Preparation of Rat Whole-kidney Acellular Matrix via Peristaltic Pump

Rongfu Liu,[1]* Jiasheng Gao,[2] Yufeng Yang,[1] Weixin Zeng[1]

Purpose: To design a whole-kidney a cellular matrix scaffold using peristaltic pump perfusion and to ascertain the retention of extra cellular proteins by the scaffold.

Materials and Methods: Male Sprague-Dawley (SD) rats weighing 200-250 g were used. Intravenous catheters were inserted into the renal artery followed by perfusion of decellularization solution using a peristaltic pump. After decellularization, the acellular matrix was observed under a microscope after hematoxylin and eosin (H&E) staining and a fluorescence microscope after 4′,6-diamidino-2-phenylindole (DAPI) staining. Immunohistochemistry was used to identify the composition of kidney acellular matrix.

Results: The result of H&E and DAPI staining demonstrate the removal of cellular material in kidney a cellular matrix. Immunohistochemistry confirmed the conservation of the natural expression of extra cellular matrix proteins including collagen types I and IV, fibrin and laminin.

Conclusion: Peristaltic pump perfusion enables successful preparation of renal a cellular matrix, to retainthe criticalproteins of natural extra cellular matrix. The resulting kidney a cellular matrix represents an ideal natural scaffold for renal tissue engineering.

Keywords: disease models, animal; kidney; metabolism; rats; in vitro techniques; infusion pumps.

INTRODUCTION

Acute or chronic renal failure is associated with high morbidity and mortality.[1,2] Current treatments include hemodialysis and renal transplantation. However, dialysis only partly compensates for the loss of renal functionat a high medical cost. The biggest challenge with kidney transplantation is the shortage of human organs,[3] which warrants development of new strategies for intervention. Currently, renal tissue engineering represents a feasible approach.

The key challenge in renal tissue engineering is to build ascaffold, which contains the essential functional composition for transplantation. A scaffold comprising natural biological tissues accurately removes all the cellular precursors but still retains the necessary signals for the extra cellular matrix generation. Acellular matrix refers to the reservoir of extra cellular matrix (ECM) with its three-dimensional structural integrity and biological activity after depleting the cellular components. The acellular process can be completed using detergents such as sodium dodecyl sulfate (SDS) or TritonX-100, which cause membrane rupture or fracture of the connection between the cell and ECM. Until now a variety of acellular tissues or organ matrices have been successfully used in clinical or preclinical studies including the dermis,[4] heart,[5] small intestinal submucosa,[6] ligament[7] and bladder.[8,9] This study adopts peristaltic pump perfusion for the preparation of rat whole-kidney acellular matrix and simultaneously identifies the matrix composition. The identification focuses on retention of the main natural protein ingredients of ECM by the scaffold. The ingredients may play a key role in cell adhesion, migration, and proliferation for cell implantation.

MATERIALS AND METHODS

1. Experimental Materials

1.1 AnimalResources

Twenty male Sprague-Dawley (SD) rats (male, 200-250 g) were purchased from the Experimental Animal Center of People's Liberation Army Military Academy of Medical Sciences.

1.2 Reagents

1% SDS, 3% Triton X - 100, 5 mM calcium chloride, 5 mM magnesium chloride, 4′,6-diamidino-2-phenylindole (DAPI) staining solution, type I collagen, type IV

[1] Department of Urology, the First Affiliated Hospital of Xiamen University, Xiamen 361003, China.
[2] Department of Urology, the First People Hospital of Jiujiang City, Jiujiang 332000, China.
*Correspondence: Department of Urology, the First Affiliated Hospital of Xiamen University, No.55 Zhenhai road, Xiamen 361003, China.
Tel: +86 592 2139714. E-mail: lliurf@126.com.
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collagen, fibrin, laminin protein and four types of antibodies were obtained from Wuhan Boster Biological Technology Co. Ltd. (Wuhan city, China).

1.3 Equipment

The BT600L traffic intelligent peristaltic pump was acquired from The Baoding Rafer Fluid Technology Co. Ltd (Baoding city, China), along with 24 gauge (0.7 mm × 19 mm) indwelling needle, ophthalmic surgical instruments, and microscope.

2. Experimental Methods

2.1 Kidney Samples and Renal Artery Catheter

Rats were anesthetized by intraperitoneal injection of 0.5 mL of 2% sodium pentobarbital and fixed to a surgical platform to remove and disinfect the skin. The abdominal cavity was opened along the midline, and the left kidney was stripped of the surrounding fat using a small forceps. After freeing the entire left kidney and the renal pedicle, it was stripped bluntly again along the renal pedicle till the abdominal aorta to identify the ureter, renal artery, and renal vein. The tiny renal artery and vein were distinguished from the rear of the kidney. The pulsating renal artery, with its thick white wall, was located in the front. The renal artery was separated from the renal vein with the tweezers from the middle and gently lifted for intubation. The micro-peristaltic pump was started to let the liquid flow from the catheter at a speed of 2 mL per minute. The catheter was then slightly and slowly replaced into left renal artery from the beginning. A part of the kidney suddenly changed from brownish red to white, indicating successful renal artery intubation. A thin string was used to ligate the place of cannulation to hold the needle in position. Finally, we divided the joint between ureter, renal vein, renal artery, and abdominal aorta and then fixed the kidney above the glassware for perfusion.

2.2 Micro-peristaltic Pump Perfusion

The perfusion fluid on the left side of the micro-peristaltic pump was connected with the kidney on the right with a hose. The micro-peristaltic pump was opened and the flow speed was adjusted at an average level of 2 mL per minute. The perfusion was performed in the following sequence: heparinized phosphate buffered saline (PBS) solution for 30 min; 3% Triton X-100 solution for 30 min; deionized water for 15 min; 5 mM calcium chloride and 5 mM magnesium chloride solution for 30 min, respectively; deionized water for 15 min; 3% Triton X-100 solution for 30 min again; deionized water for 15 min; 1% SDS for 12 h; deionized water for15 min followed by PBS-containing penicillin and streptomycin for 48 h. The shape of the kidney color was observed. When the perfusion was conducted with 1% SDS alternating with PBS, the perfusion fluid was changed every 4h.

2.3 Detection of the Scaffold of Renal Acellular Matrix

During the preparation of renal acellular matrix, the specimen was initially removed and the left kidney of the same SD rat as acellular matrix group was used while the right kidney served as a control. The acellular matrix was perfused in the order mentioned above and the control group was stored in PBS solution containing green streptomycin. The cell matrix group and the control group specimens were transferred into 4% paraformaldehyde and fixed for 48 h. Different gradients of alcohol were used for dehydration for 2 h, respectively. The specimens were placed into I and II cylinders of xylene for 20 min, respectively, and then immersed in wax for 2 hat 60°C, followed by slicing of the paraffin-embedded specimens.

2.4 H&E Staining

The H&E staining protocol requires dewaxing of the sample slices with xylene, and then dehydration using graded ethanol followed by washing using distilled water. The sections were sequentially transferred and washed after each step as follows: into hematoxylin-stained nucleus for 1 min; 1% hydrochloric acid solution for 10 s; 1% ammonia complex blue for 30 s; and 0.5% eosin solution for 2 min. After subjecting to ethanol dehydration the specimens were mounted and the acellular matrix micro structure was visualized under the microscope to ensure the absence of residual cell debris. Five non-overlapping cortical and medullary views of each specimen were randomly selected and photographed (×200).

2.5 DAPI Staining

The paraffin sections were dewaxed with xylene, dehydrated with graded ethanol, washed three times with PBS and diluted with DAPI staining solution at a ratio of 1:1000. The specimens were visualized under a fluorescence microscope after DAPI staining to ensure the absence of nuclear remnants in the acellular matrix.

2.6 Detection and Identification of Acellular Matrix Components by Immunohistochemistry (IHC)

Paraffin sections were placed in xylene for dewax ingandin graded ethanol for dehydration. After addition of 0.3% H_2O_2 for 10 min to remove endogenous peroxidase, they were transferred to a container filled with citrate buffer, followed by microwave heating (92°C-95°C, 10 min). After addition of goat serum for 30 min, and diluted primary antibody solution (collagen type I, collagen IV, fibrin, and laminin from rabbit, diluted at the ratio of 1: 200 respectively) the specimens

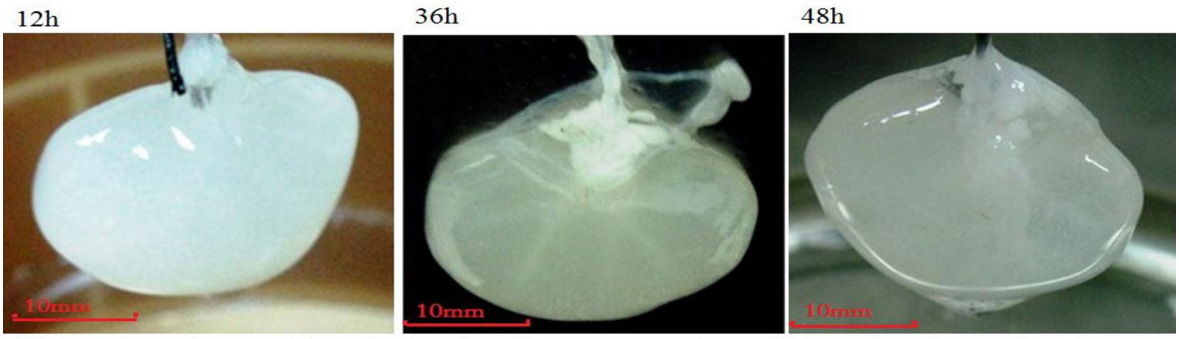

Figure 1. Altered kidney color after sequential perfusion of acellular fluid.
12 h: Color of the kidney invitro changes from bright red to milky white; **24 h:** the kidney gradually turns translucent white with a segmented and lobulated internal structure; **36 h:** the whole kidney is translucent white with a clear branch-like renal structure.

were incubated overnight at 4°C. A goat anti-rabbit secondary antibody solution was added and left for 30 min at 37°C. After washing with PBS, horse radish peroxidase (HRP) was used to label the fluid for 30 min at 37°C. After washing the sample slices, 3,3'-diaminobenzidine (DAB) chromogenic substrate solution was added for 5 min. It was followed by hematoxylin staining of the nucleus, and sealing by resin, and stored at room temperature. Five random fields were selected from each specimen and photographed (× 200 times).

RESULTS

1. General Observation of Acellular Matrix

After 12 h, the color of the kidney invitro changed from bright red to milky white (**Figure 1**). At 24 h, it gradually turned into translucent white with a segmented and lobulated internal structure. At 36 h, the whole kidney was translucent with a clear branch-like structure. After the perfusion was completed, the acellular matrix was soft and flexible under gentle pressure.

2. Histology of Acellular Matrix

Figure 2 illustrates the microstructure of the H&E-stained acellular matrix. No residues of cells or nuclear debris were seen in the renal cortex and medulla of the acellular matrix group. The three-dimensional network structure of the acellular matrix was closely connected. The structure of the glomerular vascular basement membrane and tubular base membrane of the internal stripped cells remained intact. The nucleus and the normal renal structure were clear. The results demonstrate that the micro-peristaltic pump perfusion at a low flow rate removed the cells and nuclei of kidney but still retained the integrity of the ECM structure.

3. DAPI Staining of Nucleus

Figure 3 shows the result of DAPI staining. After the appearance of blue fluorescence in acellular matrix, the

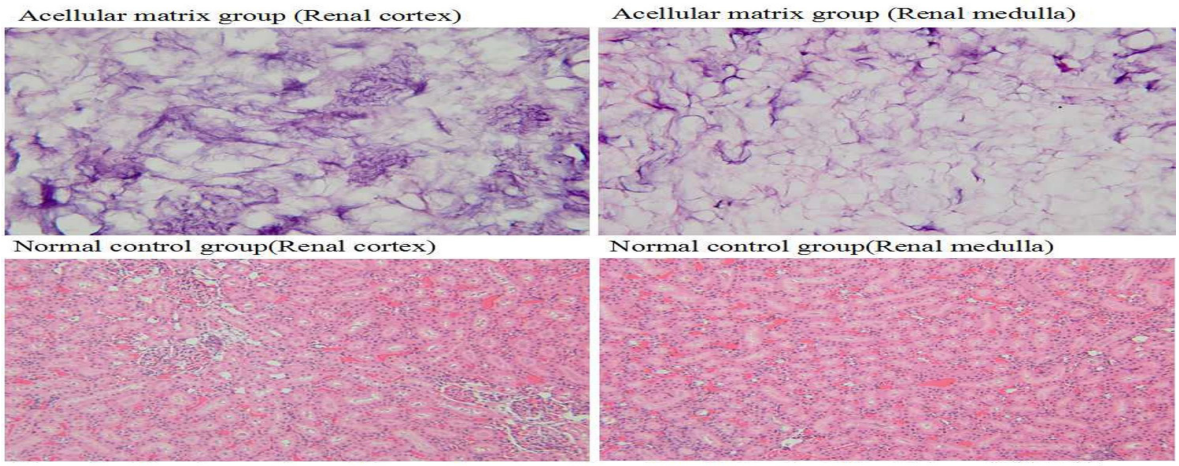

Figure 2. Hematoxylin and eosin staining of renal cortex and renal medulla (20×10).
The figure shows the absence of cellular residues and nuclear debris in the renal cortex and renal medulla of the acellular matrix group and the mesh structures of the glomerular tubule remain intact. The following diagram illustrates the renal cortex and medulla of the normal control group. The glomerular structure, tubular organization and the nucleus are clear.

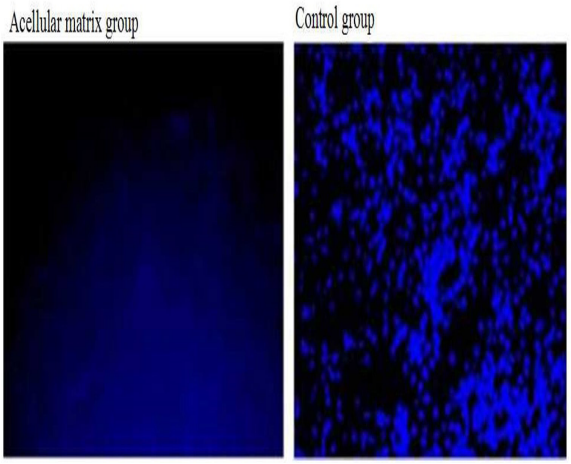

Figure 3. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei (40×10).
After the appearance of blue fluorescence in acellular matrix, a faint blue fluorescence can be seen without any round blue nuclei following DAPI staining. The control group was covered by cells with a strong blue fluorescence.

basement membrane was connected to network structure. The control group was covered by cells with a strong blue fluorescence.

4. IHC of Acellular Matrix Components

Immunohistochemical staining shows the key protein expression in ECM including the type I and IV collagen, fibrin, and laminin (**Figure 4**). The expression of four proteins was similar to the natural acellular tissue. The type I and IV collagens show significant expression in glomerular vascular basement membrane but less in the tubular basement membrane. Fibrin and laminin were highly expressed in both glomerular and tubular basement membrane, revealing the ECM network. Immunohistochemical results show that the acellular matrix retained the key component of the natural ECM.

DISCUSSION

Using invitro studies of rat kidney acellular matrix and identification of the components of the 3D kidney scaffold, the sequential perfusion of detergent 3% TritonX-100, 5 mM calcium chloride and magnesium chloride and 1% SDS with micro-peristaltic pump completely removed kidney cells. The identification of acellular matrix composition by IHC confirms the expression of the main components of ECM including collagen type I, collagen type IV, laminin, and fibrin. Renal tissue engineering includes seed cells and scaffolds. The selection of an ideal material for scaffold is an important step in kidney tissue engineering. An ideal material for scaffold is characterized by the following properties: 1. good biocompatibility to ensure regeneration of host tissue without immune rejection; 2. three-dimensional structure, which is similar to the shape of the internal organs; 3. cellular adhesion and growth; and 4. biodegradation. Acellular matrix scaffold represents such an ideal scaffold since it retains the natural expression of proteins in the ECM and contains an internal structure that matches the target organ. The key step in the study is to develop a full kidney acellular matrix scaffold. The report of Nakayama and colleagues[10] confirmed that acellular rhesus monkey kidney stent was an ideal scaffold. Their results suggest that the kidney was sliced into layers and the best acellular method was selected. The most efficient acellular process appeared with 1% SDS solution at 4°C. Ross and colleagues[11] demonstrated that rat kidney acellular matrix can be successfully prepared through the sequential perfusion of 3% Triton X-100, DNA nuclease, followed by 3% Triton X-100 and 4% SDS. Liu and colleagues[12] reported that the acellular matrix of the whole kidney can be prepared through the sequential perfusion of 1% SDS and 1% Triton, which confirms the success of renal acellular matrix from a histological perspective. Our experiment uses a new autoperfusion method to prepare the decellularized scaffolds by sequential perfusion of 3% Triton X-100, 5 mM calcium chloride and magnesium chloride (to remove endogenous nuclease), 1% SDS at a flow rate of 2 mL/min with micro-peristaltic pump. The results of H&E and DAPI staining show that preparation of the whole kidney acellular matrix scaffold was successful, without retaining any residual cellular organelles except for the

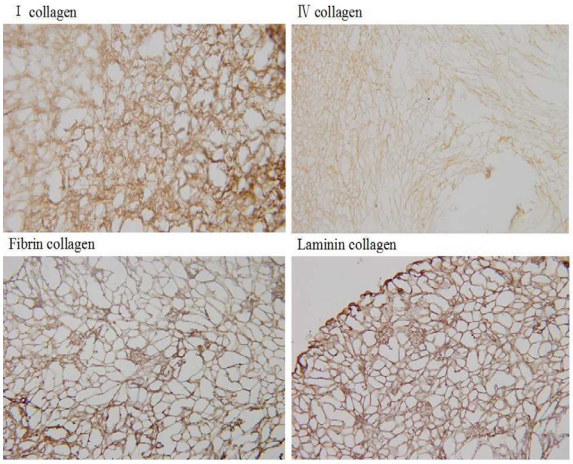

Figure 4. Immunohistochemical identification of acellular matrix components (40×10).
Type I and IV collagens were prominently expressed in glomerular vascular basement membrane but less prominently in the tubular basement membrane. Fibrin and laminin were highly expressed in both glomerular and tubular basement membrane, and the network structure of the extracellular matrix was clearly seen.

original cells and the ECM meshwork.

ECM is present in all tissues and organs as a reticularnetwork of fibrin, collagen, glycoproteins (laminin), and proteoglycans (basement membrane). ECM not only provides mechanical support, but also mediates cell signaling, cytokine and growth factor activity, playing an important role in cellular repair and regeneration.[13] The report of Rossand colleagues[11] shows that EC M maintains a complex anatomical structure and plays a key role in cell differentiation. Vaccination of the acellular kidney with embryonic stem cells (ES) confirms that ECM induced ES cells to differentiate into renal cells. Our study also confirms acellular matrix expression of collagen type IV, laminin, and fibrin by IHC and also the conservation of important ECM proteins, which play a key role incellular adhesion, proliferation, and differentiation.

CONCLUSIONS

Experiments show that the whole kidney acellular matrix scaffold, which retains the natural extra cellular matrix protein, can be successfully prepared using a micro-peristaltic pump perfusion. It provides the best possible micro-environment for adhesion, proliferation, and differentiation of seeded cells for further studies. In conclusion, the acellular matrix scaffold represents an idealnatural scaffold for renal tissue engineering.

CONFLICT OF INTEREST

None declared.

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