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Experimental study on transplantation of autologous minced muscle with human umbilical cord mesenchymal stem cells for urethral reconstruction

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Abstract. – OBJECTIVE: To investigate the feasibility and efficiency of the autologous minced muscle-derived regenerate as a living patch graft for the urethral reconstruction with the assistance of human umbilical cord mesenchymal stem cells (hUC-MSCs) in the rabbit model.

MATERIALS AND METHODS: The hUC-MSCs and minced-muscle–derived regenerate tissue was prepared and used as potential autologous patch graft for the reconstruction of the defective rabbit urethra. Animal with autologous free skeletal muscle piece was observed as control. Histopathological and immunohistochemical staining methods were adopted to evaluate the regenerating effect; the reconstructive effects in New Zealand rabbit were observed in both groups by retrograde urethrography and urinary microscopy.

RESULTS: The expression of desmin and anti-human specific nuclear antigen (ANA) were positive for co-culture microsomes *in vivo* and *in vitro*; after the reconstructive surgery, histopathological studies revealed fibrous connective tissues and abundant muscle fibers constituted the main body of the patch-grafted urethra. Urethrography and urethroscopy showed no urethral obstruction, stenosis, fistula or diverticula anomaly existed in experimental group.

CONCLUSIONS: Our preclinical study showed that the hUC-MSCs and minced-muscle-derived regenerate could be used as an autologous pre-vascularized living patch graft for urethral reconstruction.

Key Words:

Human umbilical cord mesenchymal stem cells (hUC-MSCs), Minced muscle, Autotransplantation, Urethral reconstruction.

Introduction

The repair and regeneration of bone, articular cartilage, and muscle become a major challenge in biomedical research. One of the most promising approaches is represented by mesenchymal stem cell based tissue engineering[1-5]. A large number of animal studies have investigated the *in vitro* and *in vivo* regeneration ability of MSCs for various tissue repairs[6], multi-potent mesenchymal stromal cells (MSCs) hold effective promise for tissue engineering and regenerative medicine[7,8], these cell products, which indicate a fully differentiated cell, are critically important to produce the biomechanical and physiological properties required in many tissue engineering applications[7]. With the development of stem cells in basic and clinical application research[9], preliminary result has obtained in the application of treatment of urinary stress incontinence and bladder tissue engineering construction with stem cells[10-12], but still limited by repair material or its heterologous.

Many scientists found that human umbilical cord (hUC) may be an excellent alternative source of bone marrow stem cells because these cells are younger than other adult stem cells[13,14,15]. Importantly, UC stem cell transplants are less prone to rejection issue than either bone marrow or peripheral blood stem cells and their immunosuppressive properties make them a suitable candidate for urethral repair cell therapy. Human umbilical cord mesenchymal stem cells providing a readily available source for cell replacement therapy[16]. The differentiation ability of hUC-MSCs in conjunction with their immunosuppressive effect makes them an ideal candidate for various cell transplantation therapies[17]. The umbilical cord blood (UCB) has been used as an alternative source since 1988[18]. The hUC-MSCs have been used successfully as an alternative allogeneic donor source to treat a variety of pediatric genetic, hematologic, immunologic, and oncologic disorders[19-21]. Fresh cord blood is also a promising source of non-hematopoietic stem cells.

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In our studies, we prepared hUC-MSCs and minced-muscle-derived regenerate tissues as the autopatch grafts to reconstruct artificial urethral defect in rabbit and explored the therapeutic application of human umbilical cord in urethral defect.

Materials and Methods

Material

Human umbilical cords were obtained from local maternity hospitals after normal deliveries. Tissue collection for research was approved by Ethics Review Committee of the General Hospital of PLA.

Harvesting and Culture of hUC-MSCs

Remaining bloods of umbilical vessels were washed with phosphate-buffered saline (PBS) after been minced to 1-2 mm^3 fragments, human umbilical cord were transferred to a 50 ml centrifuge tube with 50 ml PBS, after density gradient centrifugation, then were plated at a 15 cm cell culture flasks after re-precipitate with □-MEM (Minimum Essential Medium) culture medium, cultured in growth medium, maintained in a humidified atmosphere with 5% CO_2 at 37°C. After 5 days of culture, the medium was replaced, changed once every three days thereafter. Once 80%-90% confluence had been reached, adherent cells were treated by trypsin for expansion.

To determine their immune phenotype, the surface expression of typical marker proteins was analyzed using fluorescence-assisted cell sorting (FACS) flow cytometry. For this, cells were labeled with the following antihuman antibodies: CD29, CD31, CD34, CD45, SH3 (CD73), CD106, and HLA-ABC, type with negative and blank served as control. All antibodies were purchased from Becton Dickinson (San Diego, CA, USA). Cells were stained in single label and then analyzed by flow cytometry with a FACScan system (Becton Dickinson). Osteogenic and adipogenic differentiation analysis were conducted as described[22]; the transwell migration assay of hUC-MSCs was conducted to analysis migration ability[23].

Preparation of hUC-MSCs Muscle Microsomes

Thirty four adult New Zealand rabbits (17 male and 17 female), weighed from 3.0-3.5 kg, were used in this study, 24 of them were assigned to the experimental group and 7 of them were assigned to control group. All the research was followed by the review board of experimental animal welfare ethics review committee of the General Hospital of PLA.

Twenty five mg of muscle from left inside of thigh were taken from experimental rabbits, cultured in DMEM (Dulbecco Modified Eagle Medium) at 4°C, washed by PBS then minced to size less than 1mm^3, transferred to sterile EP (electropolished) tubes and preserved at 4°C. The suspended rabbit musculature particles were added to a 24 well plates, which cultured adherent hUC-MSCs, DMEM medium contained no hUC-MSCs severed as control. Differentiation and growth of these hUC-MSCs muscle microsomes were monitored with images captured using inverted microscope day by day.

Immunohistochemical Analysis of hUC-MSCs Muscle Microsomes

After cultured in 24 well plates for 3, 5 and 7 days, slides contained hUC-MSCs muscle microsomes were fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and permeabilized with 0.1% Triton X-100 (Sigma). Desmin antigen and human specific anti-nuclear antibody (ANA) were applied to analysis: The monoclonal mouse anti-human Desmin (Abcam, Cambridge, MA, USA) were incubated with slides for 1 hour at room temperature, goat anti-mouse IgG conjugated to alkaline phosphatase, severed as secondary antibodies (Southern Biotech, Birmingham, AL, USA), incubated for 1 hour. The typical positive parts were counterstained with Vector Blue Kit, which located in the cytoplasm; As for human specific anti-nuclear antibody (ANA), mouse anti-human nuclear antigen monoclonal antibody was chosen as the primary antibody (MAB1281, Millipore, Billerica, MA, USA), and Goat anti-rat IgG antibody served as the secondary antibody, the positive parts counterstained with brown orange color and located in the nuclei of cells.

Reorganization Process of Muscle Microsomes

After hUC-MSCs muscle microsomes were cultured for 7-10 days in vitro, Sumianxin II (compound preparation of xylidinothiazoline, EDTA, dihydroetorphine hydrochloride and haloperidol) was injected intramuscular to these rabbits in sterile environment, after success anesthesia induction, area of 0.5 × 0.5 patched muscle microsomes was implanted to ventral subcutaneous lacuna of rabbit penile. After incubated for 2 weeks and 3 weeks, the hUC-MSCs muscle

microsomes of animal lacuna were removed and stained by HE and immunohistochemical assay to observe the reorganization of these hUC-MSCs muscle microsomes, 3 animal from experimental group and 3 from control group were used in this part.

Operation Process of Urethral Defect Repair

A total of 28 New Zealand rabbits were divided to 2 groups, 21of them in experiment group, 7 of them in control group. The experiment group was divided into 3 sub-groups, 7 of each subgroup, urethral repair result of each group was evaluated at 2, 4 and 12 weeks after operation by X ray retrograde urethrography examination and urinary microscopy.

Our prophase experiments have confirmed that lacking of stem cell assistance, muscle particles cannot form new tissue beneath the penis skin, so muscle from animal hind limb skeletal were cut to 0.5 × 0.5 × 0.3 cm slices in control group as autologous repairing material for urethral defect repair.

Operation process was summarized as follows: 3 weeks after transplantation, the animal underwent a second operation – urethral defect repair, after anesthesia effected, followed the original ventral skin graft incision of penile, blunt separated hUC-MSCs muscle microsomes along with ventral corpus spongiosum side, released graft, while maintaining the graft and the ventral side of the penis skin gap not touched or destructed; The released graft was hemispherical, or a flat sheet, attached to the ventral side of the animal from inner surface of penis. If the released graft is hemispherical, the top of hemispherical graft should be cut off; The cut off part of the graft was immediately divided into two parts, frozen at –80°C or fixed in 4% neutral buffered formalin solution for histopathological observation. After flushed with saline and cleaned white-gray necrotic tissue of graft central, the remainder was cut into 0.5 × 0.5 cm round patch, flat graft can be used directly after simple debonding and cutting, the animal ventral urethral tissue were excised with full thickness to prepare of a 0.5 × 0.5 cm urethral defect (process showed in Figure 1). The Vicryl suture 7-0 was used to suture the urethral defect graft cut from autologous cultured hUC-MSCs muscle microsomes, a total of 6 interrupted suture needle were conducted in this operation. The incision of penile ventral skin was suture d by conventional interrupted suturing, no indwelling stent was laid in urethra, and no drainage lay around the anastomotic stoma.

Statistical Analysis

Data are presented as mean ± SD. Comparisons of continuous variables between two groups were performed by a one-way ANOVA. If the F distribution was significant, a *t*-test was used to specify differences between groups. $p < 0.05$ was considered statistically significant. The SPSS software package (SPSS Inc., Chicago, IL, USA) was used for the statistical tests.

Results

Immuno-phenotype Differentiation Potential and Migration Analysis of hUC-MSCs

The immune-phenotype analysis result showed hUC-MSCs samples expressed CD29, CD73, CD166, and HLA-ABC markers and hemic markers such as CD31, CD34, CD45 and CD106 were not found; all hUC-MSCs samples could differentiate into adipose and bone cells as

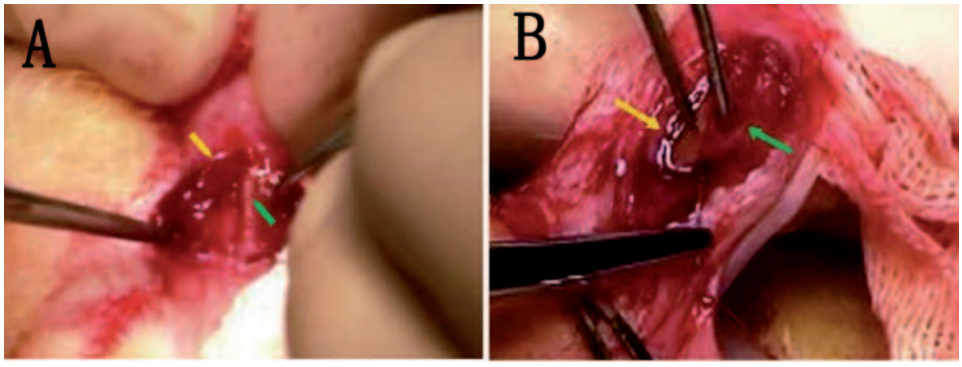

Figure 1. Operation process of urethral defect repair. **A**, hUC-MSCs muscle microsomes from animal subcutaneous penile was hold by tweezers (*yellow arrow*); **B**, hUC-MSCs muscle microsomes from animal subcutaneous penile was hold by tweezers (*green arrow*) and urethral defect (*yellow arrow*).

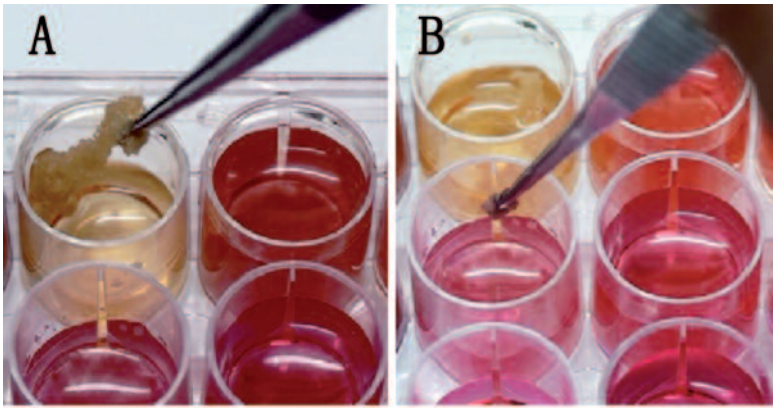

Figure 2. The growth observation result of hUC-MSCs muscle microsomes. **A**, hUC-MSCs and muscle microsomes adhered together into one; **B**, pure cultured muscle microsomes have no adhesion phenomenon.

demonstrated by the presence of fat globules and mineral deposition. The experiment group and control group all have the trafficking ability, but experiment group shows much more cell number (39.73 ± 5.92) % on trans-well polycarbonate membrane with significant difference ($p < 0.01$) compared with control group of (12.46±3.40) %.

The Growth Observation Result of hUC-MSCs Muscle Microsomes

The culture medium of hUC-MSCs muscle microsomes become yellow for the nutrition was absorbed quickly and hUC-MSCs and muscle microsomes adhered together into one (Figure 2 A), while pure culture of muscle microsomes

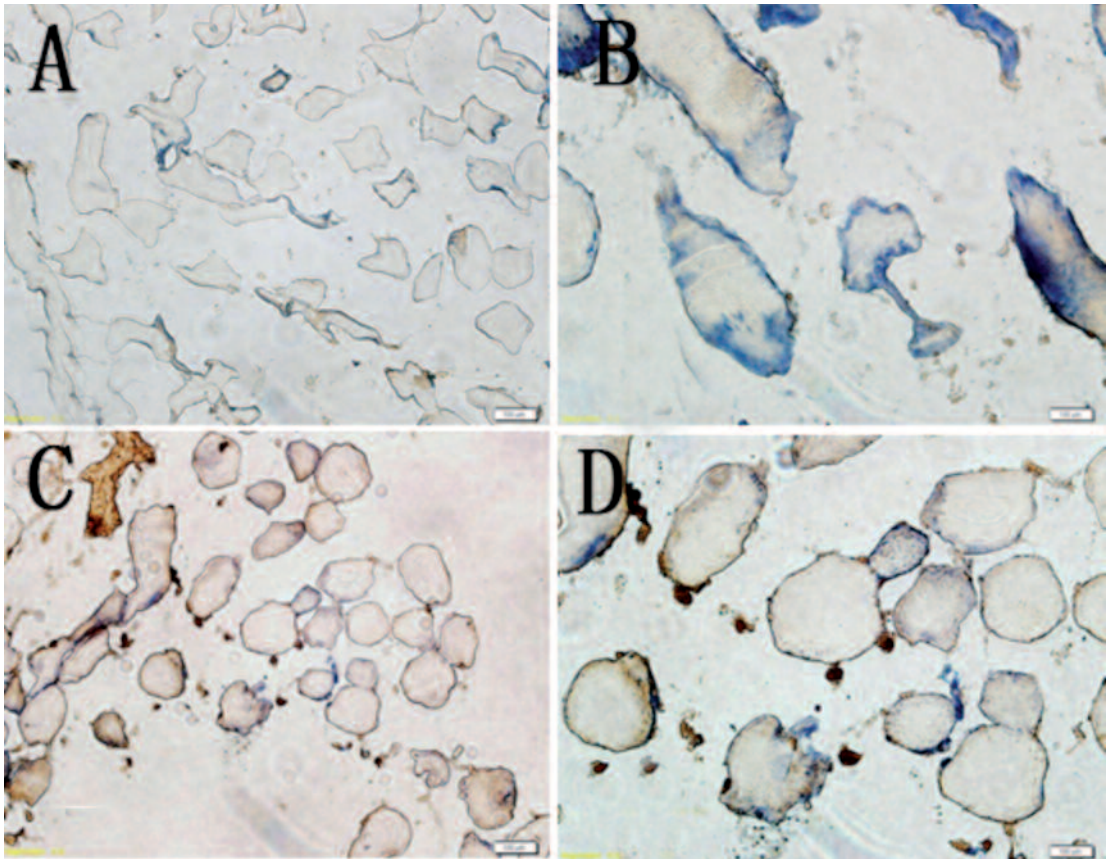

Figure 3. In vitro immuno-histochemical analysis of hUC-MSCs muscle microsomes (200×). **A** and **B**, desmin expression was visible, mainly located in the muscle membrane medial, distributed uneven and no ANA positive expression showed in pure isolated muscle microsomes. **C** and **D**, Desmin expression in hUC-MSCs muscle microsomes co-culture system was rare, mainly located in the vicinity of the muscle membrane. Small amount of ANA positive brown showed in ANA-desmin double stained specimens, around these sites has blue desmin expression; ANA expression was visible in many grid structure of co-culture system.

have no adhesion phenomenon (Figure 2 B), and medium color change is not significant, which showed the metabolism was slow.

In vitro Immuno-Histochemical Analysis

In pure isolated muscle microsomes culture specimens, desmin expression was visible, but the expression was mainly located in the muscle membrane medial, distributed uneven and no ANA positive expression showed in these specimens (Figure 3 A and B).

Desmin expression in hUC-MSCs muscle microsomes co-culture system was rare, mainly located in the vicinity of the muscle membrane. Small amount of ANA positive brown showed in ANA-desmin double stained specimens, around these sites has blue desmin expression, but was not typical. ANA expression was visible in many grid structure of co-culture system, around which showed more desmin expression positive (Figure 3 C and D). Co-expression of desmin ANA were positive, which suggested marker of desmin cell components derived from human umbilical cord mesenchymal stem cells.

In vivo Reorganization Result of Muscle Microsomes

After incubated in animal penile ventral subcutaneous lacunar for 2 weeks, hUC-MSCs muscle microsomes developed into the nascent massive tissue, the regeneration tissues adhered to penile skin tightly and were hard to be peeled. The necropsy central part of regenerated tissue was visible; the gray necrosis could be removed easily after washed by sterile saline.

HE staining result showed under normal optical microscope, a large number of inflammatory cells existed in regenerated tissue center portion and accumulated; a small amount of eosinophilic long fusiform or round fibroblast-like cells existed. The regenerated tissue surrounding a portion of the visible expression of desmin, and positive color part showed like annular (Figure 4 A and B); HE staining after cultured 3 weeks *in vivo* showed a clear structure of strip-shaped collagen fibrous component of grid, existed close to the ventral side of the penis skin; endothelial-like cells with different diameter were visible within the regenerating tissue (Figure 4 C and D). We observed moderate polymorphonuclear cell infiltration in second week after operation, no obvious gap between repair of partial and normal urethra in overlaying of urothelial tissue was observed, and immune-histochemical staining showed the positive expression of desmin in 4^{th} and 12^{th} weeks (Figure 5); Primary urethral repair parts in control group were

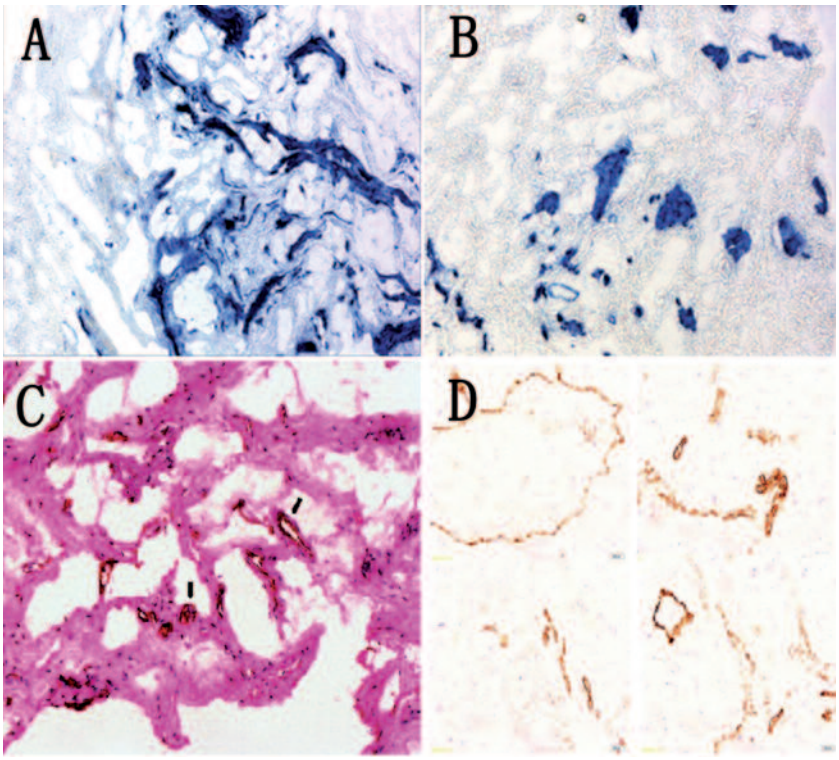

Figure 4. *In vivo* immuno-histochemical analysis of hUC-MSCs muscle microsomes after cultured 3 weeks. **A**, The regenerated tissue surrounding a portion of the visible expression of desmin, and positive color part showed like annular, mainly located in strip-shaped collagen fibrous component of grid (100×); **B**, the unregularly distribution of positive Desmin expression existed in grid-like area (100×). **C** and **D**, expression of CD31 in hUC-MSCs muscle microsomes (frozen section, 200×), **C**, Diaminobenzidine stain and hematoxylin counterstain result of small vessel like structure formed by CD31 positive cells (*black arrows*); **D**, Diaminobenzidine stain result.

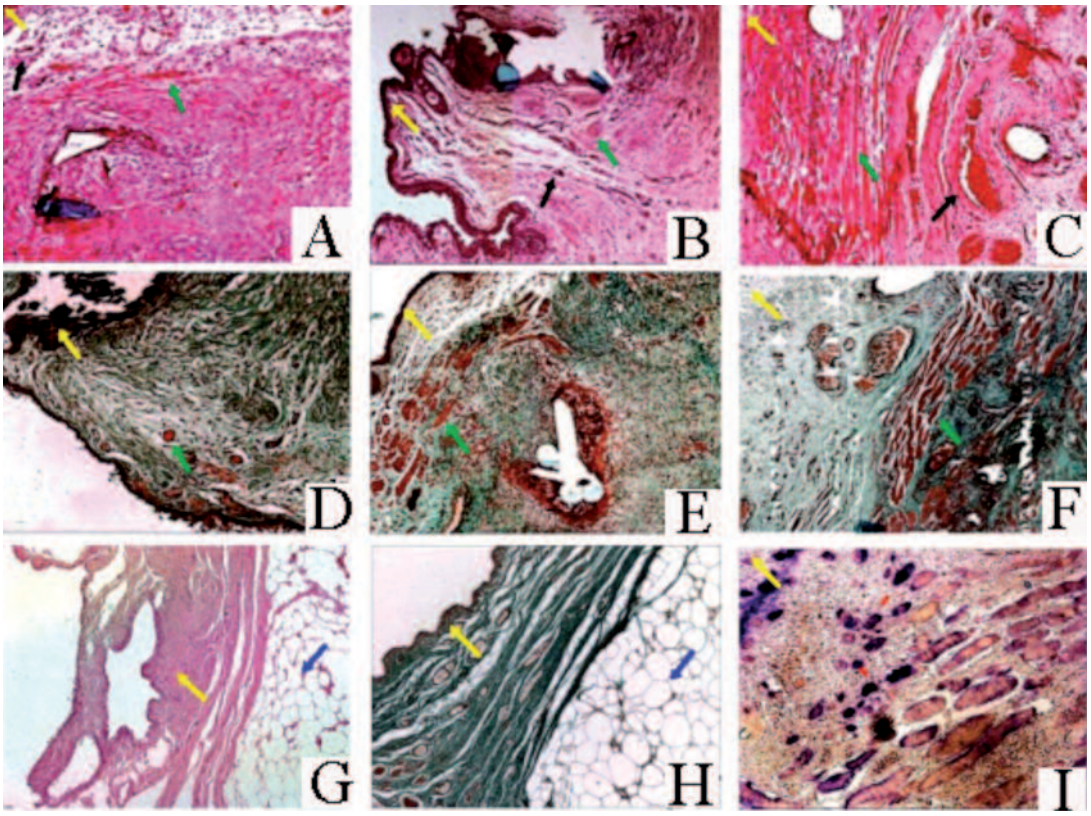

Figure 5. optical microscope morphology result of *in vivo* reorganization, G and H, control groups, others, experimental groups. **A**, Urethral repair after 2 weeks, the repair of urethral was covered by urothelial (yellow arrow) small vessels in repair tissue (*black arrow*), muscle fibers (*green arrow*); **B**, Urethral repair after 4 weeks; **C**, Urethral repair after 12 weeks; **D**, Urethral repair after 2 weeks, II collagen fibers (*green arrow*) increased; **E**, Urethral repair after 4 weeks, II collagen fibers (*green arrow*) decreased; **F**, Urethral repair after 12 weeks, II collagen fibers (*green arrow*) decreased (**G** and **H**) HE and Masson 's stain result in control group, adipose tissue existed in the urethral repair, occupied the original urethral defect (Paraffin section, 100×) (i) Muscle fibers were densely distributed in the repair of urethral sub-mucosa (*yellow arrow*), red arrow pointed to positive desmin distribution.

packed by fibrous adipose tissue from the transplanted muscle.

Urethral Defect Repair Result

All experimental animal survived in predetermined observation period, urethrography and urethral endoscopy results (Figure 6) showed the urethral patency in experiment groups were satisfied; we observed the presence of urethral stricture in control group, except one animal died of postoperative fistula.

Discussion

Urethral injury is a common complication of pelvic trauma; it occurs in as many as 24% of adults with pelvic fractures[24]. Unless urethral injuries are associated with major bladder trauma, they are rarely life-threatening in the acute phase. However, they may lead to significant long-term morbidity, strictures have been reported in 31%-69% of patients after a complete disruption of the bulbous urethra[25]. Incontinence and impotence are other well-recognized associated problems. In select patients with urethral strictures in whom genital skin is insufficient alternative tissues are needed for urethral reconstruction.

Human MSCs, with particular reference to Wharton's jelly MSCs, have an important role as immune-modulators and their multilineage differentiation potential makes their use in tissue regeneration and repair possible[26]. This versatile and tunable differentiation potential of hUC-MSCs in conjunction with their ability modulate the recipient's immune system makes them a promising cell source in regenerative tissues[27].

Our preclinical work demonstrated that MSCs from human umbilical cord are therapeutic in urethroplasty and represent a promising source

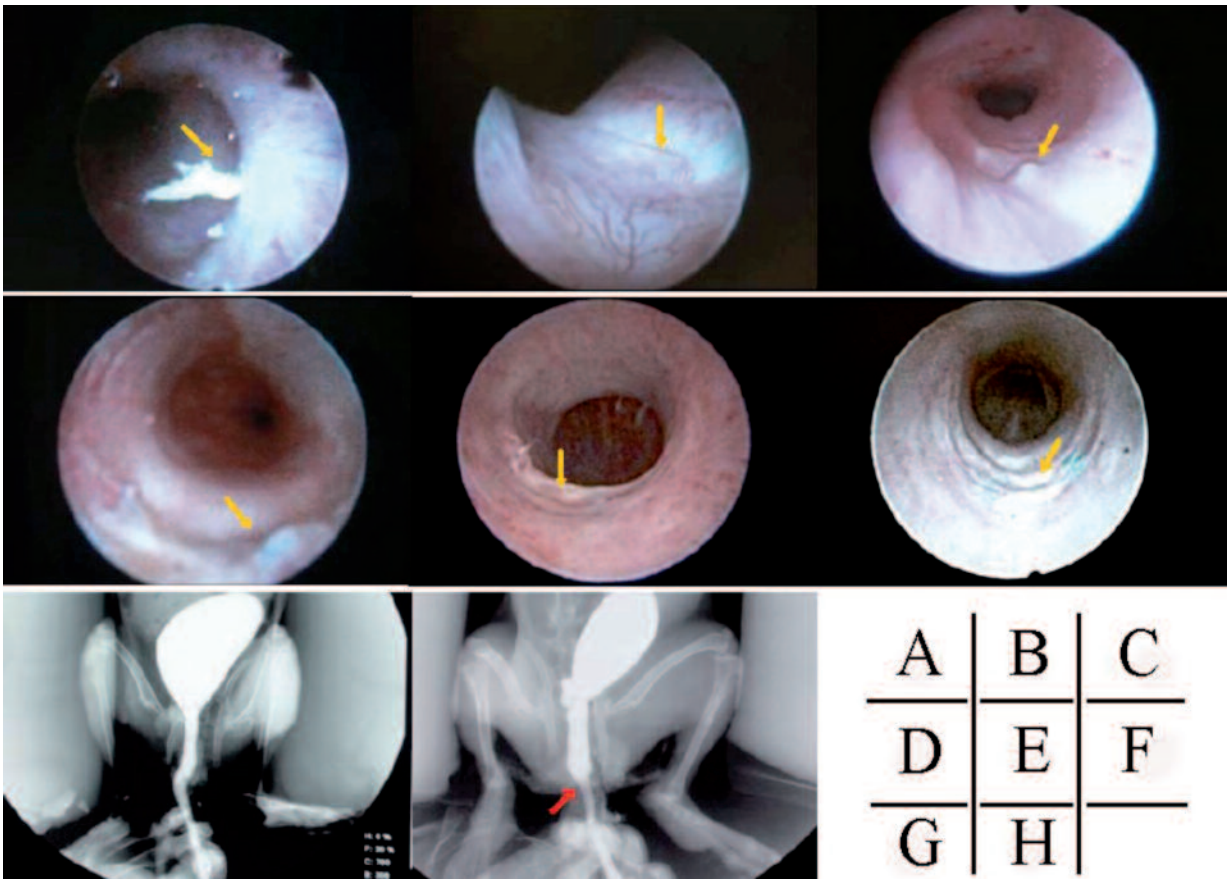

Figure 6. Urethral X angiography and urethral endoscopy results. Representative images of (**A**) urethral morphology of endoscopic on 2th week after operation in control group (*yellow arrow point* to primary urethral defect); **B**, 4th week endoscopic result in control group; **C**, 12th week endoscopic result in control group; **D**, urethral morphology of endoscopic on 2th week after operation in test group; **E**, 4th week endoscopic result in test group; **F**, 12th week endoscopic result in test group; **G**, urethral contrast examination of test group on 12th week; **H**, urethral contrast examination of control group on 12th week (*red arrow point* to urethral stricture).

for progenitor cells with the potential to repair and regenerate solid tissues[4].

Conclusions

The main deficiency in our study was the lack of morphological observation of typical sponge like structure in repaired urethral wall, how to reconstruct the corpus spongiosum tissue will be the direction of further study. With the result of this part of work, this regeneration might be developed into a corpus spongiosum-like reconstructive material given some modifications. This possibility will be the focus of our future studies.

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