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Biomedical Materials

NOTE

Collagen scaffolds tethered with bFGF promote corpus spongiosum regeneration in a beagle model

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Abstract

Regeneration of the corpus spongiosum helps prevent complications following urethral reconstruction, but currently there is a lack of effective therapeutic methods in clinic. In previous studies, we fabricated a fusion protein collagen-binding domain (CBD)-basic fibroblast growth factor (bFGF) that specifically binds to and releases from collagen biomaterials. We demonstrated that CBD-bFGF could promote angiogenesis and tissue regeneration *in vivo*. In this study, we established a beagle model with extensive urethral defects, and reconstructed the defects with collagen biomaterials that were unmodified or modified with CBD-bFGF. The results demonstrate that CBD-bFGF promotes corpus spongiosum regeneration resulting in improved outcomes following urethral reconstruction. Modifying collagen biomaterials with CBD-bFGF may represent an effective strategy for urethral substitution in urethral reconstruction.

Introduction

The corpus spongiosum plays an important role in urination. Both congenital and acquired factors, such as trauma and inflammation, can induce injury to the spongiosum tissue. During recovery, if normal vasculature of the affected urethra is not restored, complications such as urethral stricture, urethral diverticula, and urethrocutaneous fistula can develop [1]. Autologous tissues are the primary source materials used for urethral reconstruction; however, in some cases, these tissues may not meet the clinical requirement. Moreover, the source pool of autologous tissues is very limited. Thus, the use of artificial biomaterials for urethral reconstruction has become an attractive alternative strategy for urethral reconstruction.

In urethral reconstruction, provision of an adequate blood supply increases the chances of a successful surgical outcome [2], while significantly decreasing complications [3, 4]. The ideal material for reconstruction should be fully vascularized and morphologically resemble the spongiosum tissue. Promoting regeneration of spongiosum tissue improves the outcome of urethral reconstruction. Basic fibroblast growth factor (bFGF) is a glycoprotein that is abundant in eukaryotic organisms and plays prominent roles in several pathophysiological processes [5, 6]. Being a potent chemotactic and mitogenic factor,

bFGF can stimulate proliferation and differentiation of a number of different cells, including endothelial and smooth muscle cells, and plays a role in inducing angiogenesis [7]. Indeed, bFGF has been shown to be among the most potent angiogenesis inducing growth factors, and researchers have already attempted to explore a role for bFGF in mediating urethral injuries and reconstruction. Burdzińska *et al* demonstrated that preincubation with bFGF significantly increased the abundance of muscle-derived cells in engrafted tissue upon transplantation into the urethral wall [8]. Kim *et al* reported that bFGF could promote smooth muscle regeneration and exerted a good therapeutic effect against urinary incontinence [9].

Previously, we used bFGF and a collagen-binding domain (CBD) to construct the fusion protein CBD-bFGF, which could specifically bind type I collagen [10], accelerate cellularization of collagen biomaterials, increase neovascularization, and promote regeneration [11–16]. From these observations, we hypothesized that CBD-bFGF could promote spongiosum tissue regeneration in a urethral reconstruction scenario. To test this hypothesis, we modified collagen biomaterials with CBD-bFGF, and evaluated the performance of the system in an extensive urethral defect beagle model.

Materials and methods

Experimental protocol

Ten healthy mature male beagles were randomized into two groups, the CBD-bFGF and control groups. To develop a model of urethral defect, a 5 cm segment of the corpus spongiosum in all test animals was removed. Thereafter, the urethral defect was reconstructed with tubularized collagen scaffolds, either unmodified or modified with CBD-bFGF in the control and CBD-bFGF groups, respectively. 6 months after reconstruction, the regenerated corpus spongiosum was functionally evaluated by retrograde urethrography and urodynamic studies, histological examination of hematoxylin-eosin (HE)-stained sections, and immunofluorescence examination.

Animals

The beagles (age 12–14 months; body weight 10–13 kg) were purchased from Sichuan Institute of Musk Deer Breeding. During the course of the experiment, animals were housed individually in a controlled temperature (18 °C–29 °C) and humidity (30%–70%) dog housing facility. At baseline, all animals had normal urethral structure and urinated smoothly. The operation for establishing the model, as well as the reconstructive surgery, was performed after two weeks of adaptation. All of the animal experimental protocols met the criteria specified by the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the Animal Experimentation Ethics Committee, of the Third Military Medical University.

Preparation of biomaterial scaffolds

Preparation of the CBD-bFGF protein was carried out as previously described [10, 14]. The gene of CBD-bFGF that contained a His_6 Tag was amplified by polymerase chain reaction and inserted into vector pET-28a, the recombinant plasmids of pET28a-CBD-bFGF were transformed into *Escherichia coli* BL21 (DE3), and the recombinant protein was induced to express with IPTG. Then the recombinant proteins were purified through nickel column chromatography.

The collagen membranes were derived from bovine skin treated with methanol:chloroform (1:1) solution. Then ultrasonic machine was used to wash out the unwanted tissues. For removing cellular components, the samples were treated with 0.2% Triton. After freeze-dried, the membrane possessed a porous stereoscopic structure, which was assessed under scanning electron microscopy (SEM). Then it was cut into 5 cm × 3 cm slice and sterilized by 12 kGy Co^{60} irradiation before usage. Furthermore, the tensile strength of the wet membrane was measured using a tensile testing machine (Instron ElectroPuls E1000, United Kingdom). The test speed was 2 mm min^{-1}.

Preoperatively, the collagen scaffolds were constructed by suturing the collagen membranes into a tube using a synthetic absorbable suture (VCP304H, ETHICON), according to the diameter of the host urethra during the reconstructive surgery. The water absorption volume of the collagen scaffold was ascertained to be 133.3 μl cm^{-2}, with complete wetting and no leakage. We then dissolved 150 μg of CBD-bFGF in 2 ml of sterile phosphate-buffered saline (PBS), which was dripped into the tubularized collagen scaffold following the construction of the tubularized collagen scaffold according to host urethra area, with a specified CBD-bFGF dosage of 10 μg cm^{-2} collagen membrane, and refrigerated at 4 °C until grafting.

Surgical procedures for urethral reconstruction

After two weeks of acclimatization, a model with extensive urethral defects was set up surgically and the defective corpus spongiosum was reconstructed in the same surgical session. Following 6 h of preoperative fasting, the beagles were anesthetized with pentobarbital sodium (30 mg kg^{-1}) by intraperitoneal injection, placed supine, and antibiotic prophylaxis was initiated with penicillin G sodium (5×10^4 U, IM) 15 min before the surgery. After shaving, the skin around the ventral penis was disinfected with Entoiodine.

Through an upside down 'U' incision, the ischiocavernosus and bulbocavernosus were incised longitudinally to expose the corpus spongiosum, which was then dissected from the cavernous body of the penis. A 5 cm full-thickness segment of the entire corpus spongiosum between the baculum and bulbous urethra was

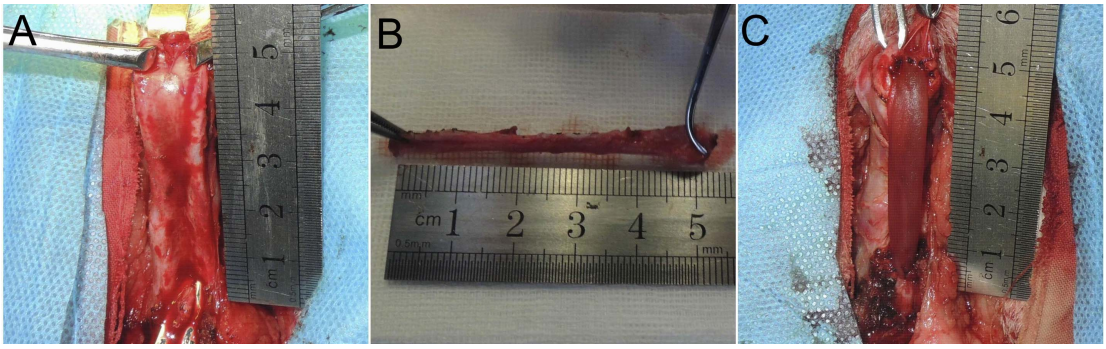


Figure 1. Surgical procedures of urethral reconstruction. A 5 cm full-thickness segment of corpus spongiosum between the baculum and bulbous urethra was excised (A), (B). Tubularized collagen scaffold, unmodified or modified with CBD-bFGF for the collagen or collagen-CBD-bFGF group, respectively, was anastomosed to the native urethra with a synthetic absorbable suture (VCP304H, ETHICON; (C)).

excised and a tubularized collagen scaffold, unmodified or modified with CBD-bFGF for control or test groups, respectively, was anastomosed to the native urethra with a synthetic absorbable suture (VCP304H, ETHICON) based on its group (figure 1). A 12 F (French catheter scale, one French (1 F) means the circumference round a catheter is one millimeter) catheter was inserted into the bladder via the native and reconstructive urethra as a drainage tube, fixed to the external urethral orifice with sterile silk sutures, and retained *in situ* for 14 d. All animals were maintained individually in a postoperative recovery cage at 27 °C–29 °C until they regained consciousness. Penicillin G sodium (5×10^4 U kg^{-1} d^{-1} IM) was administered as antibiotic cover for 5 d postoperatively. Wounds were covered with sterile gauze and disinfected with entoiodine daily until they healed.

Postoperative observation

The wound, general wellbeing, food intake, and urination of all experimental animals were monitored postoperatively. Following catheter removal, urination, including urine stream, urination time, and frequency of voiding, was observed to check for dysuria, which is symptomized by a thinner urine stream, longer urination time, and urinary frequency.

Urethra radiographic evaluation

Retrograde urethrography was used to evaluate the morphology of the urethral lumen, and was performed both preoperatively and 6 months after urethral reconstruction. Each beagle was anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg kg^{-1}) and placed in an oblique position on an examination table. A 3-cavity catheter was inserted into the distal urethra and fixed with 2 ml sterilized distilled water. Ioversol (Jiangsu Hengrui Medicine Co., Ltd) contrast solution was gently injected into the urethra via the catheter until it was full, and an x-ray was taken.

Urethra functional evaluation

Urodynamic studies, including filling cystometry and urethral pressure profilometry (UPP), were used for functional evaluation of the neo-urethra and were performed both preoperatively and 6 months after urethral reconstruction. Each beagle was anesthetized with an intraperitoneal injection of chloral hydrate (300 mg kg^{-1}) and placed supine on the examination table. A urodynamic catheter (6 F, French catheter scale, one F means the circumference round a catheter is one millimeter) was inserted into the bladder via the urethra to drain the bladder, which was then filled with sterile physiological saline at a rate of 20 ml min^{-1}. Intravesical and intra-abdominal pressures, measured via a small balloon catheter inserted into the rectum, were recorded during the filling phase.

After the filling cystometry, the bladder was drained again and sterile physiological saline was infused into the urethra through the side-hole channel at a rate of 2 ml min^{-1}; then, the catheter was withdrawn at a rate of 2 mm s^{-1} with simultaneous recording of the UPP.

Histological evaluation

Histological evaluation of tissues was conducted after the animal were euthanized (pentobarbital overdose; 90 mg kg^{-1}), which was performed subsequent to the retrograde urethrography and urodynamic studies. The animals were placed supine, and the entire urinary system from the kidneys to the urethral orifice was exposed. All gross specimens were examined *in situ* and data were recorded, including the length of the neo-urethra, the extent of scar tissue, rigidity of the scar, and the dilatation of the urinary tract, prior to harvesting of the specimen, which included the neo-urethra and adjacent autogenous urethra. Then the specimen was cut into two parts longitudinally. Half of the tissue was fixed in formalin for 24 h, paraffin embedded, cut into 2.5 μm thick sections, stained with HE, and observed using light microscopy. The other half of the specimen was cut into 5 μm thick sections and frozen in liquid nitrogen. Tissue regeneration,

Table 1. Primary antibodies used for immunofluorescence staining.

Primary antibody	Host	Dilution	Catalog, company
Polyclonal antibodies against Von Willebrand factor*	Rabbit	1:400	ab6994, Abcam
Monoclonal [AE1/AE3+ 5D3] antibodies against pan cytokeratin**	Mouse	1:100	ab86734, Abcam
Monoclonal [4A4] antibodies against alpha smooth muscle actin***	Mouse	1:200	ab119952, Abcam

Note. * Was the marker of vascular tissue, ** was the marker of epithelial tissue, *** was the marker of smooth muscle tissue.

including vascular tissue, urothelium, and smooth muscle, was evaluated by immunofluorescence. The frozen sections were placed for 30 min at room temperature, fixed in acetone for 10 min at 4 °C, dried for 20 min at 37 °C, and washed three times with PBS. To reduce the nonspecific binding, the sections were incubated with 10% sheep serum for 60 min. Then the sections were incubated with primary antibody overnight at 4 °C (antibodies to identify specific tissues and their dilutions are listed in table 1). Next morning the sections were washed with PBS three times and incubated with secondary-fluorescence for 60 min at 4 °C. DAPI staining was used to identify nuclei, and the immunofluorescence-treated sections were visualized by confocal scanning laser microscopy (Leica TCS SP5, Germany).

Five sections at same position of neo-urethra were selected to evaluate tissue regeneration. Using LAS AF Lite (2.3.0 build 5131, an affiliated software of the confocal scanning laser microscopy), we measured the thickness of epithelial layer at five corresponding location in each section at 400× magnification, and calculated the mean thickness of epithelial layer to evaluate regeneration of epithelial tissue (the unit is micron). The quantitative analysis of vascular tissue was performed with Image-Pro Plus 6.0. Five high power fields (×400) at corresponding location of each section, stained with polyclonal antibodies against Von Willebrand factor, were converted to Gray Scale 8 (8 bit gray scale), and then the pictures were inverted. Finally, the fluorescence density of each field was counted. The mean density was calculated to evaluate regeneration of vascular tissue (the density was counted from the whole field). The quantitative analysis of smooth muscle tissue was performed with Image-Pro Plus 6.0 as above too. The dyeing area of each field was counted, the mean area was calculated to evaluate regeneration of smooth muscle tissue (the area was counted from the whole field).

Statistical analysis

The quantitative data were described as mean ± standard deviation. Differences of means among groups were tested by one-way analysis of variance (One-Way ANOVA), and multiple comparisons between the pairwise groups were conducted with Student–Newman–Keulsmethod to reduce false positive. Survival analysis of Kaplan–Meier method was used to calculate mean survival time without dysuria of the collagen-CBD-bFGF group and the collagen group, and Log-rank test was use to compare the difference of survival time without dysuria between the two groups. A p-value < 0.05 was considered statistically significant according to statistics convention.

All the statistical analysis were performed with the Statistical Package for Social Sciences version 17.0 (SPSS 17.0).

Results

Characterization of collagen membrane

The collagen membranes that were used in urethral reconstruction were purified from fresh bovine aponeurosis, and exhibited a 3-dimensional structure similar to native collagen. Both sides of the collagen membranes were not waterproofed, and SEM revealed that the collagen membranes were poriferous (figure 2), which would be conducive to the widespread dissemination of CBD-bFGF throughout the collagen fibers.

Meanwhile the stress of three membrane samples randomly selected was measured. As shown in figure 2(C), the ultimate average stress of the wet collagen membrane was 6.32 ± 0.42 MPa.

Clinical observation

Two weeks after urethral reconstruction, the catheter was removed from the neo-urethra. All the beagles had autonomous urination. As time went on, some of the beagles presented symptoms of difficult urination, including arduous urination, prolonged urination, retention of urine, followed by vomiting and dispirited humor. Prolonged urination was one objective indicator of difficult urination; once it was observed, we considered that the beagle was suffering with urination difficulties. When a beagle was observed vomiting and appeared dispirited, we consider that the beagle was suffering with severe urination difficulties. Over time, all five beagles in the collagen group presented with symptoms of difficult urination, whereas only two beagles in the collagen-CBD-bFGF group presented with symptoms of difficult urination. Usually, these symptoms presented earlier and more severe in the unmodified collagen group. We analyzed the survival time without dysuria, in both groups, with Kaplan–Meier survival analysis. The mean survival time without dysuria after urethral reconstruction in the collagen-CBD-bFGF group was 127.4 d, which was significantly longer than that of the collagen group

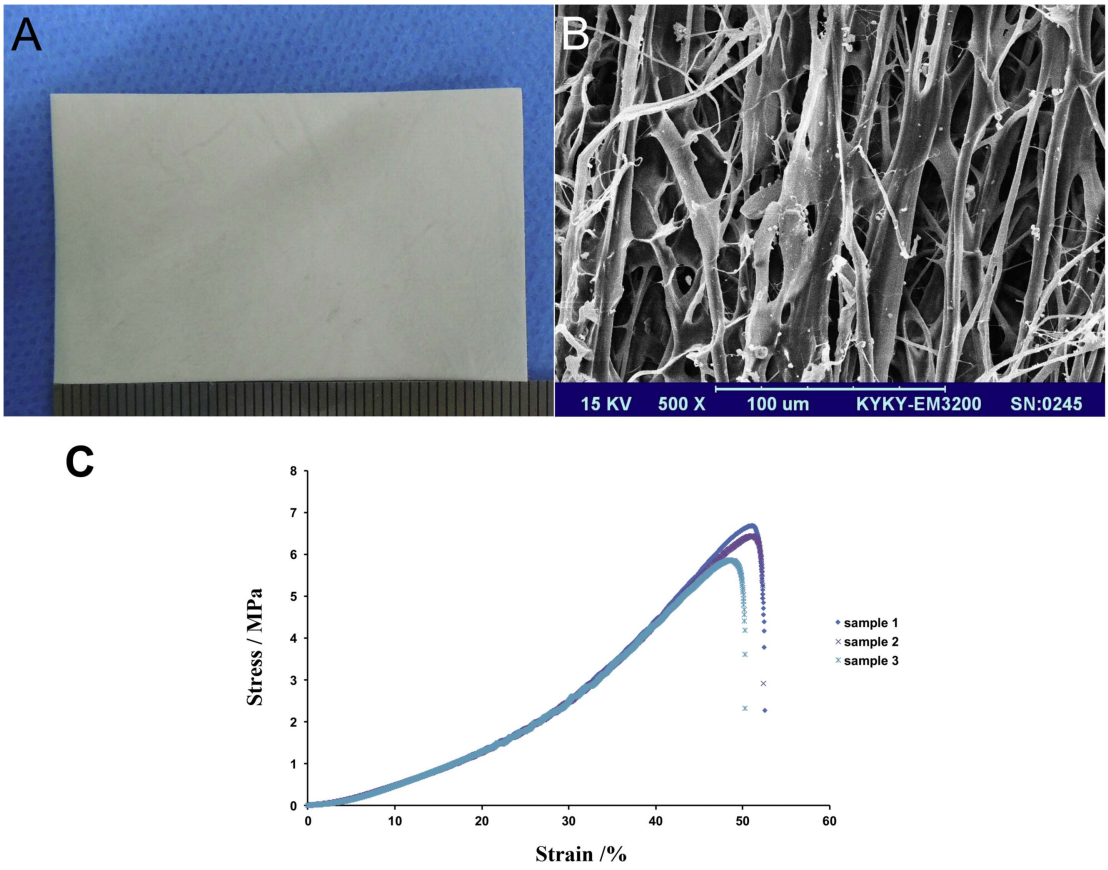


Figure 2. The structure of collagen membranes. The collagen membranes were similar in structure to native collagen. Both sides of the collagen membranes were not waterproofed (A), SEM revealed the collagen membranes were poriferous (B). The ultimate stress of the wet membrane (C).

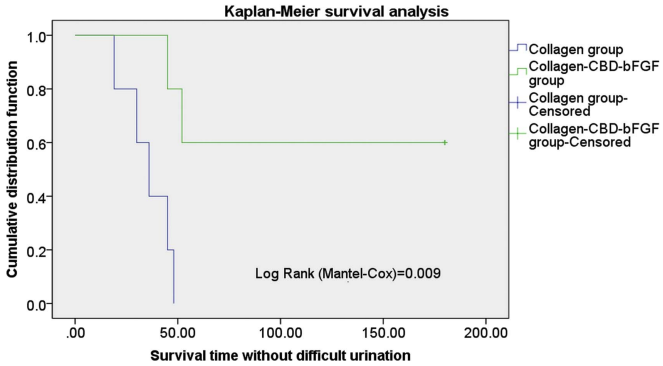


Figure 3. Kaplan–Meier survival analysis of beagle survival time without dysuria after urethral reconstruction. The mean survival time without dysuria after urethral reconstruction in the of collagen-CBD-bFGF group was 127.4 d, which was significantly longer than that of the collagen group (35.6 d; $P < 0.01$).

(35.6 d, $P < 0.01$; figure 3). This result indicates that the inclusion of CBD-bFGF could delay shrinkage of the neo-urethra. Furthermore, in the collagen group, three beagles developed urethral fistulas, and the other two underwent cystostomy procedures due to severe urination difficulties. In the collagen-CBD-bFGF group, one beagle developed a urethral fistula, while another underwent cystostomy due to severe urination difficulties, while the other three beagles showed no symptoms of dysuria during the observation period. These results indicate that the incorporation of the CBD-bFGF component could improve the outcome of urethral reconstruction.

Radiographic evaluation

Retrograde urethrography showed that, at baseline, the lumina of normal urethra were open and smooth, with a mean diameter of 6.31 ± 0.59 mm following reconstruction. However, 6 months after urethral reconstruction, the lumina of the neo-urethra in both groups

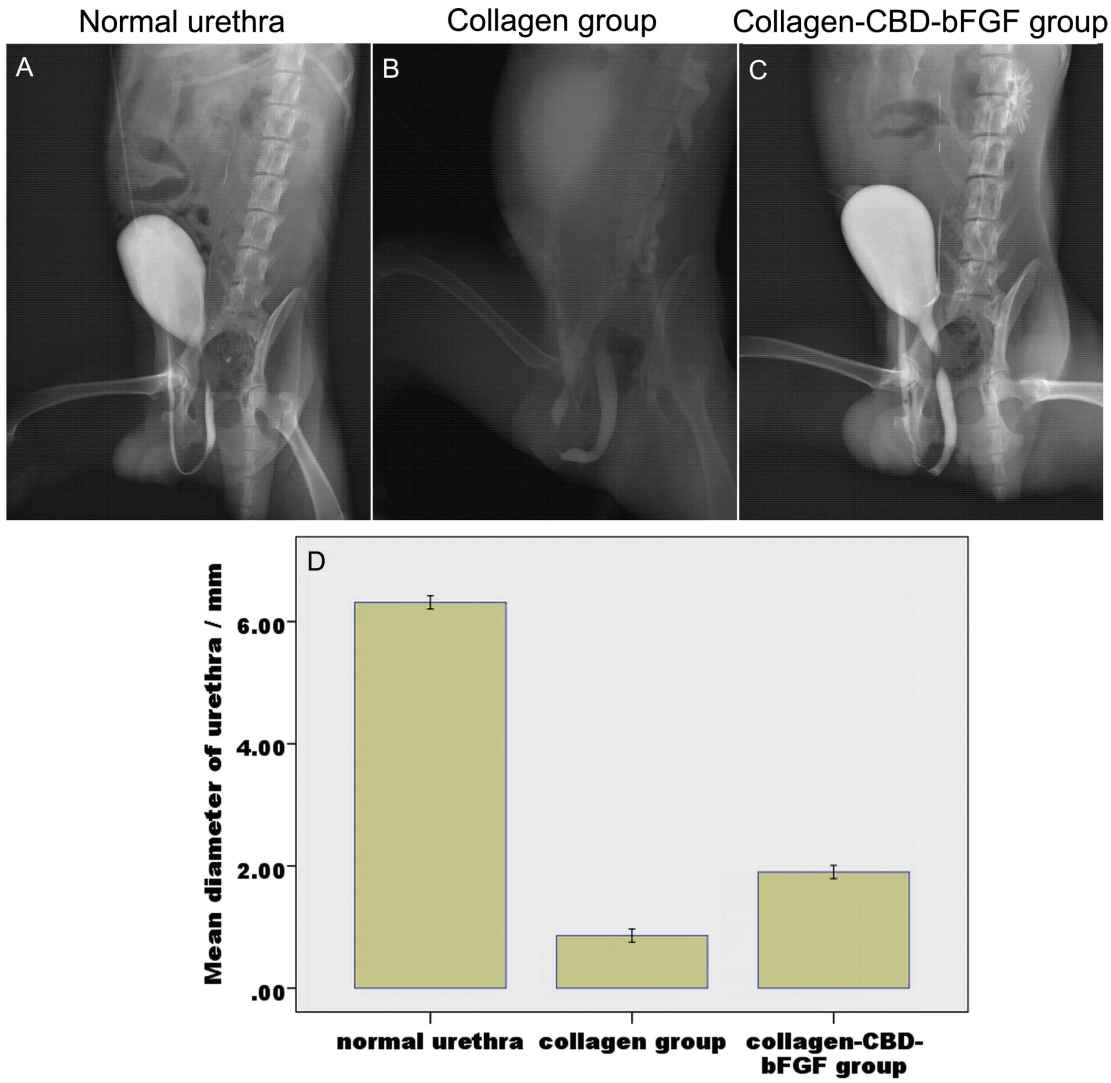


Figure 4. Urethra morphologic examination with retrograde urethrography. The lumina of normal urethra were open and smooth (A), the lumina of the neo-urethra in both groups showed varying degrees of defects and rigid marginated narrowing (B), (C), but the mean diameter of the neo-urethra in the collagen group was significantly less than that of the collagen-CBD-bFGF group ((D), $P < 0.01$).

exhibited varying degrees of defects and rigid marginated narrowing. The mean neo-urethra postoperative diameter of the collagen group (0.86 ± 0.21 mm) was significantly less ($P < 0.01$) than that in the collagen-CBD-bFGF group (1.90 ± 0.36 mm). Moreover, three beagles in the collagen group and one in the collagen-CBD-bFGF group suffered from urethral fistulas (figure 4).

Urethra functional evaluation

At baseline, a urodynamic catheter (6 Fr) could be inserted into the bladder via the normal urethra. However, 6 months after urethral reconstruction, all five beagles in the collagen group and two beagles in the collagen-CBD-bFGF group suffered urination difficulties and could not be catheterized via the neo-urethra because of severe urethral strictures. Therefore, this evaluation could not be completed in all of the beagles included in the study. We conducted a functional evaluation of the urethra in the three beagles of the collagen-CBD-bFGF group that exhibited normal urination behavior. The data shows that the average neo-urethral pressure was 54.67 ± 14.58 cm H_2O, which was significantly higher than the pressure of the normal urethra at the corresponding section (5.73 ± 0.82 cm H_2O, $P < 0.01$; figure 5(A)). Filling cystometry tests revealed that the average maximum cystometric capacity in these three beagles was 53.40 ± 8.30 ml, which did not differ statistically from that of the normal urethra (49.65 ± 7.21 ml, $P > 0.05$; figure 5(B)).

Gross observation

Post-sacrifice, we dissected the entire urinary system of all candidate beagles, and discovered varying amounts of scar tissue surrounding the neo-urethra in both groups. Usually, more severe difficulties in urination was accompanied by more extensive scaring around the neo-urethra. We measured the length of the neo-urethra, and found that the mean length of the neo-urethra in the collagen-CBD-bFGF group was

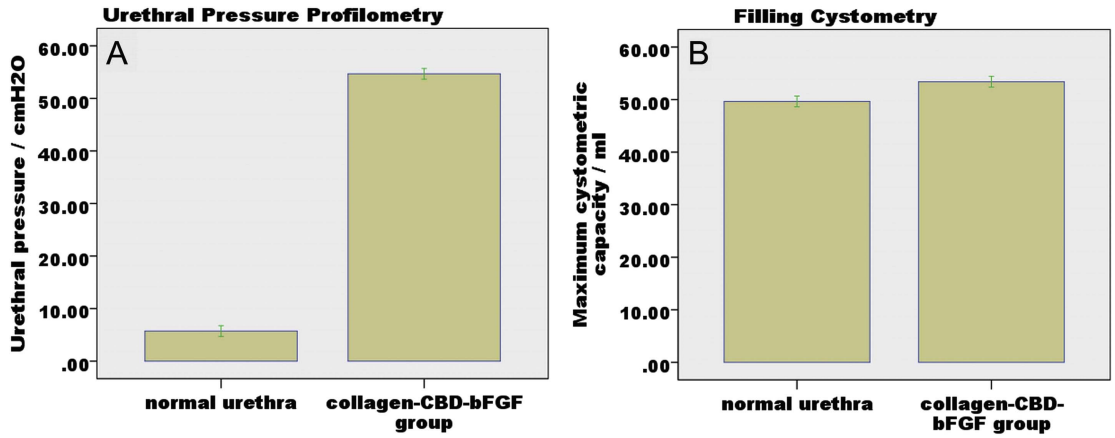


Figure 5. Urethra functional evaluation; urodynamic studies. Urethral pressure profilometry demonstrated that the mean neo-urethra pressure was significantly higher than preoperative urethral pressure ((A), $P < 0.01$), while filling cystometry showed that there was no significant difference in bladder capacity and compliance before and after urethral reconstruction ((B), $P > 0.05$).

Table 2. Mean length of the neo-urethra at 6 months postoperatively.

Group	*N*	Mean length/cm	Standard deviation
Collagen group	5	2.88	0.37
Collagen-CBD-bFGF group[a]	5	4.36	0.25

[a] The mean length of neo-urethra in collagen-CBD-bFGF group was significantly longer than that of the collagen group 6 months postoperatively ($P < 0.05$). The incorporation of the CBD-bFGF component seemed to mitigate shrinkage of the neo-urethra due to scar contracture.

significantly longer than that in the collagen group ($P < 0.01$; table 2), indicating that CBD-bFGF is helpful in mitigating shrinkage of the neo-urethra due to scar contracture. Meanwhile, we noted that the neo-urethra lumen in the collagen group was usually narrower than in the collagen-CBD-bFGF group; this finding is consistent with the retrograde urethrography results. Urethral fistulas were found in three beagles of the control group and one beagle of the collagen-CBD-bFGF group that had suffered with urination difficulties. Various degrees of upper urinary tract dilation were observed in beagles that suffered from urination difficulties but had not undergone cystostomy, whereas no upper urinary tract dilation was observed in beagles that did not have urination difficulties or those that underwent cystostomy procedures (figure 6). No urethral calculi were observed in any of the beagles.

Hematoxylin and eosin staining

Data from HE staining shows that the urethral cavosurface of the normal urethra in beagles was covered by stratified epithelium, with orderly cell arrangement. The epithelial layer was surrounded by the corpus spongiosum, which was comprised of abundant vascular tissue and a little smooth muscle tissue, which were the major structural components of the normal urethra. The epithelial layer of the neo-urethra in the collagen-CBD-bFGF group appeared smooth and well arranged, similar to that of a normal urethra, at 6 months postoperatively. However, the epithelial layer of the neo-urethra in the collagen group appeared coarse and disordered, and the thickness of the layer was usually less than that of the collagen-CBD-bFGF group, 6 months postoperatively. There was no typical spongy tissue observed in the neo-urethra from both groups, but the number of neo-urethral blood vessels in the collagen-CBD-bFGF group tissues was more than in the collagen group. The lumina of new blood vessels in some sections of the collagen-CBD-bFGF group were dilated and resembled little sinusoids. Urethral fistulas were discovered in three beagles of the control group and one of the collagen-CBD-bFGF group (figure 7).

Regeneration of vascular tissue

To evaluate vascular tissue regeneration, the frozen sections were stained with polyclonal antibodies against Von Willebrand factor. Vascular tissues emitted a bright red fluorescence under confocal laser scanning microscopy (figure 8). In the normal urethra, vascular tissues were in a circular arrangement around the urethral lumen in cross-sections, and were parallel to the urethral mucosa in longitudinal sections, which were arranged regularly, and constituted the main body of the corpus spongiosum. The mean density of blood vessels in the normal urethra was 0.049 ± 0.007/field. However, in the neo-urethra of both study groups, vascular tissues were not well arranged, as observed for normal urethra, and the mean densities of blood vessels in both groups were significantly lower than in the normal urethra ($P < 0.01$). However, the mean density of blood vessels in the collagen-CBD-bFGF group was 0.038 ± 0.006/field, which was significantly higher than that of the collagen group (0.024 ± 0.005/field, $P < 0.01$). This

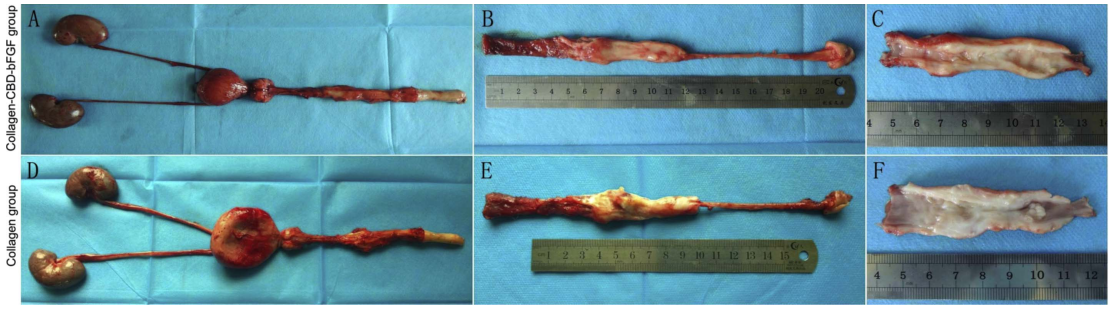


Figure 6. Gross observation of reconstructed urethra. Varying amounts of scar tissue surrounding the neo-urethra was observed in both groups. Usually, there was more scar tissue surrounding the neo-urethra in the collagen control group (D), (E), (F) than in the collagen-CBD-bFGF group (A), (B), (C).

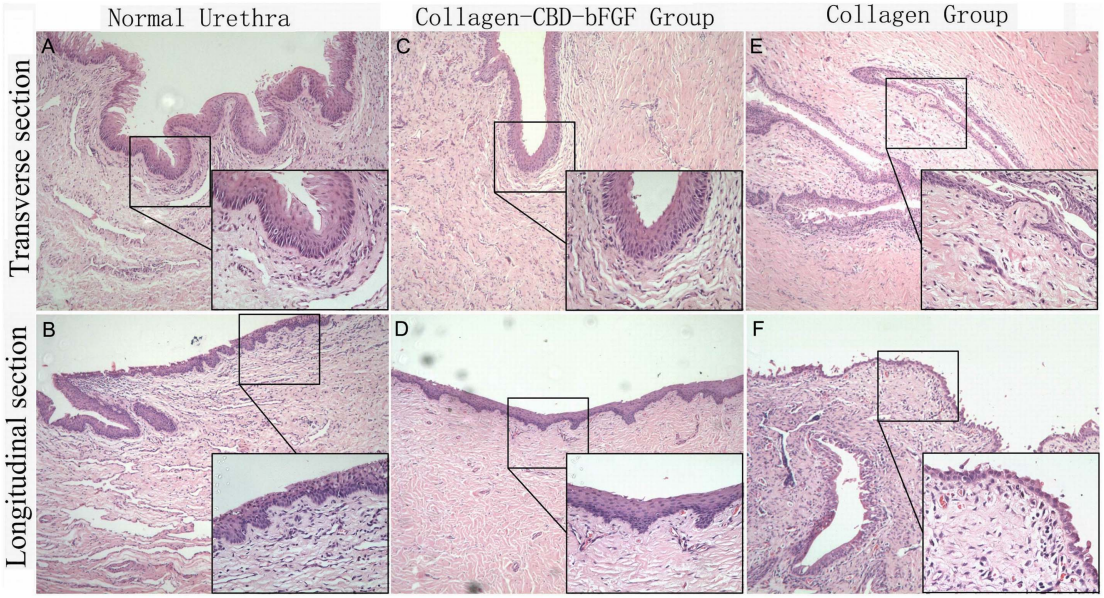


Figure 7. Hematoxylin and eosin staining. The epithelial layer in the neo-urethra of the collagen-CBD-bFGF group (C), (D) was similar to that of the normal urethra (A), (B), while in the collagen group (E), (F) it was usually coarse, disordered, and thinner than that of the collagen-CBD-bFGF group. No typical spongy tissue was observed in the neo-urethra of both groups, but the number of blood vessels in the collagen-CBD-bFGF group (C), (D) was higher than in the collagen group (E), (F).

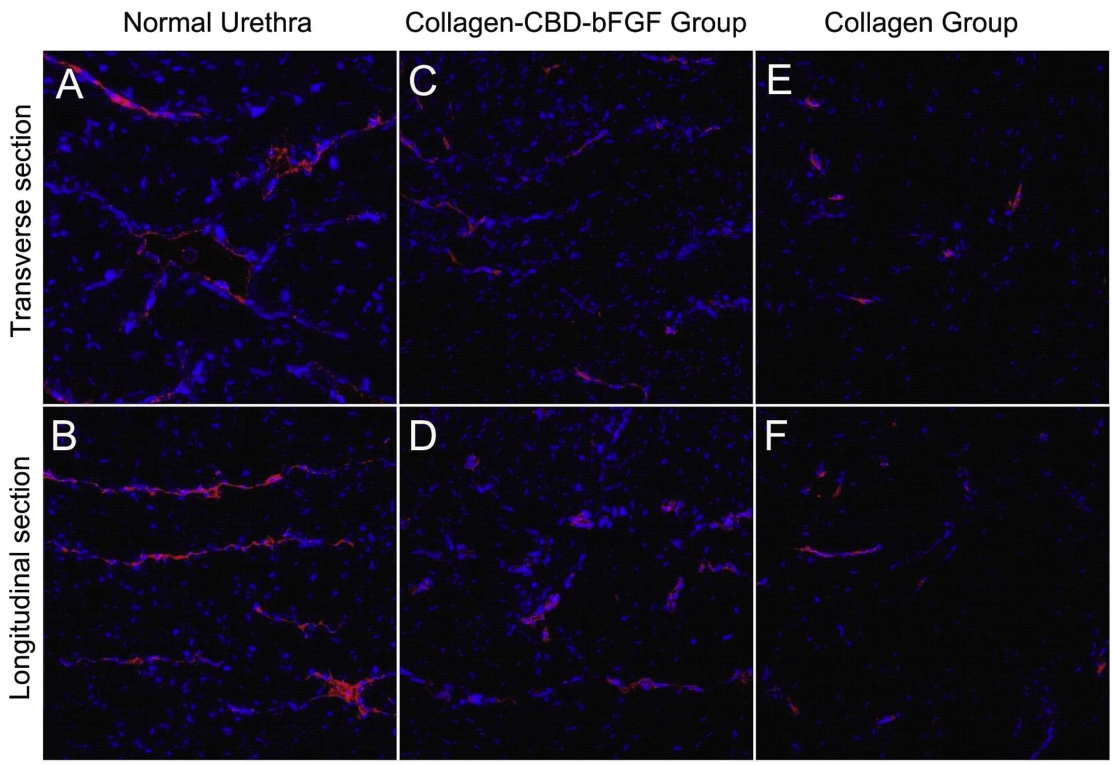
result reveals that CBD-bFGF significantly promoted vascular tissue regeneration.

Regeneration of other tissues

We identified epithelial and smooth muscle tissues with mouse monoclonal antibodies against pan cytokeratin and alpha smooth muscle actin, respectively (figure 9). Epithelial tissues emitted bright green fluorescence and were arranged in order both in the normal urethra and neo-urethra of the collagen-CBD-bFGF group, but were usually loose and disordered in the neo-urethra of the collagen group. The mean thickness of the neo-urethra epithelial layer in the collagen-CBD-bFGF group was $94.93 \pm 8.83\ \mu$m, showing no statistical difference with the normal urethra ($97.87 \pm 7.92\ \mu$m, $P > 0.05$), but was significantly thicker than that in the collagen group ($40.78 \pm 5.52\ \mu$m, $P < 0.01$). Smooth muscle tissues emitted a bright green fluorescence (figure 9). The mean smooth muscle tissue area of the neo-urethrae in the collagen-CBD-bFGF group was $44.66 \pm 8.99\ \mu$m, which was significantly lower than that of the normal urethrae ($73.86 \pm 7.97\ \mu$m, $P < 0.01$) but was significantly greater than that of the collagen group ($8.34 \pm 3.08, P < 0.01$).

Discussion

The corpus spongiosum is a conglomeration of sinusoids under the urethral epithelium. Although usually known as an erectile tissue, it plays an important role in urination. Any injury to the corpus spongiosum potentially results in fibrosis and scar formation. With scar contracture, the size of the urethral lumen reduces and, consequently, voiding problems emerge. Reconstructing the normal structure of the corpus spongiosum should reduce the incidence of urethral stricture. For patients suffering due to a long urethral stricture, an effective treatment



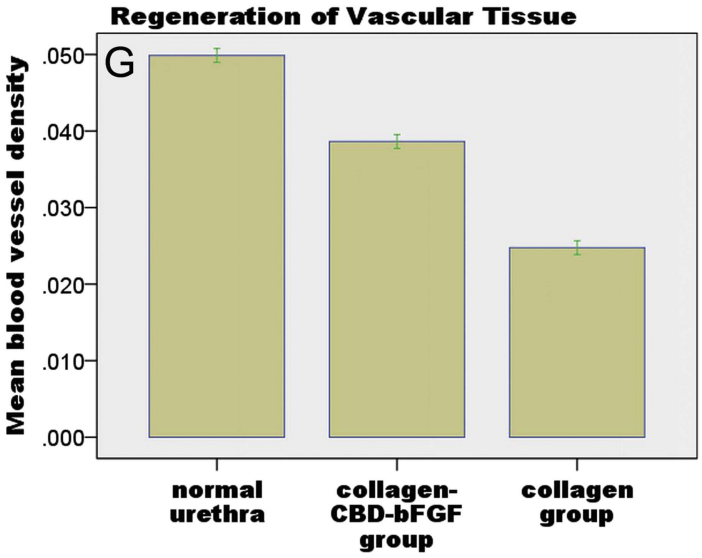


Figure 8. Regeneration of vascular tissues was evaluated with immunofluorescence staining, the primary antibody was polyclonal antibodies against Von Willebrand factor (ab6994, Abcam). In normal urethrae, vascular tissues were arranged regularly, and constituted the main body of the corpus spongiosum. In the neo-urethra of both groups, vascular tissues were not well arranged. However, the mean density of blood vessels in the collagen-CBD-bFGF group was significantly higher than that of the collagen group ($P < 0.01$).

is excision of scar tissue and reconstruction of the urethra with a substitute material. Autologous tissues, such as grafts from the buccal, bladder, colonic mucosa, and penile skin, are the main substitutes for urethral tissue clinically [17–19]. However, these materials usually do not have sinusoid-like structure similar to the corpus spongiosum and cannot restore the rich vascular supply of the urethra. When these substitutes are used clinically, complications occur frequently and the risk of complications increases with time [20, 21].

Thus, artificial biomaterials that can promote regeneration of the corpus spongiosum are becoming increasingly attractive in urethral reconstruction, and some methods involving these have been implemented. There are two methods generally used to promote regeneration of the corpus spongiosum in urethral reconstruction. One involves modifying biomaterials with bioactive molecules. Nuininga *et al* described that molecularly defined tubular type I collagen biomatrices increased neovascularization in a rabbit urethral reconstruction model [22]. Wang *et al* treated rabbit anterior

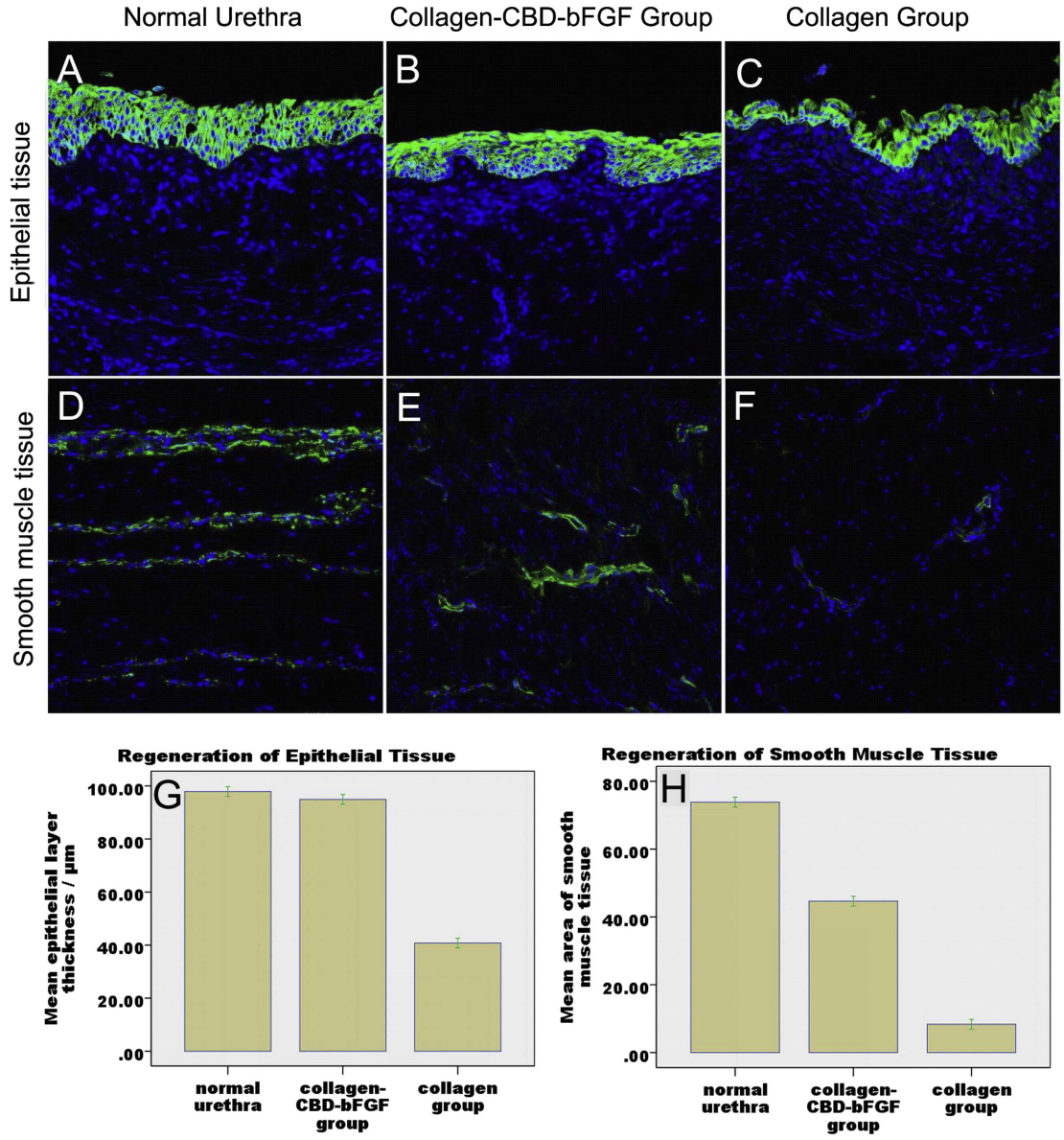


Figure 9. Regeneration of epithelial and smooth muscle tissues was evaluated with immunofluorescence staining, the primary antibodies were monoclonal [AE1/AE3 + 5D3] against pan cytokeratin (ab86734, Abcam) and monoclonal [4A4] antibodies against alpha smooth muscle actin (ab119952, Abcam), respectively. The mean thickness of the neo-urethra epithelial layer in the collagen-CBD-bFGF group (B) was not statistically different than that of normal urethrae ((A), $P > 0.05$), but was significantly thicker than that in the collagen group ((C), $P < 0.01$). The mean neo-urethra smooth muscle tissue area of the collagen-CBD-bFGF group (E) was significantly lower than that of the normal urethra ((D), $P < 0.01$), but was significantly greater than that of the collagen group ((F), $P < 0.01$).

urethral stricture with continued sustained release of vascular endothelial growth factor (VEGF) through a poly lactic co-glycolic acid nanosphere-modified bladder acellular matrix graft stent, and demonstrated that they could significantly promote angiogenesis of the repaired tissue [23]. The other method involves seeding cells into the biomaterials. Various types of cells have been tested in urethral reconstruction, including bone marrow mesenchymal stem cells, urethral epithelial cells, smooth muscle cells, urine-derived stem cells, adipose-derived stem cells, oral keratinocytes, and so on [24–30]. It is generally believed that cell-seeded biomaterials could promote tissue regeneration more effectively. Given the vehement controversies surrounding the clinical application of cell therapy [31], the former method may be more viable in clinical practice.

CBD TKKTLRT is a heptapeptide derived from mammalian collagenase that can specifically bind to collagen [32, 33]. On this basis, we previously constructed a fusion CBD-bFGF protein. *In vitro* studies showed that the bioactivity of CBD-bFGF was similar to that of commercial bFGF, but its collagen-binding ability was significantly higher than the latter. When binding to collagen biomaterials, it can be consistently released without altering its bioactivity over long periods [10, 11]. Further experiments showed CBD-bFGF could promote tissue regeneration, including in the abdominal wall, bladder, bile duct, uterine horn, and

nervous tissue, and facilitate functional organ recovery [12–16, 34]. In particular, we noted that CBD-bFGF could promote neovascularization in all of these studies. All of this evidence indicates CBD-bFGF is an ideal candidate for promoting corpus spongiosum regeneration.

In this study, dog models of extensive urethral defects were established to evaluate the potential effectiveness of CBD-bFGF in corpus spongiosum regeneration. We investigated and compared the vascularization and cellularization of collagen scaffolds with or without CBD-bFGF. There were more vascular tissues in neo-urethrae of the collagen-CBD-bFGF group than those of the collagen group, which indicates that CBD-VEGF contributed to the vascularization of the neo-urethrae in this study. The lumina of these new blood vessels, in some sections of the collagen-CBD-bFGF group, were dilated, and morphologically resembled little sinusoids, which is the typical structure of corpus spongiosum. This should facilitate improved neo-urethral blood supply, promote cellularization and other tissue regeneration, and improve neo-urethral function. Vascularization is essential for tissue regeneration and the functional recovery of a reconstructed urethra; based on this, neo-urethra from the collagen-CBD-bFGF group presented better cellularization potential than in the collagen group. There were more epithelial cells and smooth muscle cells in the neo-urethrae of the collagen-CBD-bFGF group. Notably, the thickness of the epithelial layer and its morphology in neo-urethra of the collagen-CBD-bFGF group was similar to normal urethra. The regeneration of smooth muscle tissue in the collagen-CBD-bFGF group was inferior to epithelial tissue; the mean smooth muscle tissue area in the neo-urethra in the collagen-CBD-bFGF group was significantly lower than that of normal urethra, but was greater than that of the collagen group. This could be due to greater neovascularization in the collagen-CBD-bFGF group, which would increase vascularity in the neo-urethra. Rich vascularity is conducive to tissue regeneration, as proven in many other studies [35, 36]. Data from clinical observation further verified that CBD-bFGF could improve the morphology and function of the neo-urethra.

Corpus spongiosum is the structural and functional basis of the urethra. Generally, research of urethral reconstruction usually focuses on the regeneration of urethral epithelium. As an alternative approach, we believe that improving regeneration of the corpus spongiosum is conducive to long-term functional recovery of a reconstructed urethra. In this study, we demonstrated that CBD-bFGF could promote regeneration of the corpus spongiosum and improve the function of the neo-urethra. This could further improve the outcome of urethral reconstruction. Jody and colleagues replaced a critical 1 cm urethral segment in rabbits that was modified with growth factors (including FGF-2), and demonstrated rapid urethral regeneration [22]. We further confirmed that collagen biomaterials modified with CBD-bFGF could promote corpus spongiosum regeneration in larger animals with extensive urethral defects. Therefore, in urethral reconstruction, incorporation of bFGF may effectively prevent complications and achieve better outcomes following urethral reconstruction. Experimental data were all collected within 6 months postoperatively in the both studies, and longer observation studies are required to verify the long-term effect of CBD-bFGF in urethral reconstruction. Furthermore, the mechanism by which CBD-bFGF promotes corpus spongiosum regeneration needs to be elucidated for the effective use of CBD-bFGF in urethral reconstruction.

Conclusion

We evaluated the role of CBD-bFGF in urethral reconstruction. Compared to unmodified collagen biomaterials, collagen biomaterials modified with CBD-bFGF could markedly promote corpus spongiosum regeneration and improve the outcome of urethral reconstruction. This may represent a novel and valid method for urethral substitution in urethral reconstruction.

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References

[1] Cavalcanti A G, Yucel S, Deng D Y, McAninch J W and Baskin L S 2004 The distribution of neuronal and inducible nitric oxide synthase in urethral stricture formation *J. Urol.* **171** 1943–7
[2] Guan Y, Ou L, Hu G, Wang H, Xu Y, Chen J, Zhang J, Yu Y and Kong D 2008 Tissue engineering of urethra using human vascular endothelial growth factor gene-modified bladder urothelial cells *Artif. Organs* **32** 91–9
[3] Erol A, Baskin L S, Li Y W and Liu W H 2000 Anatomical studies of the urethral plate: why preservation of the urethral plate is important in hypospadias repair *BJU Int.* **85** 728–34
[4] Sun D, Yang Y, Wei Z, Xu Y, Zhang X and Hong B 2014 Engineering of pre-vascularized urethral patch with muscle flaps and hypoxia-activated hUCMSCs improves its therapeutic outcome *J. Cell. Mol. Med.* **18** 434–43
[5] Yu P J, Ferrari G, Galloway A C, Mignatti P and Pintucci G 2007 Basic fibroblast growth factor (FGF-2): the high molecular weight forms come of age *J. Cell. Biochem.* **100** 1100–8
[6] Grose R and Dickson C 2005 Fibroblast growth factor signaling in tumorigenesis *Cytokine Growth Factor Rev.* **16** 179–86
[7] Przybylski M 2009 A review of the current research on the role of bFGF and VEGF in angiogenesis *J. Wound Care* **18** 516–9
[8] Burdzinska A, Bartoszuk U and Orzechowski A 2009 Preincubation with bFGF but not sodium ascorbate improves efficiency of autologous transplantation of muscle-derived cells into urethral wall *Urology* **73** 736–42

[9] Kim I G, Oh S H, Lee J Y, Lee J Y and Lee J H 2011 Bioactive porous beads as an injectable urethral bulking agent: *in vivo* animal study for the treatment of urinary incontinence *Tissue Eng.* A **17** 1527–35
[10] Zhao W *et al* 2007 Vascularization and cellularization of collagen scaffolds incorporated with two different collagen-targeting human basic fibroblast growth factors *J. Biomed. Mater. Res.* A **82** 630–6
[11] Shi C *et al* 2011 Regeneration of full-thickness abdominal wall defects in rats using collagen scaffolds loaded with collagen-binding basic fibroblast growth factor *Biomaterials* **32** 753–9
[12] Chen W *et al* 2010 Bladder regeneration by collagen scaffolds with collagen binding human basic fibroblast growth factor *J. Urol.* **183** 2432–9
[13] Li Q *et al* 2012 Extrahepatic bile duct regeneration in pigs using collagen scaffolds loaded with human collagen-binding bFGF *Biomaterials* **33** 4298–308
[14] Li X *et al* 2011 Regeneration of uterine horns in rats by collagen scaffolds loaded with collagen-binding human basic fibroblast growth factor *Biomaterials* **32** 8172–81
[15] Cui Y *et al* 2014 Collagen scaffolds modified with CNTF and bFGF promote facial nerve regeneration in minipigs *Biomaterials* **35** 7819–27
[16] Ma F, Xiao Z, Chen B, Hou X, Dai J and Xu R 2014 Linear ordered collagen scaffolds loaded with collagen-binding basic fibroblast growth factor facilitate recovery of sciatic nerve injury in rats *Tissue Eng.* A **20** 1253–62
[17] Chawla A, Reddy S and Thomas J 2008 Combined dorsal plus ventral double buccal mucosa graft in bulbar urethral reconstruction *Indian J. Urol.* **24** 271
[18] Kim B S and Kwon T G 2015 Urethral reconstruction using autologous vein grafts for the management of urethral strictures *Curr. Urol. Rep.* **16** 467
[19] Xu Y M, Qiao Y, Sa Y L, Zhang J, Fu Q and Song L J 2009 Urethral reconstruction using colonic mucosa graft for complex strictures *J. Urol.* **182** 1040–3
[20] Mathur R, Aggarwal G and Satsangi B 2011 A retrospective analysis of delayed complications of urethroplasty at a tertiary care centre *Updates Surg.* **63** 185–90
[21] Barbagli G *et al* 2014 Long-term followup and deterioration rate of anterior substitution urethroplasty *J. Urol.* **192** 808–13
[22] Nuininga J E, Koens M J, Tiemessen D M, Oosterwijk E, Daamen W F, Geutjes P J, van Kuppevelt T H and Feitz W F 2010 Urethral reconstruction of critical defects in rabbits using molecularly defined tubular type I collagen biomatrices: key issues in growth factor addition *Tissue Eng.* A **16** 3319–28
[23] Wang J H, Xu Y M, Fu Q, Song L J, Li C, Zhang Q and Xie M K 2013 Continued sustained release of VEGF by PLGA nanospheres modified BAMG stent for the anterior urethral reconstruction of rabbit *Asian Pac. J. Trop. Med.* **6** 481–4
[24] Wang K *et al* 2012 Fibrin glue with autogenic bone marrow mesenchymal stem cells for urethral injury repair in rabbit model *Tissue Eng.* A **18** 2507–17
[25] Raya-Rivera A, Esquiliano D R, Yoo J J, Lopez-Bayghen E, Soker S and Atala A 2011 Tissue-engineered autologous urethras for patients who need reconstruction: an observational study *Lancet* **377** 1175–82
[26] Micol L A, Arenas da Silva L F, Geutjes P J, Oosterwijk E, Hubbell J A, Feitz W F and Frey P 2012 *In vivo* performance of high-density collagen gel tubes for urethral regeneration in a rabbit model *Biomaterials* **33** 7447–55
[27] Orabi H, AbouShwareb T, Zhang Y, Yoo J J and Atala A 2013 Cell-seeded tubularized scaffolds for reconstruction of long urethral defects: a preclinical study *Eur. Urol.* **63** 531–8
[28] Bhargava S, Patterson J M, Inman R D, MacNeil S and Chapple C R 2008 Tissue-engineered buccal mucosa urethroplasty-clinical outcomes *Eur. Urol.* **53** 1263–9
[29] Wu S, Liu Y, Bharadwaj S, Atala A and Zhang Y 2011 Human urine-derived stem cells seeded in a modified 3D porous small intestinal submucosa scaffold for urethral tissue engineering *Biomaterials* **32** 1317–26
[30] Li H *et al* 2014 Epithelial-differentiated adipose-derived stem cells seeded bladder acellular matrix grafts for urethral reconstruction: an animal model *Tissue Eng.* A **20** 774–84
[31] Hermeren G 2012 Ethical challenges for using human cells in clinical cell therapy *Prog. Brain Res.* **200** 17–40
[32] de Souza S J and Brentani R 1992 Collagen binding site in collagenase can be determined using the concept of sense–antisense peptide interactions *J. Biol. Chem.* **267** 13763–7
[33] de Souza S J, Madaio M P, Juliano Neto L and Brentani R R 1994 A monoclonal autoantibody against a complementary peptide recognizes interstitial collagenase *Immunomethods* **5** 172–6
[34] Shi Q *et al* 2014 Collagen scaffolds modified with collagen-binding bFGF promotes the neural regeneration in a rat hemisected spinal cord injury model *Sci. China Life Sci.* **57** 232–40
[35] Goerke S M, Obermeyer J, Plaha J, Stark G B and Finkenzeller G 2015 Endothelial progenitor cells from peripheral blood support bone regeneration by provoking an angiogenic response *Microvascular Res.* **98** 40–7
[36] Kim M S, Lee H K, Kim S Y and Cho J H 2015 Analysis of the relationship between liver regeneration rate and blood levels *Pak. J. Med. Sci.* **31** 31–6