uroplakin III but negatively for calretinin and vimentin. By 3 and 6 months, the layers of the epithelium increased to 3-5 lays, which is similar to native urothelia. Conclusions: This initial study showed that autologous free peritoneal grafts can be effectively used in bladder mucosa reconstruction. At 4 weeks after the operation, the peritoneal mesothelium regenerated completely into the uroepithelium. The newly formed tissue was histologically similar to native bladder mucosa.

Keywords: Bladder reconstruction, mucosa, rabbit model, peritoneum

Introduction

Surgical repair is required when a bladder is affected by malignancies, trauma, or mucosal lesions, and some nonurological tissues need to be used in bladder reconstruction [1]. The most commonly-used organs in such cases are the intestine and colon [2]. However, bladder reconstruction with the intestine and colon may be problematic because of the mismatched state of two tissues: urinary tissue excretes materials, while gastrointestinal tissue absorbs materials. This state may lead to infections, metabolic complications, and other side effects. To prevent adverse outcomes of bladder reconstructive surgery, researchers has been encouraged to find alternative tissue sources for bladder reconstruction [3]. Although great developments have been made in the field of tissue engineering technologies, many challenges remain in clinical applications [4]. Therefore, it is necessary to develop new techniques in reconstructing the bladder wall that are appropriate for clinical applications. The peritoneum contains mesenchymal stem cells that are similar to bone marrow stem cells and that have been used in generating biomaterials [5]. As an autologous graft, the peritoneum has been successfully used in urethral and ureteral reconstruction [6-8], demonstrating good adaptability and biocompatibility with urinary tissue. We believe that the bladder mucosa can also be efficiently reconstructed using autologous peritoneal free grafts.

In this study, we aimed to research the practicability of bladder mucosa reconstruction with autologous free peritoneal grafts and to evaluate an animal model to determine the potential usefulness of this strategy.

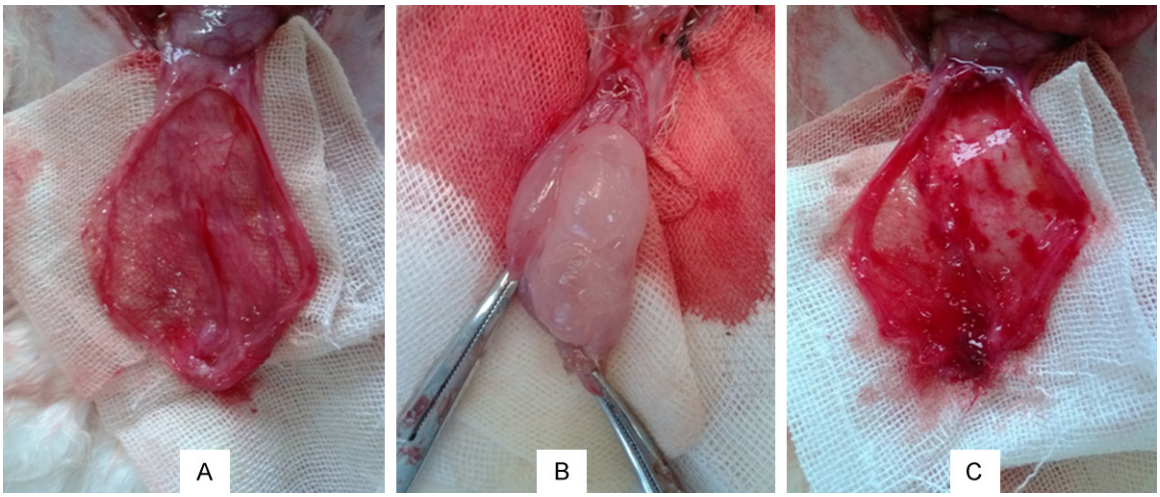

Figure 1. A. The bladder wall before the mucosa denudation. B. The bladder mucosa was elevated by injecting physiologic saline into the lamina propria. C. The bladder wall after the mucosa denudation.

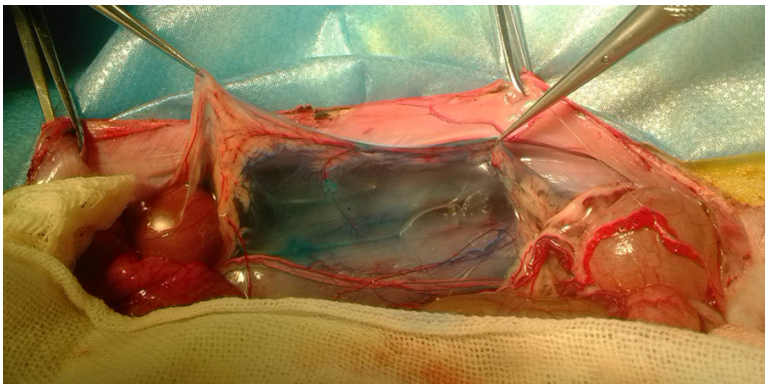

Figure 2. A sheet of tissue of approximately 50 × 40 mm that was composed of the posterior peritoneum was obtained.

Materials and methods

This study was conducted at the First Hospital of Tsinghua University, and authorization was provided by the Ethics Committee of First Hospital of Tsinghua University. All the experimental protocols were approved by the Ethics Committee of First Hospital of Tsinghua University, and all the research methods were carried out in accordance with the relevant guidelines and regulations.

Functional research

32 adult male New Zealand rabbits, weighing on average 2.5 kg (range 2.0-3.0 kg), 4-6 months of age, were randomly assigned to 3 groups: the experimental group (n=12), the sham-operation control group (n=12), and the mucosal denudation group (n=8). All the rabbits received general anesthesia with 10% chloral hydrate (2 ml/kg, IVGTT). In each rabbit in the experimental group, after denuding all of the bladder mucosa, the autologous free peritoneal grafts from the rabbit posterior abdominal wall were sutured to the bladder wall. After making a median cystotomy, physiologic saline was injected into the lamina propria with a No. 4.5 needle, and then the bladder mucosa was elevated and immediately denudated from the underlying muscle wall (**Figure 1**). The process was repeated until all the bladder mucosa was excised. Then, a sheet of tissue of approximately 50 × 40 mm that was composed of posterior peritoneum was obtained (**Figure 2**). The graft was attached using an interrupted suture to the bladder wall (with the side of viscera peritoneum upward) with 5-0 monofilament polypropylene mesh (Ethicon, 1/2 circle atraumatic needle). Before closing the incision, a water sac (15-20 ml) was placed into the bladder to support the wall (**Figure 3**), and it was maintained for 1 week and then removed surgically by a short cystostomy via minilaparotomy. In the sham-operation control group, after incising the bladder, the incision was directly sutured. In the mucosal denudation group, after denudat-

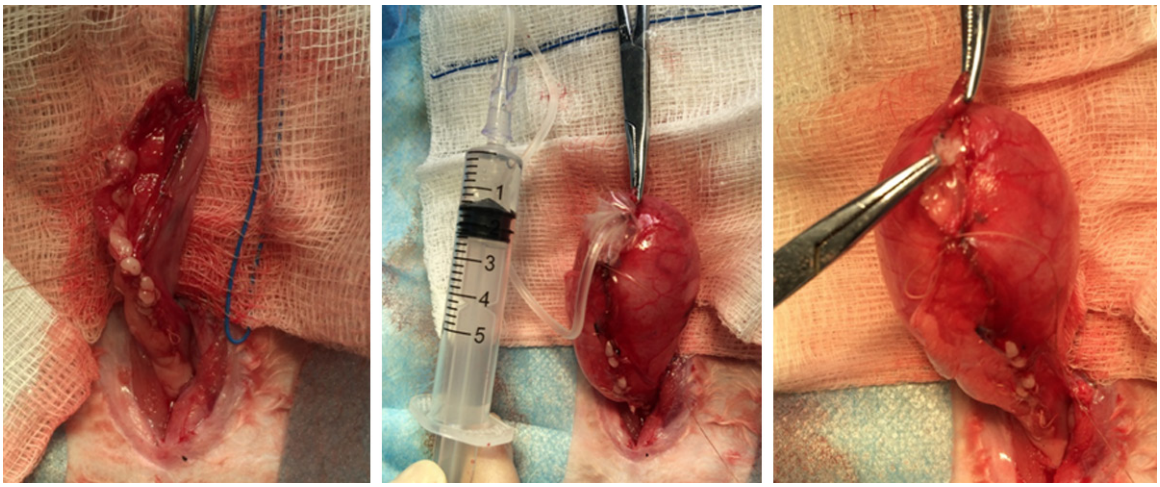

Figure 3. Before closing the incision, a water sac (15-20 ml) was placed into the bladder to support the wall.

ing all of the bladder mucosa, the incision was directly sutured. 4 rabbits were euthanized immediately, then the free bladder mucosa and the residual bladder wall were allocated for analysis. The other 4 rabbits were kept to observe their survival.

All the rabbits were raised in a standard laboratory animal environment (temperature, 21±2°C, humidity, 50±20%, fresh filtered air, and 12 h:12 h light/dark cycle), and they were monitored continuously for 6 months. They were provided fresh water and feed and were housed individually after the operation. An F6 catheter was inserted into each rabbit during the operation, and the catheters were removed 5 days later. Penicillin G sodium (10^5 U/day i.m.) was administered for 6 days after the operations.

Blood analyses, retrograde cystography, and urodynamic studies were performed under chloral hydrate anesthesia at 1, 3, and 6 months after the operation. The maximum bladder capacity, the bladder leak point pressure, and the bladder compliance were recorded using the Laborie urodynamic analyzer system (Laborie Medical Technologies Canada, ULC). And the urodynamic changes during urination could not be recorded under anesthesia.

24 rabbits were euthanized at 1, 3, and 6 months post-surgery, and their bladders were allocated for analysis. The bladder sections were fixed with 10% formalin and embedded with paraffin and hematoxylin-eosin and Masson stained. For the immunohistochemistry, the tissue sections were stained with antibodies against Uroplakin III (Sigma, St. Louis, Mo., USA), CK 7 (Dako, Carpinteria, Calif., USA), calretinin (Zhongshan, Beijing, China) and vimentin (Thermo Fisher, Fremont, Calif., USA). The bladder samples were fixed in 2.5% buffered glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded acetone series, and embedded in Spurr's resin. They were stained with aqueous uranyl-acetate and lead citrate. For the analysis, ultrathin sections were examined with a transmission electron microscope (H-9500, JEOL, Japan). And meanwhile the specimens were treated using desiccation and spray-gold, and scanning electron microscopy (SU70, JEOL, Japan) was used to study the microstructure.

Mechanistic research

Another 8 adult male New Zealand rabbits were used. All the rabbits underwent general anesthesia with 10% chloral hydrate (2 ml/kg, IVGTT). After denudating all of the bladder mucosae, the autologous free peritoneal grafts from the rabbit posterior abdominal walls were sutured to the bladder walls.

8 rabbits were euthanized at one and two weeks post-surgery, and their bladders were allocated for analysis. A cut was made in the middle of the anterior wall of the bladder. The suture was near the neck of the bladder. The gross specimen was divided into four areas

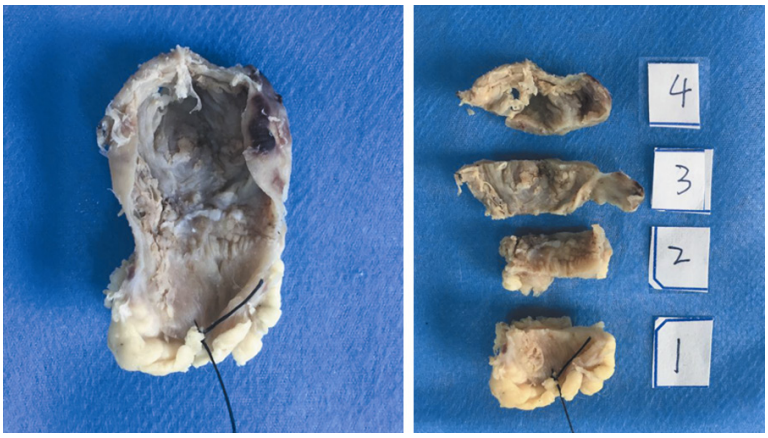

Figure 4. Area partitioning of the bladder specimen. Area 1 and area 2 were proximal to the bladder neck, and area 3 and area 4 were far away from the bladder neck.

from the bladder neck to the bottom of the bladder. Area 1 and area 2 were proximal to the bladder neck, and area 3 and area 4 were far away from the bladder neck (**Figure 4**).

The bladder sections were stained with hematoxylin and eosin. For the immunohistochemistry, the bladder sections were stained with antibodies against Uroplakin III (Sigma, St. Louis, MO, USA), CK 7 (Dako, Carpinteria, Calif., USA), calretinin (Zhongshan, Beijing, China), and vimentin (Thermo Fisher, Fremont, Calif., USA).

Statistical analysis

All the values are shown as the mean standard deviation (SD). The comparisons between the different groups were performed using one-way analyses of variance (ANOVA) with t-tests for significance. All the statistical calculations were performed using SPSS v. 22 software (SPSS Inc., Chicago, IL, USA). The significance level was set at 0.05 ($P < 0.05$).

Results

Functional research

In the mucosal denudation group, four rabbits were euthanized immediately after the operations. A histological analysis demonstrated the free bladder mucosae included mucosa, submucosa, and a small amount of muscle fibers, but no urothelia were observed in the residual bladder wall. The other 4 rabbits died 4-14 days after their operations. In the gross examinations of the specimens, severely contracted bladders and hydronephrosis were observed, and massive inflammation and necrosis of the bladder walls were found in the histological analysis.

In the experimental and sham-operation control groups, all twenty four animals survived the experiment. Their renal function and electrolyte metabolism were not significant different between the two groups at the three time points ($P > 0.05$) (**Table 1**). The urodynamic study suggested that the bladder leak point pressure was not significantly different between the two groups after 1, 3, and 6 months ($P > 0.05$), but the maximum bladder capacity and compliance were significantly lower than they were in the control group at 3 and 6 months after the operations ($P < 0.05$) (**Tables 2**-**4**; **Figure 5**).

By 6 months, a gross examination of the bladders in the experimental group revealed mucosa that appeared smooth without fibrosis or calculus and severe shrinkage. Cystography suggested that the bladder function was good, and no bladder contraction was seen in the experimental group. The shape of the bladder was stiffer, but the bladder capacity was smaller than it was in the control group at 1 month after the operation. The shapes and volumes of the bladders gradually improved from 3 months to 6 months after the operations (**Figure 6**).

By 1 month after the bladder mucosa reconstruction, no peritoneum mesothelial cells were observed in the histological analysis, and the reconstructed bladder mucosa was covered with 1-2 layers of epithelia. The immunohistochemical analyses demonstrated that the epithelium stained positively for CK 7 and uroplakin III, but no signal was observed for calretinin or vimentin (**Figures 7**, **8**). Compared with the native urothelia, the epithelia of the reconstructed bladder walls were irregular and had fewer cell layers. Neovascularization was evident beneath the mucosa, and there were some inflammatory cells and fibroblasts in the subepithelial layer, but no fibrosis was noted.

Table 1. Renal function and electrolytes in the 2 groups at each time point

	Group	Preoperative	1 month postoperative	3 months postoperative	6 months postoperative
CR (mmol/l)	EG	109.50±11.73	120.75±2.22	114.50±9.68	113.25±9.91
	CG	112.75±10.81	116.50±9.85	110.75±9.39	109.25±13.82
	p	0.917	0.191	0.944	0.550
	t	0.407	0.842	0.556	0.470
BUN (mmol/l)	EG	8.20±0.55	8.80±0.29	8.36±0.51	8.39±0.62
	CG	8.18±0.55	8.61±0.34	8.42±0.51	8.34±0.51
	p	0.965	0.557	0.913	0.298
	t	0.045	0.906	0.146	0.112
K (mmol/l)	EG	4.33±0.43	3.92±0.49	4.24±0.44	4.25±0.25
	CG	4.36±0.38	3.99±0.75	4.14±0.59	4.39±0.45
	p	0.800	0.204	0.627	0.909
	t	0.096	0.144	0.284	0.438
Cl (mmol/l)	EG	92.23±2.45	94.90±4.28	92.75±3.34	93.00±4.69
	CG	93.00±3.02	93.13±4.25	92.30±3.94	95.55±4.59
	p	0.945	0.613	0.333	0.923
	t	0.398	0.588	0.174	0.777
Na (mmol/l)	EG	139.88±3.79	139.38±2.56	138.50±5.07	141.90±4.58
	CG	142.13±2.78	139.83±6.02	139.20±4.15	139.98±5.10
	p	0.837	0.225	0.591	0.951
	t	0.957	0.138	0.214	0.562

EG: experimental group, CG: control group.

Table 2. Maximum bladder capacity in the 2 groups at each time point

Group	Maximum bladder capacity (ml)			
	Preoperative	1 month postoperative	3 months postoperative	6 months postoperative
Experimental group	23.5±2.38	12.75±2.06	15.25±0.95	16.75±0.96
Control group	23.25±2.50	14.25±1.70	20.25±0.96	21.50±1.29
Statistics	p=0.890	p=0.305	p=0.000	p=0.001
	t=0.145	t=1.121	t=7.385	t=5.911

Table 3. Bladder leak point pressures in the 2 groups at each time point

Group	Bladder leak point pressure (cm H_2O)			
	Preoperative	1 month postoperative	3 months postoperative	6 months postoperative
Experimental group	16.00±2.16	15.75±1.71	15.50±1.29	16.25±0.96
Control group	16.00±2.82	16.00±2.16	15.75±2.36	16.50±2.65
Statistics	p=0.881	p=0.862	p=0.859	p=0.868
	t=0.14	t=0.182	t=0.186	t=0.178

Table 4. Bladder compliance in the 2 groups at each time point

Group	Bladder compliance (ml/cm H_2O)			
	Preoperative	1 month postoperative	3 months postoperative	6 months postoperative
Experimental group	1.48±0.08	0.82±0.20	1.02±0.03	1.03±0.04
Control group	1.50±0.33	0.90±0.16	1.31±0.24	1.32±0.18
Statistics	p=0.911	p=0.556	p=0.049	p=0.02
	t=0.117	t=0.624	t=2.467	t=3.143

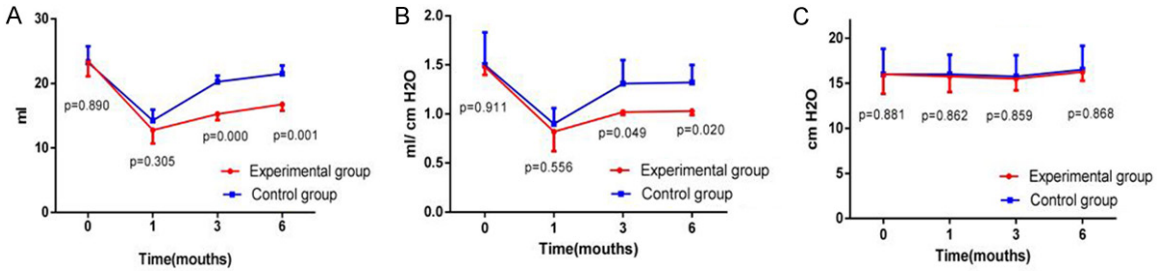

Figure 5. Urodynamic study of the bladder. A: Maximum bladder capacity. B: Bladder compliance. C: Bladder leak point pressure.

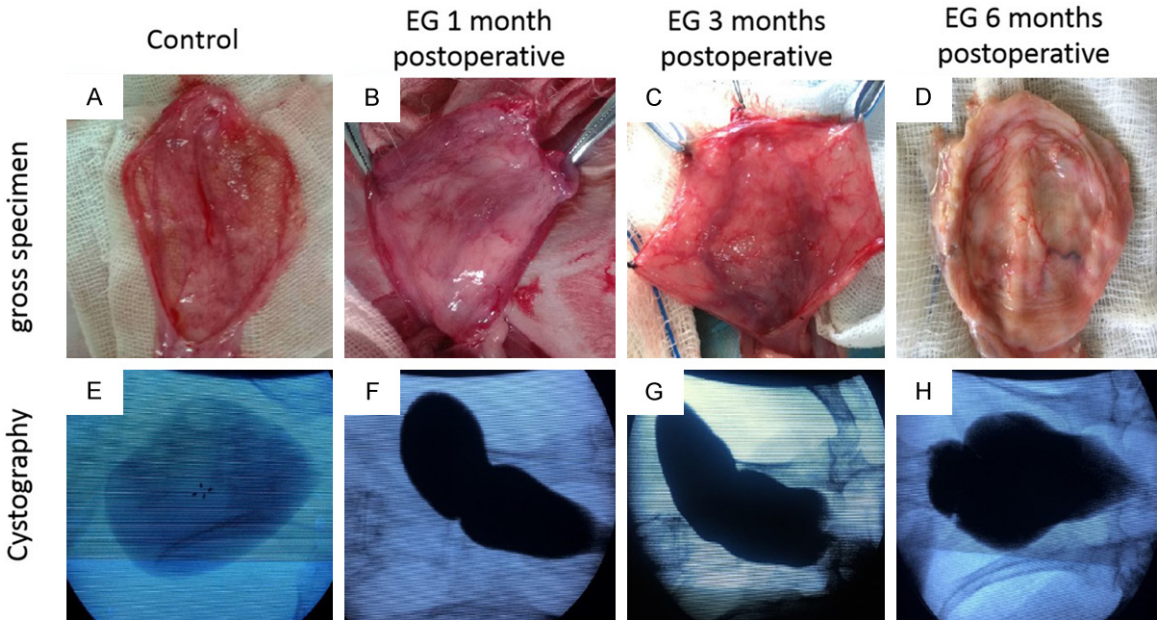

Figure 6. Gross specimen and cystography of the bladder. A: Bladder gross specimen in the control group. B-D: Bladder gross specimen in the experimental group. E: Bladder cystography in the control group. F-H: Bladder cystography in the experimental group.

By 3 and 6 months, the reconstructed bladder mucosa contained some urothelium that was similar to the native; it was covered with 3-5 layers of the epithelia, and the infiltration of the mononuclear cells in the lamina propria subsided. An immunohistological analysis of the epithelial cells demonstrated a positive expression of uroplakin III and CK 7 but no expression of calretinin or vimentin (**Figures 7**, **8**). Scanning electron microscopy showed that the reconstruction of the mucosa at 1 month after surgery showed a typical cobblestone-like morphology that was almost the same as the normal bladder mucosa at 3 months after surgery.

Mechanistic research

All 8 animals survived the experiment.

At 1 week after the operation, epithelial cells were observed in the mucosa of areas 1 and 4. An immunohistological analysis demonstrated that the cells were urinary epithelial cells that expressed both CK 7 and uroplakin III but did not express calretinin or vimentin. No epithelial cells were observed in the mucosa of areas 2 and 3, and the immunohistological examination showed no expression of either CK 7 or uroplakin III and a positive expression of calretinin and vimentin (**Figure 9**). At 2 weeks after the operations, there were multiple layers of epithelial cells in the mucosa of areas 1 and 4, and there was a monolayer of epithelial cells in the mucosa of areas 2 and 3. Our immunohistological analysis demonstrated that the cells were urinary epithelial cells with positive expressions of both CK 7 and uroplakin III but no

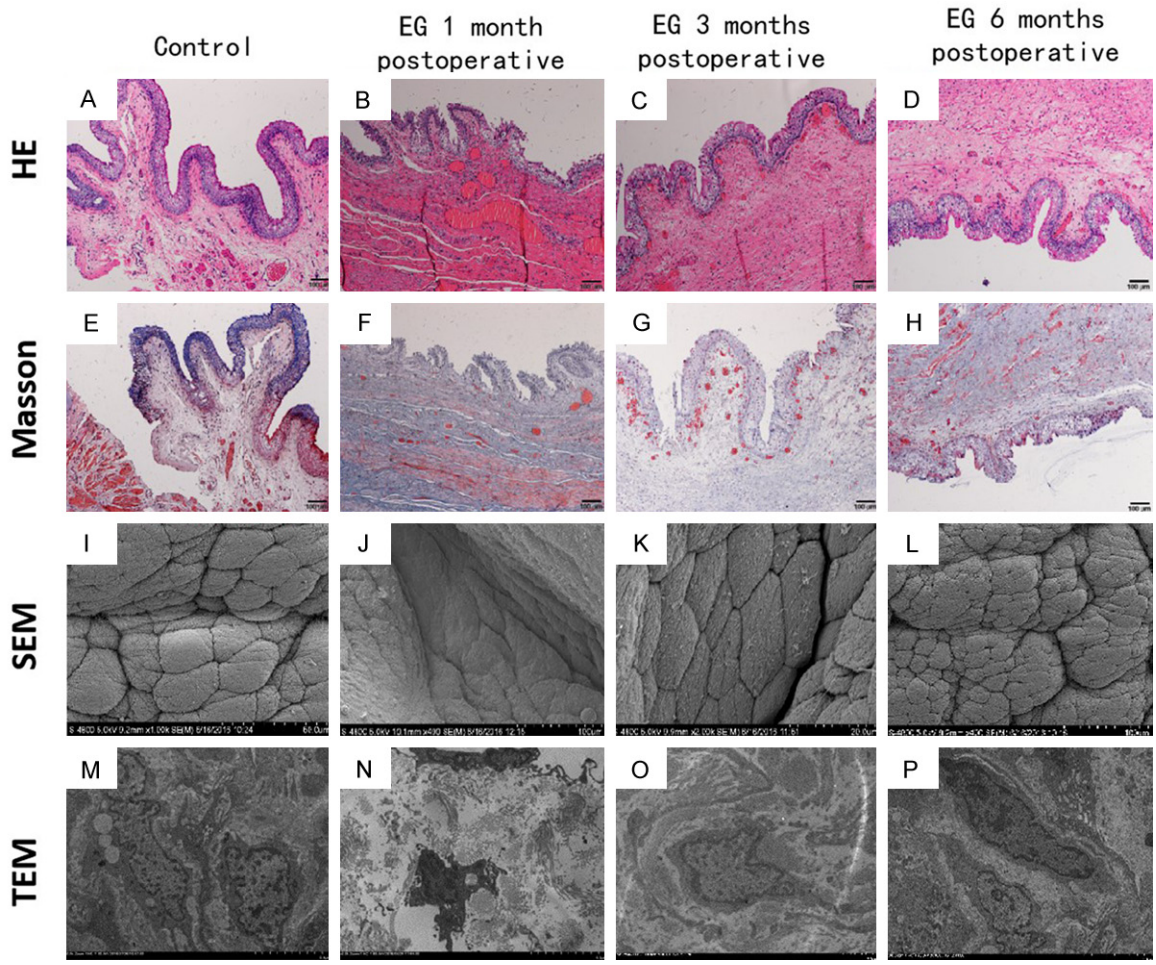

Figure 7. Histological and electronic microscope analyses of the bladder. A and E: H&E staining and Masson staining of the bladder in the control group (×100). I and M: SEM and TEM of the bladder in the control group (×2000). B-D and F-H: H&E staining and Masson staining of the bladder in the experimental group at 1, 3, and 6 months after the operations (×100). J-L and N-P: SEM and TEM images of the bladder in the experimental group at 1, 3, and 6 months after the operations (×2000).

expressions of calretinin or vimentin (**Figure 10**).

Discussion

In recent years, tissue engineering techniques in urinary tract reconstruction have been greatly developed. The key development in these approaches is the reconstruction of new urinary tract walls from stem cells expanded in vitro that have been seeded onto biodegradable scaffolds; the scaffolds are then transplanted to complete the regeneration process [3]. Different types of acellular matrices, synthetic polymers, and naturally derived materials have been widely used as biodegradable scaffolds. To date, due to relatively high technical requirements and controversial effects, there are no clinical applications in the tissue engineering of the bladder that replace the surgical treatment of bladder malignancies or chronic inflammation [9]. Considering the shortfalls in the currently-used materials and methods, an ideal graft for bladder reconstruction is still needed [10].

The peritoneum can regenerate to maintain stem cell viability and the formation of scar tissue at the injury site [11]. In some studies, autologous peritoneum is a good material to use in the repair of the pericardium, intestines, vagina, and cartilage because of the regeneration and transdifferentiation abilities of the mesothelial cells [12]. Some studies have even

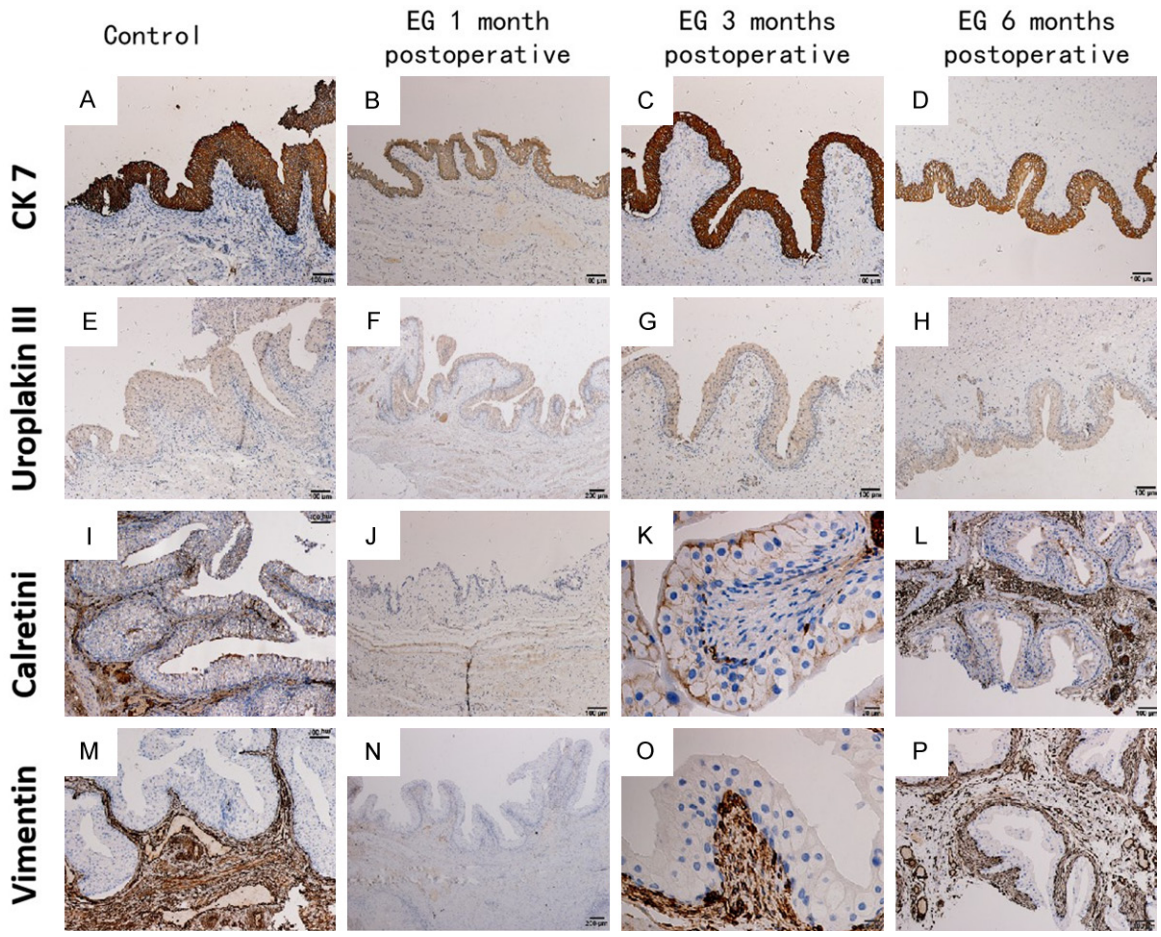

Figure 8. Immunohistochemical staining of the bladder. A, E, I and M: Immunohistochemical staining of CK 7, uroplakin III, calretinin and vimentin in the control group (×100). B-D, F-H, J-L and N-P: Immunohistochemical staining of CK 7, uroplakin III, calretinin, and vimentin in the experimental group at 1, 3, and 6 months after the operation (×100).

explored the possibility of using peritoneal cells to culture hollow organs such as the bladder, urethra, uterus, and vas deferens in the abdominal cavity [5]. The peritoneum has been applied to the urinary system [6-8], and the mesothelial cells can be differentiated into urinary transitional epithelial cells. Jing Yi-Feng used autologous free peritoneal grafts as materials for ureteral mucosa reconstruction and acquired ideal functional and histological results, showing the good adaptability and biocompatibility of the grafts to urinary tissue [13]. In this study, we attempted to use similar grafts for bladder mucosa reconstruction. The use of autologous tissue harvested from the host avoids the risk of rejection [14]. We used rabbits as an animal model because the structure and function of the rabbit bladders is very similar to that of humans [15]. Considering the fact that the only difference between the bladder wall of humans and rabbits is thinner detrusor layers relative to the overall thickness of the bladder wall, the rabbit model seems to be a satisfactory experimental representation [16].

The satisfactory artificial bladder should show native urinary bladder-like properties, such as the ability to effectively store urine at low pressure to avoid prosthesis-ureter reflux [17]. In this experiment, no signs of fibrosis were noted after the bladder mucosa reconstruction. The urodynamic analysis suggested that the maximum bladder capacity in the first month after the operations was 54.3% of the preoperative capacity, and the control group also showed the same change, which was considered to be the result of surgical trauma. Six months after the operation, the maximum bladder capacity

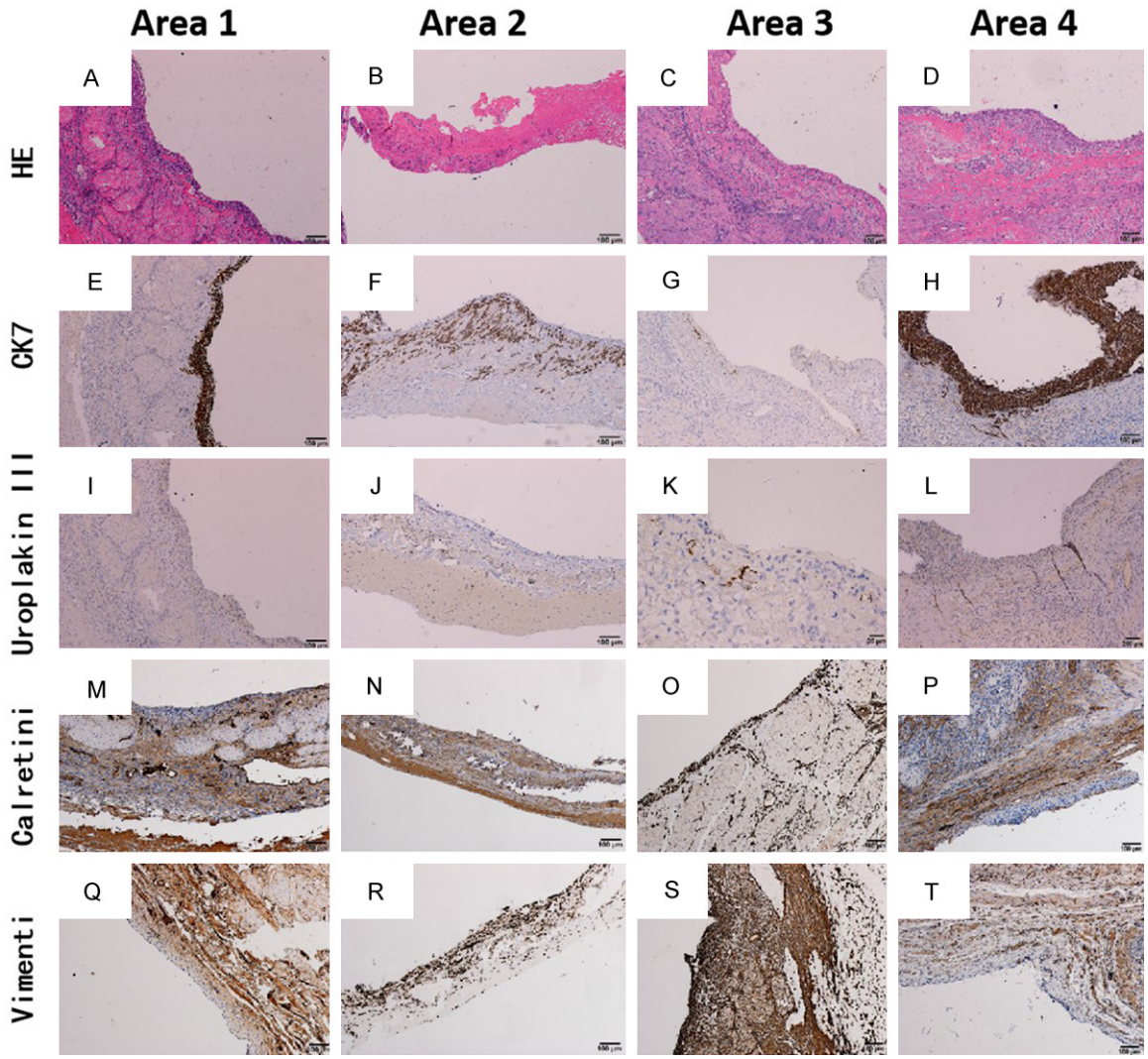

Figure 9. Histological analyses of the bladder specimens at 1 week after the operations. A-T: H&E staining and immunohistochemical staining of CK 7, uroplakin III, calretinin and vimentin in the different areas of the bladder specimens at 1 week after the operations (×100).

recovered to 71.3% of the preoperative capacity. It is believed that the maximum bladder capacity will continue to improve as the postoperative time increases. There was no significant change in the bladder leak point pressure after the bladder mucosa reconstruction, which indicated that bladder contraction function was not significantly affected. The results of the blood biochemistry analyses showed that the preoperative creatinine level was 109.50 mmol/l, and the postoperative creatinine level was 113.25-120.75 mmol/l. The results suggest that there was no effect on the renal function after the bladder mucosa reconstruction.

Urothelial cells express some epithelial markers, such as cytokeratins and uroplakins, and uroplakins are known to be terminal markers of urothelial differentiation [18]. In our experiment, the newly generated urothelium in the experimental group expressed CK 7 and uroplakin III, as did the control bladder. The results show that the protein expression profile of the newly generated urothelium in the experimental group was very similar to that of the native normal bladder epithelium. Jing Yi-Feng found that the peritoneal mesothelium can completely convert to a urinary tract transitional epithelium at 10 weeks after ureteral mucosa reconstruction by transplantation with autologous free peritoneum [13]. In our experiment, 4 weeks after the operation, a histological analysis demonstrated that the newly formed bladder mucosa tissues were completely lined by

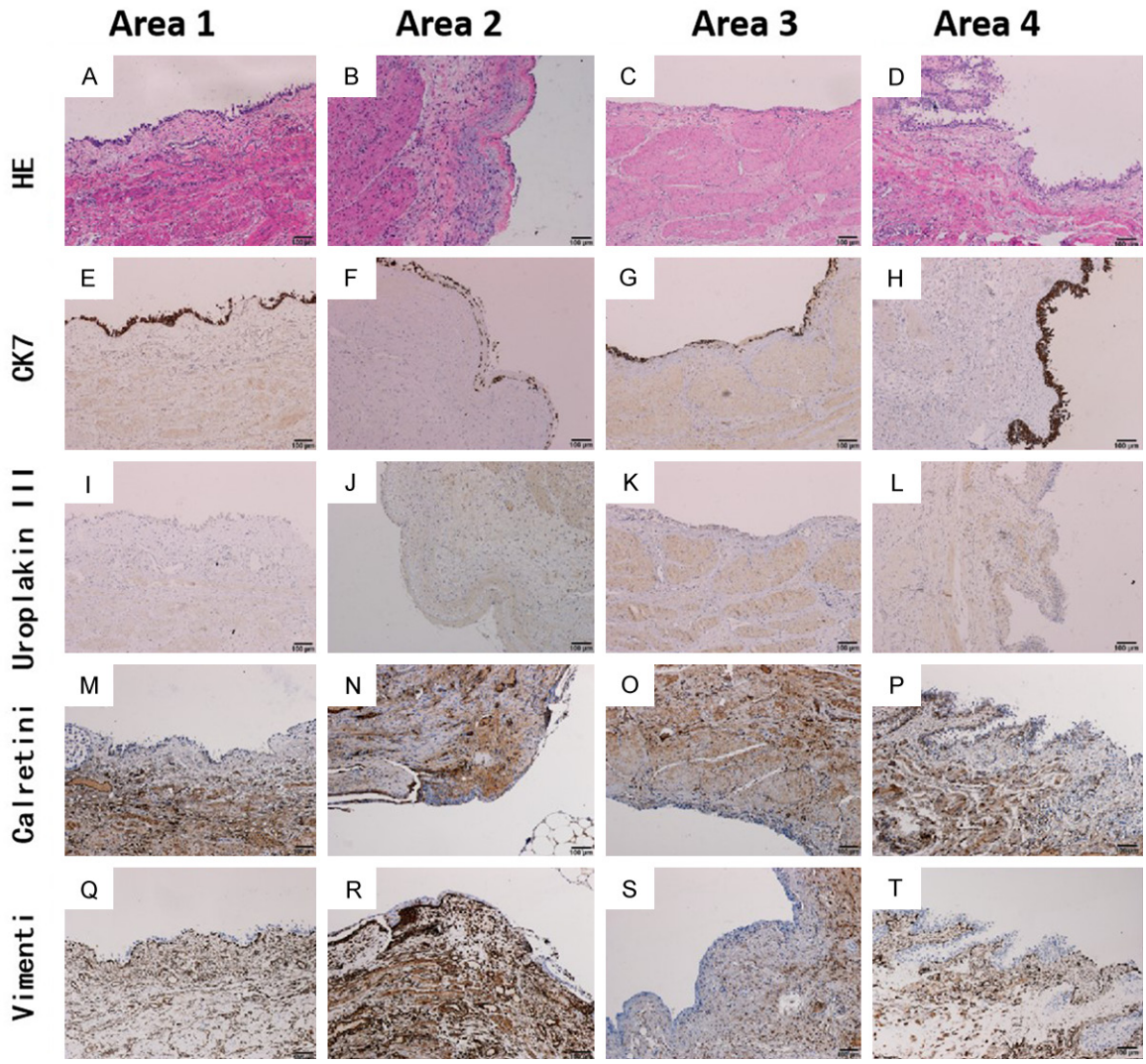

Figure 10. Histological analyses of the bladder specimens at 2 weeks after the operations. A-T: H&E staining and immunohistochemical staining of CK 7, uroplakin III, calretinin and vimentin in different areas of the bladder specimens at 2 weeks after the operations (×100).

multilayers of urothelia, and no peritoneum mesothelial cells were observed. Neovascularization was evident beneath the mucosa, and some chronic inflammatory cells and fibroblasts were present in the subepithelial layer. Our immunohistological analysis showed no expression of calretinin or vimentin, but there were expressions of both CK 7 and uroplakin III, suggesting that the peritoneal mesothelial was completely replaced by mature urothelium. Twelve weeks after the operation, the newly-formed tissues were similar to the normal bladder mucosa.

The mechanism by which the peritoneum is converted into an urothelium in the urinary tract system is not very clear. There are two hypotheses about the mechanism of conversion of peritoneal mesothelial cells into urothelial cells [6, 7]. One is the distant migration of the epithelium, and the other is the direct metaplasia of the peritoneal mesothelial cells, but the specific mechanism is not yet known. In our study, we found that regenerated urothelia arise multicentrically in the same bladder sample at the first and second weeks after the bladder mucosa reconstruction. In addition, some urothelial cells far away from the bladder neck and urethra range regenerate more actively, which opposes the distant migration theory. Therefore, we believe that urothelial regeneration in bladder mucosa reconstruction is also associated with the transformation of mesenchymal stem cells (MSCs). The specific mecha-

nism remains to be confirmed by further research. MSCs are multipotent stromal cells that can differentiate into the urothelium and secrete various bioactive molecules that can mediate tissue regeneration [19]. It is probable that TGF-b1 and bFGF play a crucial role in urothelial cell proliferation and differentiation [4, 20]. Stem cells are able to provide a means for reconstructing the urinary bladder mucosa after the bladder cancer resection and intravesical adjuvant therapy [21]. Irrespective of the mechanism of peritoneal conversion, our grafts support bladder mucosa reconstruction. Compared with conventional materials and current tissue engineering strategies, autologous free peritoneal grafts have advantages in bladder reconstruction: they take advantage of a normal biological process and are totally autologous; further, they use the bladder wall to retain the blood supply and innervation. Our study is preliminary and requires further validation. In the future, this bladder mucosal reconstruction method may be used for the surgical treatment of bladder mucosal lesions, such as non-muscle invasive bladder cancer, cystitis glandularis, and interstitial cystitis, to prevent recurrence.

Conclusions

This initial study shows that autologous free peritoneal grafts can be effectively used in bladder mucosa reconstruction. In our work, the approach that we propose in reconstructing bladder mucosa is novel and appears to be effective. However, further studies must be performed before this approach can be used in clinical practice for bladder mucosa reconstruction.

Acknowledgements

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Disclosure of conflict of interest

None.

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