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# Novel perfusion-decellularized method to prepare decellularized ureters for ureteral tissue-engineered repair

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**As the endoscopic technique is widely used in the diagnosis and treatment of diseases, the incidence of ureteral injuries increases annually. The classical surgical therapies are not always satisfactory. With the constant development of the tissue engineering technology in the field of urinary reconstruction, the ureteral reconstruction has become possible technology. In this study, a novel perfusion-decellularized protocol, which combined a perfusion system with the commonly used physical and chemical methods, was used to prepare the decellularized ureters for ureteral reconstruction and the urinary tract-derived cells (UDCs) were seeded on the surface of the perfusion-decellularized ureters (PDUs) in order to observe the cells survival, adhesion, proliferation and distribution. The data of H&E staining, DAPI staining, and the agarose gel electrophoresis demonstrated that the cellular components of PDUs were removed, and the decellularized time was shorter than previous study. In addition, compared with the native ureters, the DNA content of the PDUs was significantly decreased just two percent residue ($P < 0.05$). Scanning electron microscopy, collagen and glycosaminoglycan content assay showed that the three-dimensional (3D) ultrastructure and the compositions of the extracellular matrix (ECM) of PDUs were well preserved. When the UDCs were seeded onto the PDUs, the UDCs formed multilayer structure on the surface of the PDUs, infiltrated into the deep layer of the decellularized ureters and then formed laminated structure. In conclusion, the decellularized ureters prepared by the novel perfusion-decellularized method may be the potential surrogate for ureteral tissue-engineered repair.**



[**Key words:** Ureter; Decellularized; Perfusion system; Tissue engineering; Repair]

Ureter, as a retroperitoneal organ, is located in the deep of the body, therefore the incidence of ureteral injuries is low (1). In recent years, with the increasing use of endoscopic technique to diagnose and treat the diseases, the risks of ureteral injures were increasing. The classical surgical therapies, such as colon substitution, bladder flaps, transureteroureterostomy and renal autotransplantation could not achieve entirely functional recovery and they also might induce a series of clinical complications such as recurrent strictures, urinary leakage, even renal damages (2,3). However, tissue engineering technology could reconstruct the ureter and solve these clinical problems.

With the advance of tissue engineering technology, the exploration of ureteral regeneration has never stopped. Various types of synthetic or biological scaffolds were introduced in ureteral reconstruction (3). However, synthetic scaffolds often had a lack of in biocompatibility, peristaltic motion, and incrustation (4). Unlike the synthetic scaffolds, the biological scaffolds had unique advantages, especially when the natural acellular matrix materials removed the antigenic cellular components, the native 3D ultrastructure and the compositions of the ECM were preserved. The remained ECM had a close relationship with the cell attachment, migration, proliferation and three-dimensional spatial arrangement (5). The acellular matrix derived from different tissues was used to study the ureter regeneration, such as bladder, vessel, and ureter itself (6–9). The continuous stirring and the chemical detergents were widely used to prepare the ECM for ureteral tissue-engineered repair (4,10,11). This decellularized process took several days or even a week, and the process which was continuous stirring and the effect of detergents could destroy the organizational structure and cause the loss of bioactive components of ECM. Besides, the prepared ECM has never been evaluated systematically. Moreover, the prepared ECM should be conducive to the cells adhesion and proliferation, which was beneficial for its survival *in vivo* and functional repair.

In this study, we combined the perfusion system and the commonly used physical and chemical methods scientifically, and then explored the best decellularized time by trial and error. At last, a novel perfusion-decellularized protocol was established. Compared with the traditional methods, this perfusion process would take less time and remove more cellular components. Meanwhile the 3D structure was well preserved. The characteristics of the PDUs were also described in detail, including the structural integrity and the quantification of the biological components. Moreover, the feasibility of the PDUs for the tissue-engineered repair was evaluated clearly through seeding the UDCs on the surface of the PDUs. The UDCs could survive and proliferate in the decellularized ureters, which was beneficial for the tissue-engineered repair.

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## MATERIALS AND METHODS

**Animals and decellularization processes** Animal experiments were approved by the Animal Experimental Ethics Committee of People's Liberation Army General Hospital. The ureters were harvested from the adult beagles (7–10 kg) euthanized in connection with other experimentation, and then were kept at −80°C for at least 24 h. A perfusion system was used throughout the whole decellularization process. The ureters were thawed at room temperature. A 16-gauge needle was inserted into the ureteral lumen and then connected to a perfusion pump using silicon tubing. The ureters were flushed with 1% TritonX-100 for 7 h, and then with the deionized water for 1 h. Subsequently, the ureters were treated with 1% Sodium dodecyl sulfate (SDS) for 1 h. Finally, the ureters were rinsed with deionized water for 24 h, in order to remove the detergent completely. During the decellularization process, the perfusion speed was controlled at 1.5 ml/min. Meanwhile, the harvested ureters were treated by the commonly traditional methods (11). TritonX-100 treatment (TritonX-100 group): Ureters were placed in a 1% TritonX-100 solution with 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) together with DNase and RNase and shaken for 48 h. The solution was changed every 24 h. After the decellularized procedures, the decellularized tissue was washed with deionized water for 24 h. SDS treatment (SDS group): The ureters were treated in a solution of 0.5% SDS, 150 mM NaCl, and 10 mM EDTA in 10 mM tris-HCL (pH 8.0) and shaken for 48 h. Then the decellularized ureters were washed with deionized water for 24 h.

**Histological and immunohistochemical analysis** Native or decellularized ureters were fixed in 10% neutral buffered formalin, dehydrated with a graded ethanol and embedded in paraffin, sectioned into 5 μm slices. Haematoxylin and eosin (H&E) staining was used to obverse the cellular content and general structure of the decellularized ureters. Nucleic acids were stained with 4,6-diamidino-2-phenylindole (DAPI). Masson's trichrome staining was used to detect the collagen distribution and orientation. Alcian Blue staining was carried out for qualitative analysis of glycosaminoglycan (GAG) (12).

In the immunohistochemical (IHC) staining, we placed the slides into antigen retrieval solution and heated them until the temperature reached 95–100°C for 30 min. Endogenous peroxidases were blocked by incubation with 3% hydrogen peroxide solution. We blocked slides with 4% goat serum. Sections were incubated with primary antibody at 4°C overnight. The secondary antibody was applied for 30 min. The slides were treated with streptavidin-horseradish peroxidase complex, diaminobenzidine (DAB) solution and counterstaining with hematoxylin, mounted and imaged using microscopy (Olympus, Tokyo, Japan). The primary antibody used was rabbit anti-Collagen type I antibody (Abcam, Cambridge, MA, USA) at a dilution of 1:200. The secondary antibody was goat anti-rabbit IgG (Abcam) at a dilution of 1:100.

**Quantification of total genomic DNA** The total genomic DNA of the samples was extracted using a Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Both native ureters (n = 6) and decellularized ureters (n = 6, each group) were lyophilized and digested with proteinase K and RNase for 5 h until no visible material remained. The remaining genomic DNA was collected and quantified using the spectrophotometer (Bio-Rad, CA, USA). The size of the collected DNA fragments was exhibited using 2% agarose gel electrophoresis. The DNA quantity was express as ng/mg dry weight of the samples.

**Collagen and GAG content assay** The collagen content was determined on the content of hydroxyproline (Hyp) (13). Both native ureters (n = 6) and decellularized ureters (n = 6, each group) were lyophilized and acid-hydrolyzed. The amount of total collagen content per mg dry weight of the decellularized ureters was calculated using a Hyp-to-collagen ratio of 1:7.2. The GAG content was quantified using a GAG assay kit (Bangyi, Shanghai, China) according to the manufacturer's instructions. Final values were expressed as μg of GAG per dry weight.

**Scanning electron microscopy and porosity measurement** The native ureters (n = 4) and PDUs (n = 4) were cut longitudinally, and expanded into slices. The slices were fixed and dehydrated through an ethanol gradient, sputter coated with gold and mounted for analysis (14). The tissue samples were then viewed under the scanning electron microscope (SEM, Zeiss, Germany). The swelling ratio can be used to reflect the porosity of native ureters and PDUs (15). Both native ureters (n = 6) and PDUs (n = 6) were immersed in PBS at 25°C for 24 h to achieve fully swollen, and measured the weight (Ws). Then, the samples were lyophilized and measured the weight (Wd). The swelling ratio was expressed as (Ws-Wd)/Wd.

**Isolation and culture of the UDCs** Portions of the bladder (2.5 cm × 2.5 cm) were removed from beagles. The muscular layer was minced and digested with 1 mg/ml collagenase type IV and dispase at 37°C for 30 min (16). The enzymatic activity was neutralized by high glucose-Dulbecco's modified Eagle's medium (H-DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco). Dissociated tissue was filtered to remove debris and centrifuged at 1000 rpm for 10 min. The cell pellet was resuspended and washed twice. The remaining cells were plated onto 100-mm culture dish and cultured in H-DMEM with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C with 5% $CO_2$. The medium was replaced every 3 days. The adherent cells were more than 80% confluent, they were digested with 0.25% trypsin-0.02% EDTA, and re-plated at a dilution of 1:2. Third-passage cells were used in the subsequent experiments.

**Cytotoxicity assay of the PDUs** The PDUs were incubated in standard H-DMEM for 72 h, and the supernatant was collected as a test extract for later use (17). The UDCs were seeded in 24-well plates at a concentration of $2 \times 10^4$ cells/well, and incubated in standard H-DMEM for 24 h. The medium was then removed and replaced with the test extract. Cells cultured in standard medium served as the control group. The cell viability was observed by acridine orange and propidium iodide (AO/PI) staining, on days 1, 3 and 7. Five replicates were conducted per sample. At the same time, proliferative activity of the cells was determined by MTT assay on days 1, 3, 5, and 7.

**Recellularization of the PDUs with UDCs** The PDUs were scissored longitudinally and cut into thin slices (1 cm × 1 cm). The samples were immersed in H-DMEM for 24 h and dried using sterile filter paper. The UDCs were seeded on the thin slices at a density of$10^6$ cells/$cm^2$ in the 24-well plates. The seeded slices were incubated for 2 h before the supplemented culture medium was slowly added. The culture medium was changed every 3 days. The slices were collected and examined on days 3, 7, 14 after cell seeding. Three replicates were conducted each time. The samples were washed with PBS, fixed for 48 h in 10% formalin solution, embedded in paraffin and sectioned into 5 μm sections. The sections were stained with H&E and DAPI to observe the cells distribution in the PDUs. By IHC, the sections were stained with proliferating cell nuclear antigen (PCNA) to evaluate the cells' proliferative ability. The primary antibody was rabbit anti-PCNA antibody (Boster, Wuhan, China) at a dilution of 1:200. The secondary antibody was goat anti-rabbit IgG (Abcam) at a dilution of 1:100.

**Statistical analysis** Statistical analysis was performed using SPSS (17.0, IBM, USA). The results were expressed as mean ± standard deviation. The differences between groups were estimated using Student's *t* test. P values of less than 0.05 were considered significant.

## RESULTS

**Evaluation of the decellularization efficiency** The macroscopic images show the native ureters and PDUs. During the perfusion process, the ureters changed into white gradually (Fig. S1). H&E and DAPI staining revealed the absence of cell nuclei in the PDUs compared with the native ureters. The cellular nuclei could be found in the TritonX-100 group, while there was nothing in the SDS group (Fig. 1A). The DNA content was measured to evaluate the decellularized effect quantitatively. From the data, the significant difference could be lightly discovered between the PDUs and the native ureters (1760.8 ± 190.1 ng/mg for the native ureters and 40.3 ± 3.5 ng/mg for the decellularized ureters, $P < 0.05$). Also, there were not visible DNA bands on a 2% agarose gel (Fig. 1B). In the SDS group, the nuclei were almost completely removed (39.7 ± 4.6 ng/mg), while a number of nuclei could be detected in the TritonX-100 group (158.5 ± 27.8 ng/mg). There was no significant difference between the PDUs and the SDS group (Fig. 1C) ($P > 0.05$).

**Biologic components of the decellularized ureters** Collagen and GAG, the two main biological components of ECM, had been detected. Masson's trichrome and Alcian Blue staining showed that the collagen fiber and GAG were remained in the PDUs. In the TritonX-100 group, the collagen structure and GAG distribution became loose, while the collagen structure was severely damaged and GAG distribution was disordered in the SDS group (Fig. 2A). The IHC staining implied that the collagen type I was the main ingredient of collagen structure (Fig. S1). We used the Hyp content to calculate the collagen content. The collagen content in the PDUs did not reduced compared with that in the native ureters (284.47 ± 19.60 μg/mg dry weight in the native ureters and 265.89 ± 17.74 μg/mg dry weight in the PDUs, $P > 0.05$). However, the collagen content in the TritonX-100 group (196.57 ± 10.44 μg/mg) and the SDS group (155.82 ± 8.56 μg/mg) was significantly decreased, compared with the content of PDUs (Fig. 2B). The GAG content was tested by a GAG assay kit. The results showed that the GAG content in the PDUs decreased significantly (3.61 ± 0.16 μg/mg) dry weight in the native ureters and 1.73 ± 0.15 μg/mg dry weight in the PDUs ($P < 0.05$). The GAG content had little difference between the TritonX-100 group (0.928 ± 0.067 μg/mg) and the SDS group (0.890 ± 0.024 μg/mg)