Bioengineering Research

Decellularized Kidney Scaffold Mediated Renal Regeneration in a Rhesus Macaque Mulatta Monkey Model

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Abstract

Introduction: For regeneration of complex organs, such as the kidney, decellularized (DC) tissues are promising candidates that provide a neutral environment.

Objective: We successfully DC rhesus monkey kidneys by perfusion of trypsin/EDTA, sodium dodecyl sulfate (SDS), triton X-100, and peracetic acid/ethanol. Subsequently, we analysed the samples using quantitative and qualitative analysis.

Methods: Histological staining, DNA quantification and scanning electron microscopy (SEM) were used to investigate cellular removal evaluation. Also, ECM architecture integrity of vascular tree was assessed by x-ray fluoroscopy. The biocompatibility properties of the DC kidney tissues were evaluated by culture of human embryonic kidney 293 cells (HEK). To evaluate the renal function recovery, 30% of DC tissue was partially grafted into 70% of nephrectomized monkey kidney.

Results: Perfusion-decellularization monkey kidneys retained their essential ECM architecture, intact vascular tree, and cellular compatibility ensured clearance of cellular material, which directly impacts immunoreactivity during transplantation. Quantitative assay and immunohistochemistry demonstrated preservation of native expression patterns and integrity of ECM components, including glycosaminoglycan (GAG), collagen, fibronectin, elastin and laminin. Also, x-ray fluoroscopy confirmed the integrity and patency of vascular network. Sonography and CT scan images showed significant recovery of renal function after partial transplantation. Histological analysis confirmed increases in renal size and regeneration.

Conclusion: Monkey kidneys could be efficiently decellularized by our novel perfusion-decellularization protocol that can be scaled up for use in human organs. This method represents a step towards development of a transplantable organ using tissue engineering techniques.

Keywords: Decellularized, Kidney, Engineering, Regeneration, Scaffold
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1. Introduction

Tissue engineering and regenerative medicine have offered new hope for treatment of kidney diseases [1,2]. Decellularized (DC) extra cellular matrix (ECM) scaffolds to repair damaged tissue is considered a promising approach in the field of renal regeneration [3-6]. DC kidney scaffolds are able to act as an inductive template for organ functional recovery, allowing the injured area to be recellularized (RC) by cells [7-11]. DC scaffolds contain intact vascular trees, ECM architecture, and significant concentrations of various cytokines which hold great therapeutic promise to be used in organ structure recovery, particularly in case of highly vascular organs [12, 13]. Additionally, ECM plays a critical biochemical and physical role in initiating and sustaining various cellular functions [14]. Thus, particular physiochemical properties of DC kidney induce generation of specific cellular niches within body tissues.

Previous studies have shown that small units of engineered kidney scaffolds are able to maintain function in animal studies [15]. However, recellularization of these scaffolds with normal renal cells which have sufficient functional capacity is still a major challenge [16]. However, re-endothelialization of acellular kidney scaffolds were enhanced by antibody conjugation of vasculatures and some different approaches recently [17]. Song et al. [18] RC rat kidney scaffold grafts, though did not indicate how long is required for cells to be seeded in the scaffold. In addition, Orlando et al. [19] successfully implanted porcine DC kidney scaffolds into pigs. Moreover, Yu et al. [20] showed that grafted DC kidney scaffold into partially nephrectomized rat kidneys increased renal size and regenerated the area containing the grafted scaffold by renal parenchyma cells.

We hypothesized that transplanting a DC kidney scaffold provided by less toxic protocol that including treatment with detergents in less time and more wash would allow better regeneration in border line of the nephrectomized area. Therefore, the goal of the study was to develop less toxic DC kidney scaffolds which could induce renal regeneration.

2. Materials and methods

2.1. Preparation of DC kidney scaffolds

All animal experiments were performed in exacting state with the ethical principles and the approval of the Institutional Review Board at Royan Institute (No. EC.93.1136). For agitation decellularization, we isolated rats (n=3) and monkeys (n=2) kidneys. Nephrectomized kidneys were cannulised through renal artery for heparinization (Heparin-RotexMedica, Germany). Then, kidneys were agitated by applying rotation on a two dimensional rotator (along the x and y axes) (Stuart, SB3) with 0.5% and 1% (v/v) sodium dodecyl sulfate (SDS) at 40 rpm and rotation angle of 360 degree for 3 days. For perfusion decellularization, rabbits (n=2), sheep (n=1) and monkeys (n=9) kidneys were cannulised through renal artery with a prefilled 22 gauge cannula needle (Vasofix Braunule, Germany) and antegrade arterial perfusion of heparinized PBS at 30 mmHg arterial pressure was performed for 15-30 min to remove residual blood from the kidney. Then, heparinized kidneys were frozen at -70-80 ºC for 24-48 h. Next, we administrated decellularization solutions at 8.5 ml/min flow rate (24 rpm) in the following order: 60 min of 0.02% Trypsin/ 0.05% EDTA, 15 h of SDS 1% (Sigma) in deionized water (D.W.), and 30 min of 3% Triton X-100 (Sigma), 10 min of 0.1% peracetic acid / 4% ethanol. Finally, kidney scaffolds were washed with antimicrobial PBS or D.W. containing 200 U/ml penicillin G, 200 µg/ml streptomycin (pen/strep), 0.2 mM gentamicin and 2 mM amphotericin B (Sigma), at 8.5 ml/min constant arterial perfusion for 12 d. This DC protocol with less time treatment with SDS and more wash with PBS or D.W. is less toxic, thus less tissue is destroyed.

2.2. Histopathological analyses

H&E were used for cellular removal evaluation and assessment of the ECM architecture integrity. Kidneys and scaffolds were washed twice with PBS and then fixed with 4% paraformaldehyde for 24 h at 4ºC. The samples were then dehydrated through a series of graded alcohol solutions and xylol and embedded in paraffin. The paraffin-embedded specimens were sectioned into 5-µm thicknesses,

placed on poly-l-lysine-coated glass slides and kept in an oven at 60ºC for 12 h; Next, they were deparaffinized and dewaxed in xylene, stained with H&E and Masson's trichrome (MT) stain and observed using a light microscope. We stained the samples for collagen, fibronectin, laminin and pentachrome to assess ECM qualification.

2.3. Scanning electron microscopy (SEM), ultrasonography and intravenous pyelography (IVP)/CT scan, x-ray and fourier transform infrared (FTIR) spectroscopy

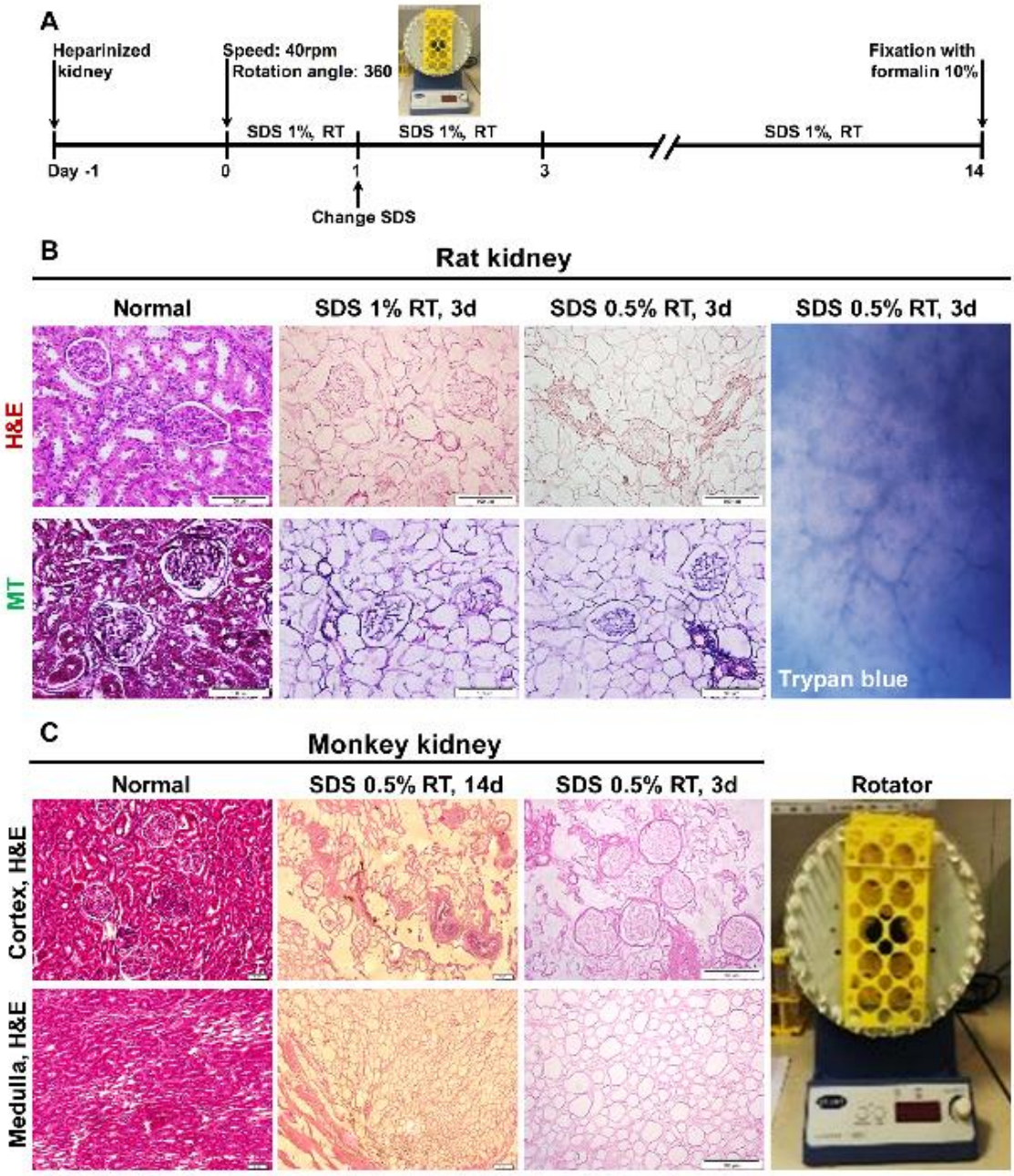
For ultra-structural evaluation, the specimens were pre-fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide for 3 h at room temperature, and then, dehydrated through a series of graded alcohol solutions. Once dried, the samples were mounted on aluminium stubs, sputter-coated with gold-palladium (AuPd), and viewed by using SEM (Tescan VE-GA-II, the Check). For dynamic renal function and recovery, we used the ultrasonography and IVP/CT scan images after partial transplantation. Iohexol (omnipaque™, iodinated a nonionic contrast medium) was used for IVP scanning. To confirm the integrity of the vascular tree and demonstrate that the fluid injected into the vasculature flowed rather than extravasate throughout the organ, x-ray fluoroscopy (Shimadzu, Flexscan L568) was performed before histological evaluation and transplantation. Visipaque™ (iodixanol) contrast agent was diluted at a ratio of 1mg/ml in distilled water and perfused through the vasculature at a rate of 20 ml/min. Surface chemistry of treated tissues was examined by FTIR spectra. Lyophilized powder from the treated tissues was prepared for FTIR spectroscopy analysis using a 102 MB BOMEM apparatus FTIR Spectrometer. The resulting IR spectra were recorded from 4000 cm-1 to 650 cm-1 wavenumbers.

2.4. Quantification of SDS, DNA, collagen, sulfated glycosaminoglycans (GAGs) and elastin

SDS was quantified using Stains-All Dye (Sigma) as previously described [21]. Briefly, lyophilized tissues were digested in 1% collagenase I (Sigma) for 24h at room temperature with gentle rotation. Digest supernatants (1µl) containing residual SDS were then added to 4 ml of a working Stains-All Dye solution, and absorbance was measured at 488 nm. DNA was quantified using the manual approach. Briefly, DNA was extracted from lyophilized tissue samples in Tris-HCl buffer following incubation with Proteinase K (200 µg/ml; Invitrogen) for 3 h at 37 °C with gentle rotation. Digest supernatants were diluted in TE buffer and then mixed with prepared reagents. DNA yield was measured by absorbance at 260 nm. Soluble collagen amount was measured using the Sircol Assay (Biocolor), as per the manufacturer's instructions. Lyophilized tissue samples (10-30 mg wet samples) were first subjected to HCl-pepsin collagen extraction at 4 °C overnight and then to overnight isolation. Next, the absorbance was measured at 555 nm. GAGs were quantified using the Blyscan Assay (Biocolor). Before measurement, GAGs were extracted using a papain extraction reagent (Sigma) (using 10-20 g of samples) and heated for 3-5 h at 65 °C and the absorbance was measured at 656 nm. Also, elastin was quantified using the Fastin Assay (Biocolor). Lyophilized tissue samples (10-20 g) were subjected to oxalic acid elastin extraction for 1 h at 100 °C and absorbance was measured at 513 nm. All concentrations were determined following generation of a standard curve and values were normalized against original tissue wet weight.

2.5. Cell culture and characterization

For human fetal kidney epithelial cells (HFKECs) culture, human fetal kidneys were transferred to wash medium (cold PBS supplemented with pen/strep). Kidneys were minced into 1-2 mm3 cubes using sterile curved surgical scissors. Then, DMEM/high glucose/10%FBS (Hyclone) medium was added, and tissue pieces were put into a 15 ml centrifuge tube, and incubated in medium containing gentamicin, pen/strep, and collagenase/dispase (0.1%) for 2-3 h. The following day, the digested tissue pieces were centrifuged at 1500 rpm for 5 min and then washed twice with medium. Afterwards, the pelleted tissue pieces were resuspended in medium containing pen/strep, L-glutamine 2 mmol/L and NEAA 1 mmol/L. Under these culture



Supplementary Figure 1. Decellularization of rats and monkeys kidney by agitation. (A) Rat kidneys were successfully decellularized by applying rotation and treatment with 0.5 and 1% (v/v) sodium dodecyl sulfate (SDS) for 3 days. (B) H&E, MT and trypan blue were used for evaluation of cellular removal and integrity of ECM architecture. Rotation-decellularization of rats' kidneys retained their essential ECM architecture, an intact vascular tree and ensured clearance of cellular material. (C) Monkeys kidney decellularization. Monkey kidneys were not decellularized after exposure to 0.5% SDS for 3 day because of their large size and were disrupted after exposure to 0.5% SDS for 14 day because of long-term treatment. Rotator equipment was shown. Original magnifications were X10 and 20; scale bar 50 and 100 µm, respectively. RT: room temperature; d: day; H&E: Hematoxylin and eosin; MT: Masson's trichrome.

conditions, the HFKECs attach to the substrate-coated plates and grow to form a confluent monolayer of bright, tightly packed cells within 5–10 days. For human umbilical vein endothelial cells (HUVECs) culture, the 2 ends of the cord were tidily cut using scalpel. Then, a cannula at each extremity of the vein was introduced and it was tightly maintained with string. The cord was washed with PBS and the collagenase (0.2 %) was injected at one end of the vein and the other end was tightly clamped with the surgical clip and the ends were protected with clean aluminium foil. The cord was incubated for 10 min in the physiologic serum at 37 °C. Upon the sterile envelope of gloves, the cord was gently squeezed and a sterile falcon of 50 ml was filled up with 10 ml of culture medium including DMEM high glucose/M199 completed with FBS 10%, pen/strep, L-glutamine 2 mmol/lit, NEAA 1 mmol/L, EGF 5 ng/ml, bFGF 10 ng/ml, VEGF 0.5 ng/ml and ascorbic acid1µg/ml. Then, the cells were collected in this falcon by washing the vein with PBS and centrifugation at 1500 rpm for 5 min. The supernatant was carefully discarded and cells were suspended in culture medium. A humidified 95% air and 5% CO2 atmosphere at 37° C was used for cell culture. For human embryonic kidney (HEK) cells preparation, HEK-293 cells were cultivated in DMEM/high glucose supplemented with 10% FBS, pen/strep and L-glutamine 2 mmol/lit at 37°C with 5% CO2 and 95% saturated atmospheric humidity. For assessment of the biocompatibility properties of DC kidney tissues, HEK-293 cell lines were seeded on DC kidney scaffold patches in flat bottom 24-well plates (400 µL/well) at a density of 30 × 103 cells/well and then incubated in a CO2 incubator, overnight. Manually, scaffold patches were prepared by cutting DC kidney off. The difference in HEK cells viability percentage between control (on plate) and test (on patch) was evaluated by a standard spectrophotometric 3-(4, 5-dimethylthiazole- 2-yl)-2, 5 diphenyltetrazolium bromide (MTT; Sigma-Aldrich, M5655) assay. HEK cells were grown in 24-well plates in 400 µl of culture medium (control) or in 24-well plates in 400 µl of culture medium on patch (Test). Next, medium was removed, 20 µl preheated (37 ◦C) MTT solution (5 mg/ml in PBS) was added and cells were incubated for 4 h at 37 ◦C. Afterwards, MTT solution was removed, and 200 µl DMSO was added to the mixture. The extinction of the solution was measured at 570 nm using a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland). Also, the biocompatibility properties of DC kidney tissues were assessed following culture of HEK-293 cells on DC monkey kidneys.

2.6. Recellularization of DC monkey kidney with perfusion and direct injection

We trypsinized 50 × 106 HUVECs and diluted in DMEM high glucose/M199 and seeded onto the acellular kidney through the arterial cannula. Then, 50 × 106 HFKECs were isolated, counted and resuspended in medium. The suspended cells were seeded on DC monkey kidney through the ureter cannula while subjecting the organ chamber to a negative 40 mmHg pressure. Cells were allowed to attach overnight, and subsequently culture perfusion resumed. We designed a custom-built kidney bioreactor as a closed system that could be autoclave-sterilized after cleaning and assembly, needing only to be opened once at the time of organ placement. Perfusion media and cell suspensions were infused through sterile access ports to minimize the risk of contamination. Media was allowed to equilibrate with 5% CO2 and 95% room air by flowing through a silicone tube oxygenator (Cole-Parmer) before reaching the cannulated renal artery at 8.5 ml/min. The ureter and vein were allowed to drain passively into the reservoir during the biomimetic culture. For cell seeding with direct injection, 20 × 106 HFKECs were seeded through 22G needle in cortical region of DC kidney and incubated for 3h and then perfused with medium and followed for 48h.

2.7. Total and partial transplantation of DC monkey kidney

After median laparotomy and systemic heparinization, left nephrectomy was done to harvest native kidney under inhalation anaesthesia. For recellularization of DC monkey kidneys with total transplantation (n=2), DC kidneys were prepared for

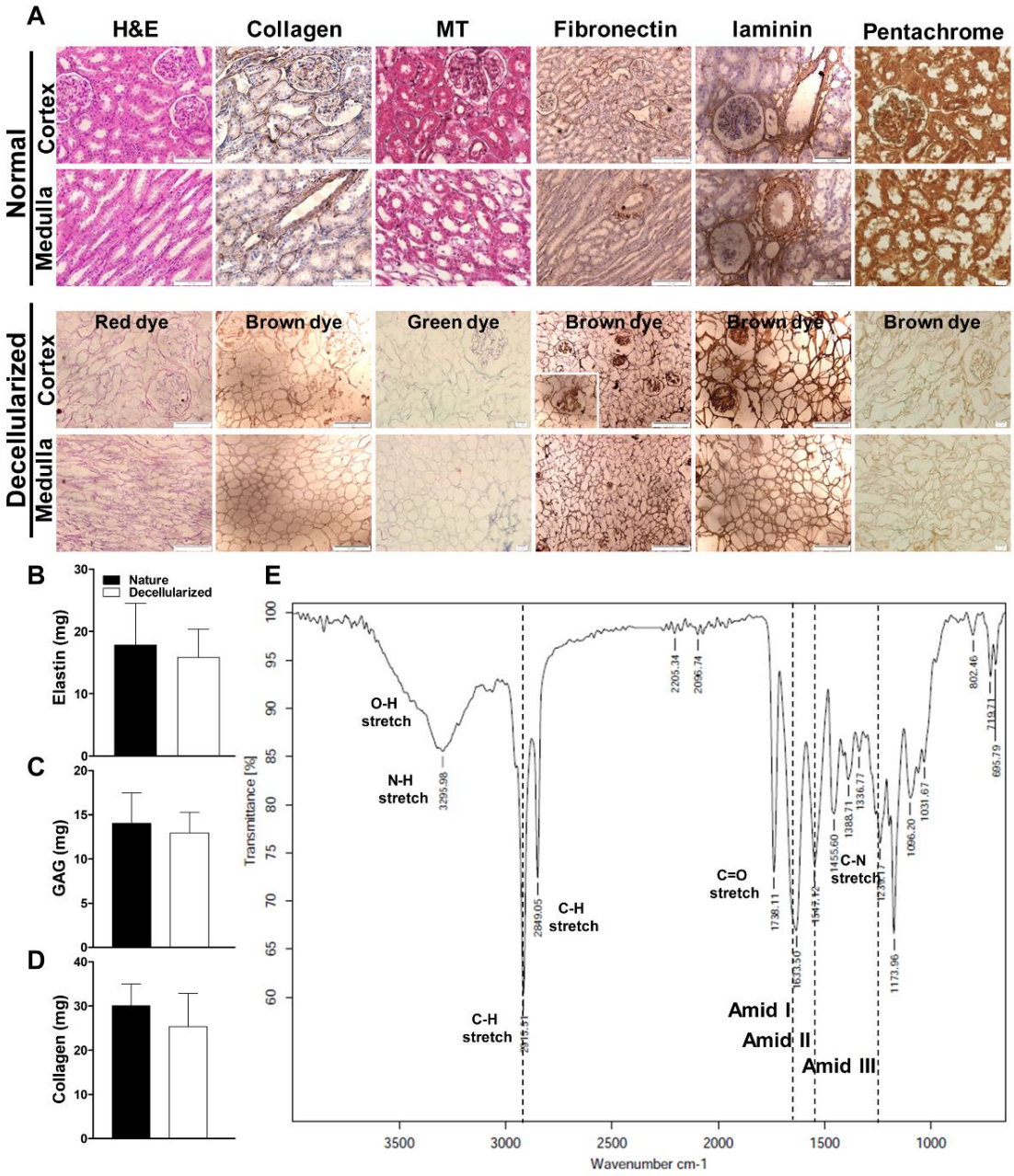


Figure 2. Quantitative and qualitative characterization of decellularized monkey kidney by perfusion. (A) Quantitative assay and immunohistochemistry demonstrated preservation of native expression patterns and integrity of ECM components, including glycosaminoglycan (GAG), collagen, fibronectin, elastin and laminin. Original magnification X20; scale bar 100 µm. (B-D) Quantitative analysis of ECM components including (B) elastin, (C) glycosaminoglycan (GAG) and (D) collagen showed no significant difference between natural and decellularized monkey kidneys by perfusion. Data are mean ± SD. (E) Surface chemistry of DC scaffolds was examined by FTIR. As shown, major functional groups containing amide I, II and III, C-N, N-H and O-H are appeared and confirmed the structure.

orthotopic transplantation by dissecting the hilar structures (artery, vein and ureter) circumferentially on ice. The graft renal artery and vein were cuffed using a cuff technique with 24-G and 20-G, respectively, FEP polymer custom-made cuffs (Smith-Medical). For in vivo experiments, six-year-old (5.5 kg) recipient monkeys inhaled 5% isoflurane and were exposed to 1% isoflurane through an endotracheal tube (I.D 3). Monkeys were placed in dorsal position on a heating pad (Sunbeam). After a caudal median laparotomy, the left recipient eternal artery and caudal vena cava were dissected circumferentially. The left external artery was clamped with micro-ligation A-traumatic haemostatic clip (mini-bulldog lemmen AESCULAP, Germany). End-to-end anastomoses were done (7/0 polypropylene suture material). Caudal vena cava (CVC) was clamped with vena cava clamps (De BAKEY-Beck) and end to site anastomoses were done. The recipient artery and vein were then unclamped and patent anastomoses were confirmed. Urine was allowed to drain passively from the ureter through a 25G angiocath (Harvard Apparatus) and monkeys were followed for 48 hours.

For recellularization of DC monkey kidney with partial transplantation (n=1), animals were anesthetized using an intramuscular (i.m.) injection of ketamine 10% (15 mg/kg) and xylazine 2% (0.4 mg/kg). After induction of anesthesia, intubation was done and anesthesia was continued using isoflurane (1%). First, 1 ml of heparin (50 U/ml) was injected through the CVC. Then, renal artery and vein were clipped with micro-ligation a-traumatic haemostatic clip (mini-bulldog lemmen AESCULAP, Germany); at this point, the timer was started to make sure renal ischemia was maintained for less than 10 min. The left kidney was transacted slightly below the renal pelvis (removing about 1/3 of the renal parenchyma). Then, the wound was grafted with lower 1/3 of DC scaffold by suturing (4-0 Vicryl suture material) the external capsules between the excised kidney and DC kidney scaffold. After reperfusion, the left kidney was monitored for leakage of blood for 20 min before closing the abdominal wall. After the surgery, the animals were given unlimited access to food and water and received subcutaneous injection of tramadol (2 mg/kg twice daily (b.i.d.) for up to 2 days) and intravenous (i.v.) injection of cefazolin (25 mg/kg three times daily (t.i.d.) for 2 days); animals were followed for 27 months.

2.8. Statistical analysis

Values were showed as mean ± standard deviation (SD). Analysis was accomplished using GraphPad Prism 7.03 (GraphPad Software Inc.). Differences in DNA content and SDS absorbance among the experimental groups were tested by one-way analysis of variance with Dunnett's or Bonferroni's multiple comparison test or two-way repeated measurements. Independent-samples t-tests were performed to identify significant differences in elastin, GAGs and collagen values between intact and DC scaffolds and in HEK viability percentage between control and test groups. A two-sided value of $P< 0.05$ was considered significantly different.

3. Results

3.1. Decellularization of rats and monkeys kidney by agitation

We successfully DC rat's kidneys by applying rotation with 0.5% and 1% (v/v) SDS for 3 days (Supplementary Figure 1A). Treatment with 1% SDS and 0.5% SDS for 3 days showed significant cellular removal in rat Kidney that were the most effective protocol. Rotation-decellularization of rat's kidneys retained their essential ECM architecture, and intact vascular tree which ensured clearance of cellular materials (Supplementary Figure 1B) as well. However, 0.5% SDS did not remove cells of monkeys' kidneys within 3 days because of their large size. On the other hand, monkeys' kidneys were disrupted after exposure to 0.5% SDS within 14 days because of long-term treatment.

3.2. Decellularization of rabbits, sheep and monkeys kidney by perfusion

We approximately DC rabbits and sheep kidneys by perfusion of 0.5% (v/v) SDS for 27h and 72h, respectively. In large kidneys such as those of sheep, DC cortex had areas where the architecture was disrupted. Also, DC sheep medulla had many

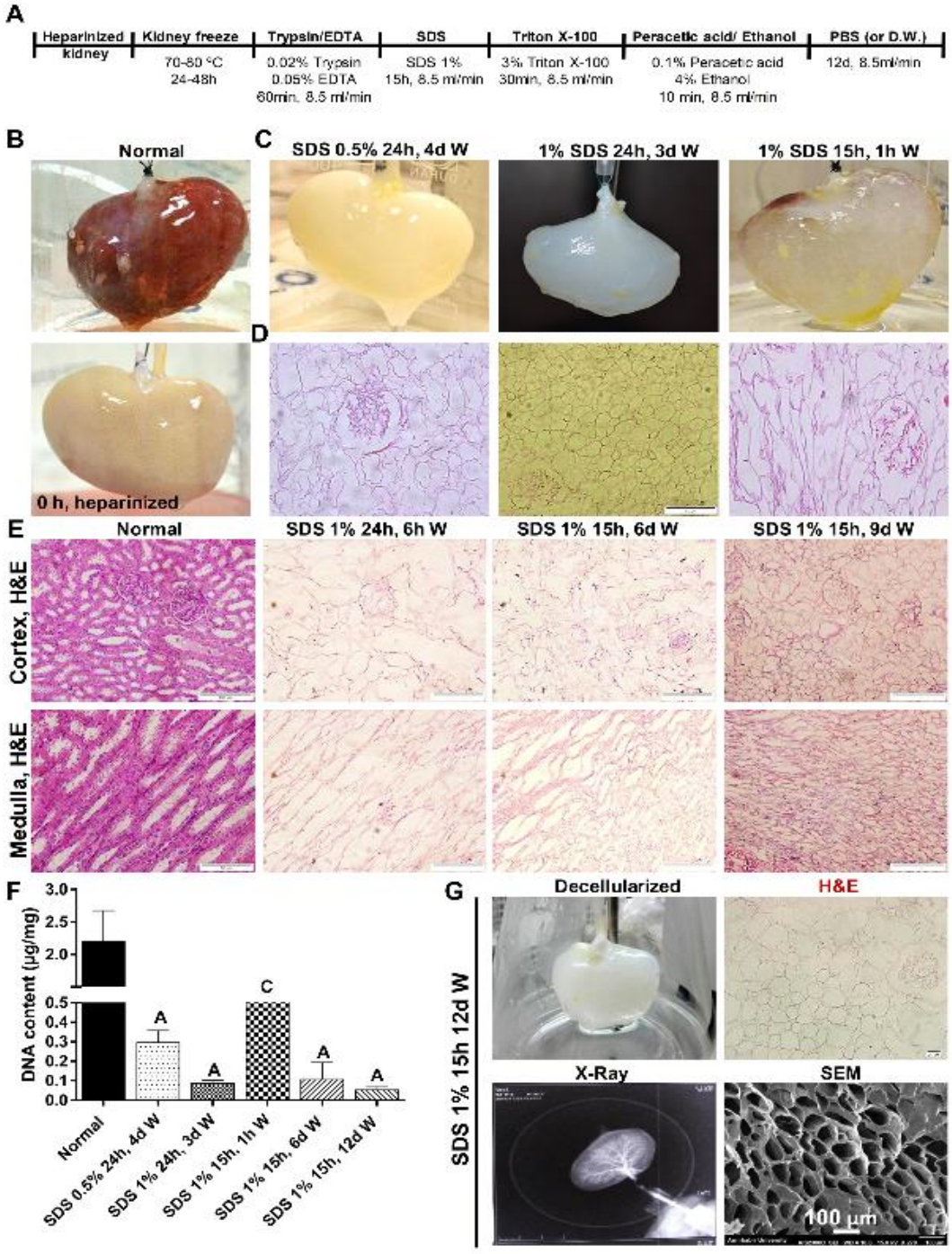


Figure 1. Decellularization of monkeys' kidney by perfusion. (A) Rhesus monkey kidneys were successfully DC by perfusion of 0.02% trypsin/0.05% EDTA, 1% (v/v) sodium dodecyl sulfate (SDS), 3% triton X-100, 0.1% peracetic acid/4% ethanol and 12-day washing with double distilled water followed by quantitative and qualitative analysis. (B) Normal and heparinized kidney. (C) DC rhesus monkey kidneys by perfusion. (D and E) H&E staining, (F) DNA quantification and (G) scanning electron microscopy (SEM) were used to examine cellular removal and integrity of ECM architecture. (G) Perfused-DC monkey kidneys retained their essential ECM architecture, an intact vascular tree and ensure clearance of cellular material. X-ray fluoroscopy on the bench confirmed the integrity and patency of vascular network. DNA count is expressed as mean ± SD. AP<0.001 and CP <0.05 show differences among different treatments and normal group. Original magnification X20; scale bar 100 µm. W: wash; d: day; h: hour; H&E: Hematoxylin and eosin; MT: Masson's trichrome; DAPI: 4',6-diamidino-2-phenylindole.

remaining cell nuclei (Supplementary Figure 2A). However, perfusion-decellularization of medium-sized kidneys such as those of rabbits retained their essential ECM architecture and ensured clearance of cellular materials (Supplementary Figure 2B). So, perfusion protocol was selected for decellularization of medium-sized kidneys such as those of monkeys. Finally, we successfully DC rhesus monkey kidneys by perfusion of 0.02% trypsin/0.05% EDTA, 1% (v/v) SDS, 3% triton X-100, and 0.1% peracetic acid/4% ethanol which followed by 12 days washing with double-distilled water (Figure 1A). H&E staining, DNA quantification and SEM were used for cellular removal evaluation and assessment of the ECM architecture integrity (Figure 1B-G). To confirm the integrity of the vascular tree, x-ray fluoroscopy was performed on the bench (Figure 1G). In this study, 15h treatment with SDS 1%, trypsin, EDTA, triton, peracetic acid and ethanol showed significant cellular removal which was the most effective protocol. Perfusion-decellularization of monkeys' kidneys retained their essential ECM architecture, and intact vascular tree, which ensured clearance of cellular material that directly impacts immunoreactivity during transplantation. Also, x-ray fluoroscopy confirmed integrity and patency of vascular network (Figure 1G).

3.3. Quantitative and qualitative characterization of monkey kidneys DC by perfusion

Quantitative assay and immunohistochemistry demonstrated preservation of native expression patterns and integrity of ECM components, including glycosaminoglycan (GAG), collagen, fibronectin, elastin and laminin (Figure 2A-D). The major functional groups containing amide I, II and III, C-N, N-H and O-H were present and confirmed the structure (Figure 2E).

3.4. Biocompatibility of DC monkey kidney with human embryonic kidney 293 (HEK) cells culture

Rhesus monkey kidneys were DC by perfusion of SDS 1% for 15 hours followed by 12 days of washing (Supplementary Figure 3A). Absorbance of residual SDS in DC monkey kidneys was measured at 488 nm. There was no residual SDS in DC monkey kidney (Supplementary Figure 3B). H&E and MT staining were used to demonstrate cellular removal and integrity of DC patch from DC monkey kidney (Supplementary Figure 3C-E). There was no significant difference in HEK viability between tissue culture plate (Control) and DC kidney patch (Test) (Supplementary Figure 3F-H). Also, the biocompatibility properties of DC kidney tissues were shown by culture of HEK-293 cells on these scaffolds by syringe through ureteral perfusion (Supplementary Figures 3I, Ja and Jb).

3.5. Characterization of HFKECs and HUVECs

HFKECs and HUVECs were expanded with cuboidal-shaped epithelial morphology in culture (Supplementary Figure 4A and C). Immunophenotypic characterization of HFKECs and HUVECs was performed by flow cytometry. HFKECs were positive for podocin (99.78%), E-cadherin (82.60), ATPase (97.53) while they were negative for CD31 (6.6%). HUVECs were positive for CD31 (71.42%), CD146 (52.39%), and CD105 (85.09) while they were negative for CD45 (3.36) (Supplementary Figure 4B and D).

3.6. Recellularization of DC monkey kidney by perfusion of HUVECs and HFKECs

To regenerate functional kidney tissue using perfusion, we repopulated acellular monkey kidneys with endothelial and epithelial cells in a perfusion bioreactor (Supplementary Figure 5). We instilled suspended HUVECs through the renal artery and HFKECs through the ureter while maintaining a negative pressure (about -40 mmHg) outside the kidney during cell perfusion (Figure 3A). Evidently, cell seeding within the kidney tubules and peritubular capillaries was very difficult to achieve (Figure 3B) thus we discontinued using perfusion cell seeding for recellularization.

3.7. Total transplantation of DC monkey kidneys without cell seeding

For recellularization of DC kidneys with total transplantation, one day after transplantation of DC kidneys without any cell seeding (Figure 3C), the DC scaffolds were morphologically intact although

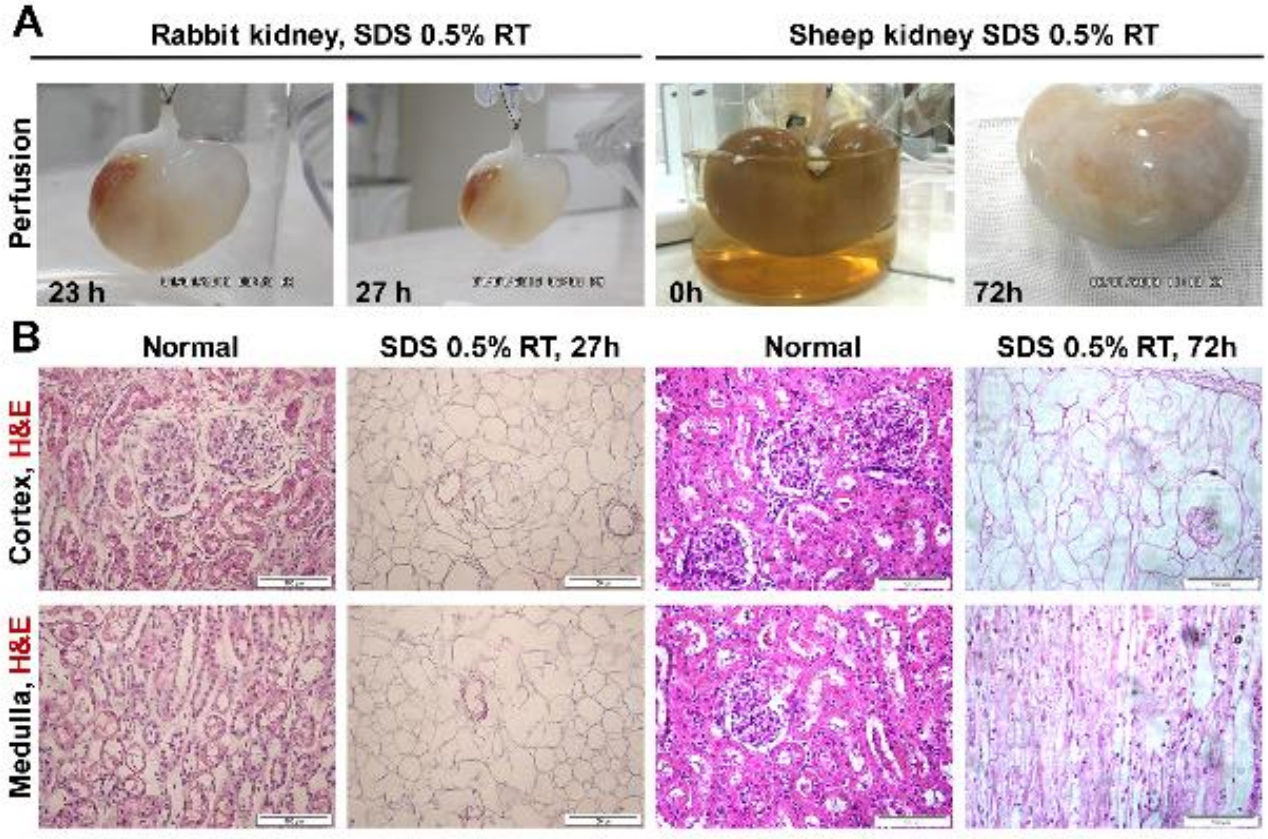
all vascular structures were obstructed with thrombi (Figure 3D).

3.8. Recellularization of DC monkey kidney with direct injection of HFKECs

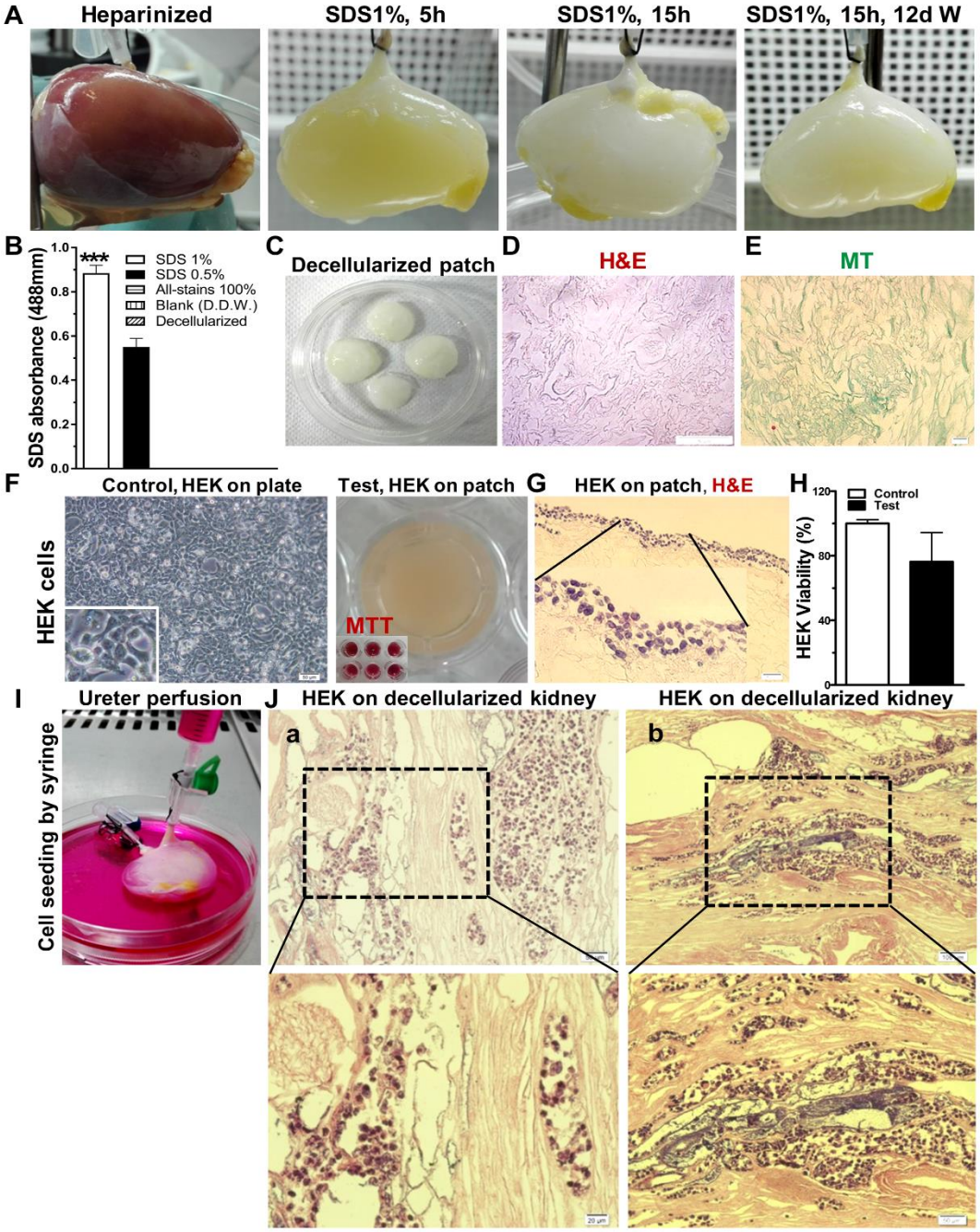
For direct injection, the HFKECs were delivered into the cortical region of renal scaffolds using a 22G needle. Also, here, cell seeding within the kidney tubules and peritubular capillaries was very difficult to achieve (Figure 3E).

3.9. Partial transplantation of DC monkey kidney

This study aimed to establish an effective cell seeding method using partial transplantation for repopulation of acellular monkey kidney scaffolds with host cells. In this study, to evaluate the renal function recovery, 30% of DC tissue was partially grafted into 70% of nephrectomized monkey's kidney (Figure 4A and B). Histological analysis confirmed increases in renal size and regeneration (Figure 4C-E). Sonography and CT scan images showed significant recovery of renal structural after partial transplantation with regeneration of the nephrectomized area by cells present in border line of the nephrectomized health residual kidney (Figure 4F and G).



Supplementary Figure 2. Decellularization of rabbits and sheep kidney by perfusion. (A) Rabbits and sheep kidneys were approximately decellularized by perfusion of 0.5% (v/v) sodium dodecyl sulfate (SDS) for 27 and 72 h, respectively. (B) H&E were used to evaluate cellular removal and integrity of ECM architecture. In large kidneys such as those of sheep, architecture of decellularized cortex was disrupted and decellularized sheep medulla showed several cell nuclei. However, perfusion- decellularization of medium-sized kidneys such as those of rabbits which are roughly the size of monkey kidney, retained their essential ECM architecture and ensured clearance of cellular material. Original magnification was X20; scale bar 100 µm. RT: room temperature; h: hour; H&E: Hematoxylin and eosin.



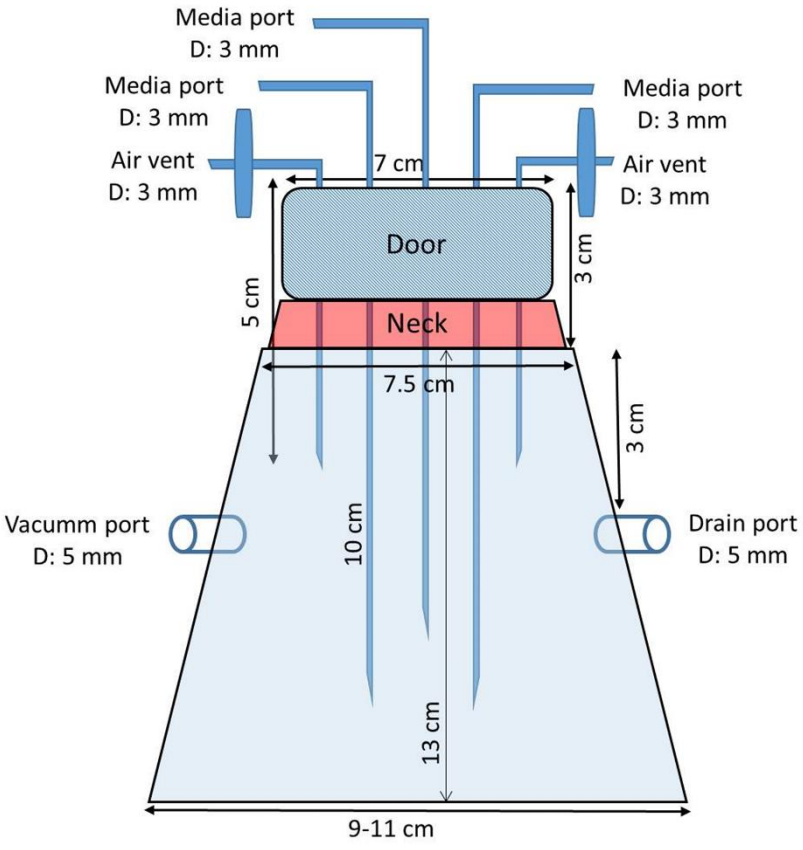
Supplementary Figure 3. Biocompatibility of DC monkey kidney with human embryonic kidney (HEK) culture
(A) Rhesus monkey kidneys were decellularized by perfusion of SDS 1% for 15 hours (h) followed by 12 days of washing (12d W). (B) Absorbance of residual SDS in decellularized monkey kidneys was measured at 488 nm. Data are mean ± SD. ***P<0.001 shows significant differences between SDS 1% and 5%. There was no residual SDS in decellularized monkey kidney. (C) Decellularized patch from decellularized monkey kidney. (D) H&E and (E) MT staining were used to demonstrate cellular removal and integrity of ECM architecture. (F-H) There was no significant difference in HEK viability (H) between tissue cultured on plate (Control) (F) and tissue on decellularized kidney patch (Test) (G). Data are mean ± SD. (I) The biocompatibility of decellularized kidney tissues was shown by culture of human embryonic kidney 293 cells (HEK) on them (a and b) by syringe through ureteral perfusion. Original magnifications were X10, 20 and 40; scale bar 50, 100 and 20 µm, respectively. W: wash; d: day; h: hour; H&E: Hematoxylin and eosin; MT: Masson's trichrome.

4. Discussion and Conclusion

We demonstrated that monkey's kidneys can be efficiently DC with our novel perfusion-decellularization protocol. Also, we reported that rotation protocol is not suitable for decellularization of large kidney (Supplementary Figure 1C). Furthermore, it indicated that partial transplantation of DC kidney might be a suitable protocol for kidney self-regeneration as reflected by the presence of the renal cells of nephrectomized host kidney in border line of the grafted kidney.

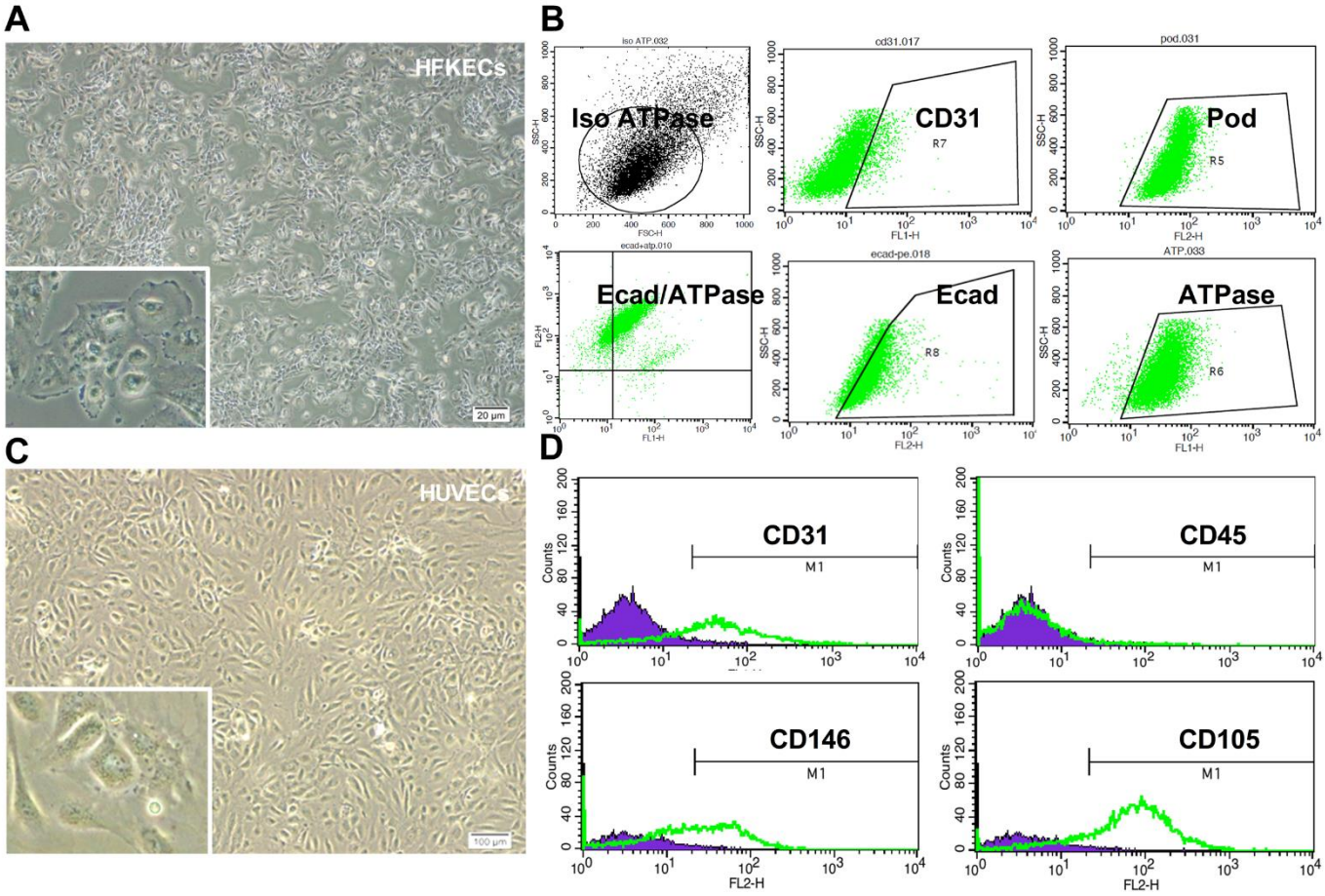
While our protocol for obtaining DC organ scaffolds appeared to be successful with integrity and patency of vascular network, none of our recellularization protocols including cell perfusion and direct injection were appropriate for recellularization (Figure 3). While, orthotopic transplantation of DC rat kidneys yielding ECM, demonstrated regeneration of vessels including artery and vein in the renal sinus following a spontaneous recanalization [22]. These findings suggest that the body itself, as a bioreactor, is a viable machine for kidney regeneration [22, 23]. Consequently, we just grafted DC kidney scaffolds into partially nephrectomized monkey kidney which resulted in long-term renal function of the model with patent renal vessel and kidney tissue reorganization.

Most of the DC kidney protocols were toxic and destroy tissue [24] and repopulation of the DC kidneys with functional renal cells is still a challenging issue [25, 26].



Supplementary Figure 5. Schematic of the bioreactor setup used for perfusion recellularization .Schematic of a whole-organ culture in a bioreactor enabling tissue perfusion through a media port attached to the renal artery. Medium circulation to a reservoir and sampling were performed through two others media ports.

In a method, the DC kidney was repopulated by human iPSC-derived PAX2 positive renal progenitor cells and endothelial cells which were subcutaneously implanted into mice [27]. In other study, intact rat kidneys were successfully DC after perfusion of SDS. Then, whole-kidney scaffolds were infused with murine embryonic stem cells (mESCs) through the renal artery. They observed loss of cells pluripotency and initiation of differentiation towards meso-endodermal lineage [11]. Moreover, DC rats' kidneys with intact intricate architecture seeded with mESCs through the artery or ureter. mESCs-derived primitive precursor cells proliferated and populated within the glomerular, vascular, and tubular structures [28]. In another similar study, DC kidney scaffolds with mouse ESCs were populated and proliferated within the glomerular, vascular, and tubular structures and were easily reperfused, tolerated blood pressure and produced urine with no blood leakage after in vivo implantation [9].



Supplementary Figure 4. Characterization of human fetal kidney epithelial cells (HFKECs) and human umbilical vein endothelial cells (HUVECs). In vitro expanded HFKECs (A) and HUVECs (C) had cuboidal-shaped epithelial morphology in culture. Original magnification was X20; scale bar 100 µm. Immunophenotypic characterization of HFKECs (B) and HUVECs (D) was performed by flow cytometry. Pod: podocin; Ecad: E-cadherin

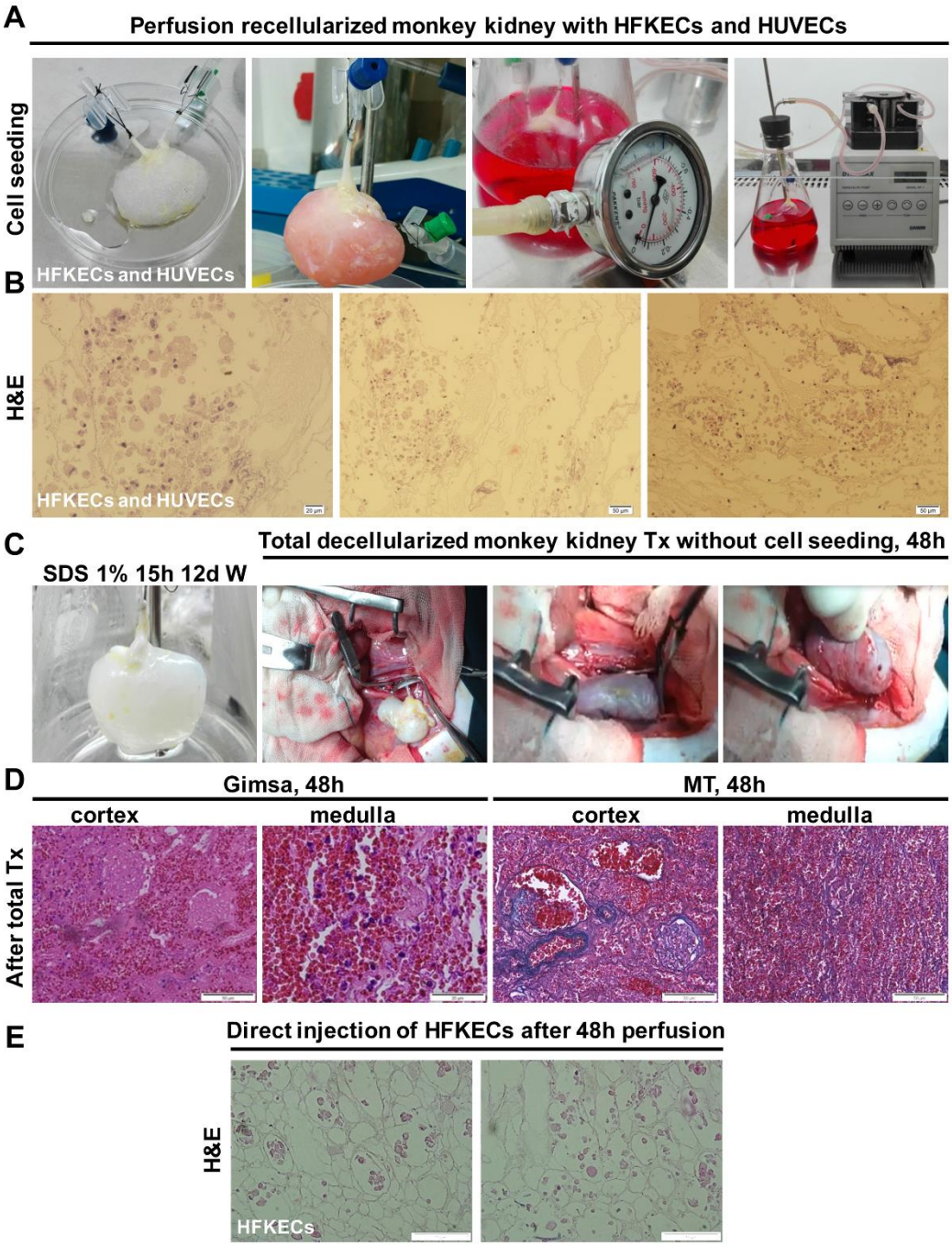


Figure 3. Recellularization of decellularized monkey kidney using perfusion, total transplantation and direct injection .(A) Acellular monkey kidneys were repopulated with HFKECs and HUVECs. Suspended HFKECs and HUVECs were instilled through the ureter and the renal artery, respectively, while maintaining a negative pressure (about -40 mmHg) outside the kidney during cell perfusion. (B) Based on these studies, it was evident that cell seeding within the kidney tubules and peritubular capillaries, is very difficult to achieve. (C) One day after total transplantation of decellularized monkey kidney, (D) the scaffolds were morphologically intact although all vascular structures were obstructed with thrombi. (E) The cells were delivered into the cortical region of renal scaffolds using a 23G needle. Also, it was evident that cell seeding within the kidney tubules and peritubular capillaries, is very difficult to achieve. Original magnifications were X10, 20 and 40; scale bar 50, 100 and 20 µm, respectively. W: wash; d: day; h: hour.

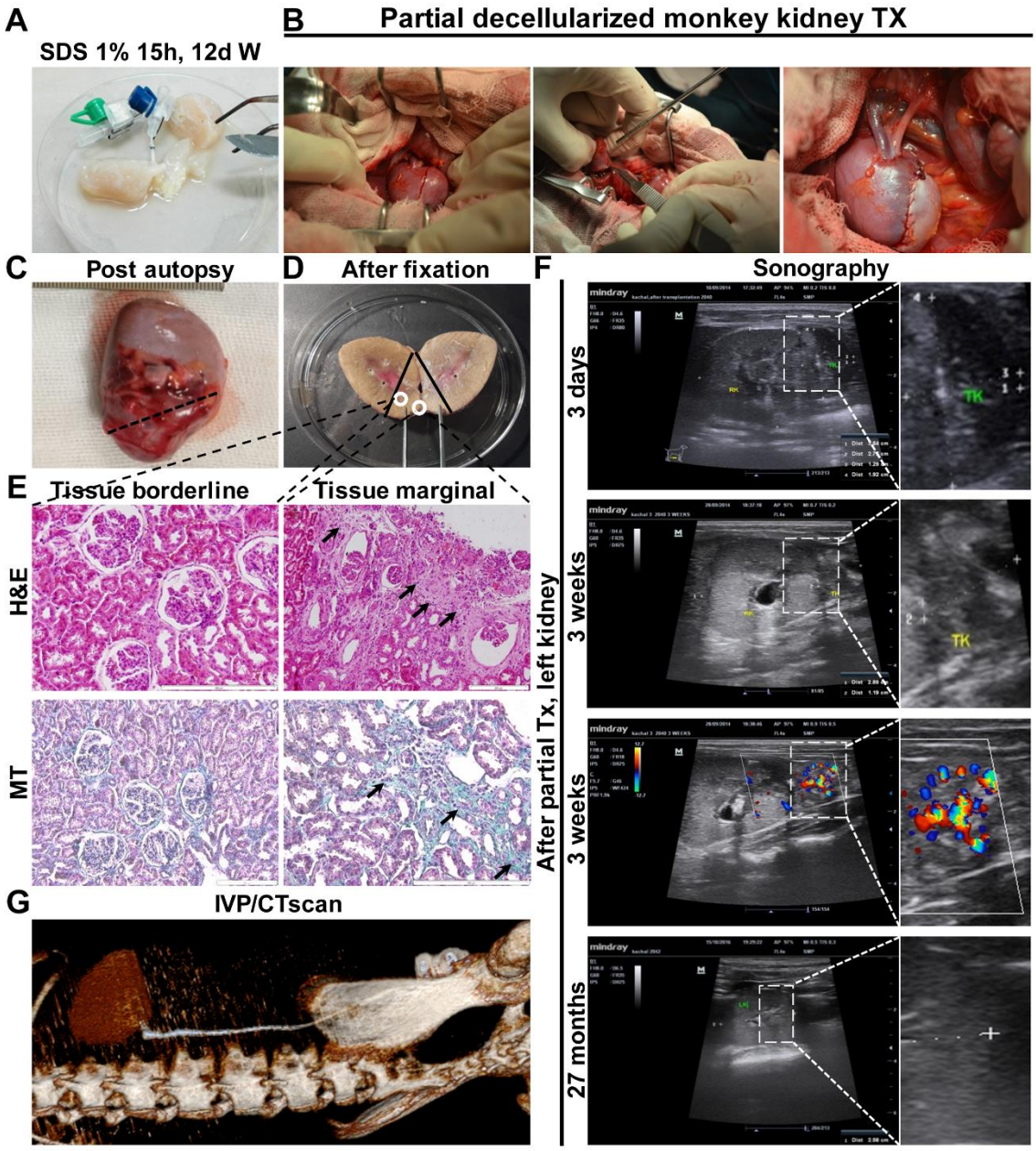


Figure 4. Partial transplantation of decellularized monkey kidney. Partial transplantation established an effective cell seeding method for repopulation of acellular monkey kidney scaffolds in border line of nephrectomized kidney with renal cells from the host. (A and B) To evaluate the renal function recovery, 30% of decellularized tissue was partially grafted into 70% of nephrectomized monkey kidney. (C and D) Histological analysis confirmed increases in renal size and regeneration. (E) The fibrosis in marginal tissue between the renal parenchyma and the grafted-decelullarized scaffold is shown by H&E and MT staining (arrow) after 27 months of partial transplantation. There was no fibrosis in regenerated tissue borderline between the renal parenchyma and the grafted-decelullarized scaffold after 27 months of partial transplantation. Original magnifications were X10 and 20; scale bar: 200 µm. (F) Sonography and (G) CT scan images showed significant recovery of renal structural and function after partial transplantation. W: wash; d: day; h: hour; H&E: Hematoxylin and eosin; MT: Masson's trichrome.

Furthuremore, whole porcine kidneys decellularization by an automated and improved method including physical and chemical steps while causing minimal damage to the collagenous ECM and human renal tubular epithelium cells were cultured on DC ECM [29]. Also, Abolbashari et al. [30] established an effective cell seeding method for repopulating DC porcine renal scaffolds that allowed the injected cells to form renal tubular structures and to impart functional capabilities. Histological and immunohistochemical analyses demonstrated that seeded cells formed tubule-like structures that expressed normal renal tubule phenotypic markers. Functional analysis has shown that cells within the kidney construct had normal renal functions.

In addition, there are some studies to address that biologic resources including collagen hydrogel, PLGA/ECM/Mg (OH)2 scaffold and DC ECM-derived hydrogels are able to facilitate the regeneration of renal tissues [31-33].

Also, Yu et al. [20] grafted some scaffold tissue into partially nephrectomized rats' kidneys and indicated an increase in renal size, and observed regenerated renal parenchyma cells in the nephrectomised area containing the grafted scaffold.

Also, following in vivo implantation of the scaffolds into the injured site after partial nephrectomy, glomeruli-like structure formation and neovascularity were observed [34]. Abolbashari et al. [17] described an endothelial cells seeding approach that permits effective coating of the vascular matrix of the DC porcine kidney scaffold using a combination of static and ramping perfusion cell seeding, while vascular patency was enhanced by conjugation of CD31 antibodies.

Finally, Remuzzi et al. [35] evaluated the potential of in vivo and in vitro kidney scaffold recellularization procedures. Their results showed that acellular scaffolds implanted in rats cannot be repopulated with host cells, thus, in vitro recellularization is necessary. In contrast to the data reported by Remuzzi et al. [35], Yu et al. [20] and Choi et al. [34] our results showed that partial transplantation is a suitable protocol for kidney self-regeneration as reflected by the presence of nephrectomized health residual renal cells in border line of nephrectomized host kidney.

This study provided the initial data for developing new regenerative medicine strategies for promoting less toxic protocol for DC kidney as well as cellular repopulation that could be used to promote renal recovery. This protocol could be scaled up for use in human organs, and this represents a step toward development of a transplantable organ using tissue engineering techniques.

Abbreviations

Decellularized: DC; Sodium dodecyl sulfate: SDS; Scanning electron microscopy: SEM; Human embryonic kidney 293 cells: HEK; Glycosaminoglycan: GAG; Extra cellular matrix: ECM; Recellularized: RC; Murine embryonic stem cells: mESCs

Conflicts of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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