Decellularization and Recellularization Processes for Whole Porcine Kidneys

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Concern over kidney disease has increased dramatically in recent years within the medical community. It is estimated that approximately one in fifteen Americans, nearly 20 million people, experience chronic kidney disease with most of those diagnosed progressing to kidney failure. The ultimate treatment available for end stage renal failure is whole kidney transplantation. However, there are very few kidneys available for patients to receive and those patients who are fortunate enough to receive an organ must remain on immunosuppressive medication for the remainder of their lives. The United States Department of Health & Human Services have reported that 18 people die every day while on the waiting list for organ donations. The treatment is fairly successful as 69% of patients who receive a kidney transplant are still alive 5 years after the transplant. Tissue engineered organs could be a promising alternative for whole organ transplantation. The overall objective is to repopulate appropriate decellularized scaffolds from pigs, which are not immunogenic, with a patient’s own cells to achieve a functional organ. Therefore, there would be an inexhaustible source of organs ready for transplantation without the risk of immune rejection.

The naturally obtained scaffolds devoid of immunogens are a potential matrix to create artificial kidneys. Repopulation of decellularized rat kidneys with renal progenitor cells has been reported in previous studies. This dissertation reports the scale-up of the previous technology and building of partially functional human-sized kidneys. In the first step, we investigated various cell lysing agents and developed an automated decellularization procedure for whole porcine kidney decellularization. We also developed a preservation method for native and decellularized kidneys to avoid spoilage before and after decellularization. We also developed a decontamination procedure for whole porcine kidneys. Finally, we recellularized whole porcine kidney scaffolds with renal epithelial cells and achieved partial repopulation of the renal structure.

Keywords: Nafiseh Poornejad, decellularization, recellularization, renal tissue regeneration, porcine kidney
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1 INTRODUCTION

Chronic kidney disease and end-stage renal failure are among the leading causes of morbidity and mortality in the United States. The U.S. Center for Disease Control and Prevention (CDCP) estimates that over 10% of adults living in the U.S. (roughly 20 million individuals) suffer from chronic kidney disease (CKD) (1). Despite an intrinsic self-healing capacity of adult kidneys, nephrogenesis is limited to embryonic development. Therefore, orthotopic transplantation is the only successful treatment for patients suffering from end-stage renal failure. Unfortunately, the number of donors is far less than patients on the waiting list. The U.S. Department of Health & Human Services reports over 122,000 individuals are on the patient wait-list, of which more than 101,000 registrants are awaiting a kidney transplant, with only 17,108 kidney transplants taking place during 2014 (2).

Different approaches are under investigation all over the world to create artificial healthy kidneys ready for transplantation without the risk of immune rejection. One idea is to use naturally obtained scaffolds (scaffolds obtained by decellularization of native organs) combined with patient-specific cells to grow whole healthy kidneys. Another idea is to build healthy kidneys from stem cells. A variety of growth factors and signaling molecules should be applied to guide the differentiation of stem cells to the right phenotype and create organoids growing into a whole healthy kidney. There are also other approaches that use injections of patient-specific stem cells into porcine embryos and result in human kidneys grown inside a pig’s body.
In decell/recell technology, artificial organs need three basic components: a scaffold, cells, and growth or signaling factors. The scaffold is the supportive structure of an organ that also carries out many other signaling functions for cell migration and differentiation. Cells are seeded onto the scaffold and by aid of growth factors and signaling molecules, the cells can potentially repopulate the entire scaffold.

This new and potentially inexhaustible technology of tissue engineering already provides blood vessels, bladders, skin and tracheas. However, none of these organs need to connect to the vasculature for perfusion of the tissue (circulatory system of the body including blood vessels) since each initially obtains required nutrients and oxygen by diffusion, and then creates the needed vasculature by angiogenesis to connect to the circulatory system, which is not enough for cell survival and leads to cell death because of ischemia. Without the circulatory system, cells can only receive oxygen and nutrients by diffusion, which extends only 1-3 mm into most tissues, limiting current tissue engineering applications to thin and small organs (3). Thicker, larger organs must receive nutrients and oxygen through a tree-like vasculature that delivers nutrients and oxygen to within less than 1 mm of the cells through numerous connected branches. Some researchers have used synthetic polymers as supportive structures and tried to vascularize them via different methods. Attempted methods for creating vasculatures for synthetic scaffolds include co-seeding of endothelial cells that spontaneously form capillary-like networks, the engineering of branching channels to mimic the vascular tree, and incorporating angiogenic peptide and growth factors into scaffolds. All of these efforts have failed to provide large organs with sufficient nutrients and oxygen (4).

Another option for scaffolds is to use naturally derived structures. These structures have an inherent vasculature system as well as essential proteins that are required for cell proliferation
and differentiation (3). Harvesting an organ from deceased donors or animals and removing the cells from its structure can obtain natural scaffolds. The ideal choice to repopulate the scaffold is to use the patient’s own cells. Collagen as the most abundant component of extracellular matrix (ECM) is highly conserved across mammalian species and because of that xenogeneic ECM (obtained from animal) can be used as a constructive scaffold in a mammalian recipient (5). This approach could solve the vasculature problem and prevent immunogenic rejection but several additional challenges remain, such as finding a donor, harvesting the scaffold, developing decellularization and recellularization methods, and endothelialization of the regenerative organ to avoid thrombosis (6). The prominent immunogens are Gal epitope (antigen expressed on the cell membrane) and DNA that both can be removed via decellularization to less than threshold needed for eliciting any immune response (5, 7).

The goal of decellularization is to remove all cells to avoid any adverse immunological response at the time of transplantation and keep the main components of the extracellular matrix such as growth factors and collagen intact. The ECM serves structural, mechanical, and signaling purposes. Signaling mechanisms involve soluble molecules coming from ECM degradation and release of soluble bioactive cryptic peptides (peptides that are hidden in the ECM structure). Depending on the tissue mass, structure, function and biomechanical characteristics of an organ, different methods of decellularization could be effective. The complexity of the structure of the kidney with its more than thirty cell types, increases the need for the ECM to be as intact as possible to increase the chance of successful recellularization (6, 8, 9).

A decellularization protocol generally begins with lysis of cell membranes using a physical treatment or ionic solution, separation of cellular components from ECM by action of an enzyme, solubilization of cytoplasmic and nuclear cellular materials by detergents, and finally removal of
cellular debris from the ECM (9). The decellularized ECM should have the following properties: it should be flexible, mechanically strong, and autologous in nature. These properties are required in order to have a construct that is functionally and morphologically similar to the native tissue, integrates with it, stays viable over time and improves renal function that was previously lost with damaged kidneys. However, the processes used so far lead to ECMs that possess different properties in terms of collagen type, content and density, glycosaminoglycans (GAGs), and susceptibility to damage from the different decellularization procedures (10).

After decellularization, the organ should be repopulated with regenerative cells under conditions in which the cells can engraft, multiply and differentiate within the decellularized organ. In one embodiment, the regenerative cells are injected or perfused into the decellularized organ (11). An ideal cell would be one that can proliferate and differentiate as much as needed. The most likely candidate to fulfill such demands is a stem or pluripotent progenitor cell (6).

In this dissertation, we focused on decellularization/recellularization technology to partially rebuild human-sized kidneys. The overall goal of this research was to scale up and improve previous technology reported for rat kidneys. We improved several processes that are required prior to recellularization.

The first step in this technology is to create the decellularized scaffold. A variety of cell lysing agents could be used to remove cellular materials from the collagenous structure; however, these materials may cause severe damage to the extracellular matrix (ECM). In the first study of this dissertation, we applied a wide range of cell lysing agents to decellularize slices of renal tissue and examined physical and chemical characterization of the resulting scaffolds to choose the most appropriate agent for decellularization of whole porcine kidneys. Then, we designed and constructed two bioreactors for perfusion of whole porcine kidneys with decellularization agents.
We developed two different processes with improved characteristics compared to previous methods. We also developed and characterized decontamination methods of whole porcine kidney scaffolds. Finally, we recellularized the whole porcine scaffold with one layer of epithelial and one layer of endothelial cells to obtain partially repopulated kidneys.
2 LITERATURE REVIEW

The massive shortage of kidneys for transplantation as well as associated immune rejection risks have driven researchers to develop bioengineering strategies for renal tissue regeneration. This review section focuses on various strategies and techniques that have been developed by labs across the world to produce kidneys that could be viable for eventual transplantation. Decellularization/recellularization techniques, organoid formation using pluripotent stem cells, de-novo organ regeneration using xenoembryos, and blastocyst complementation are some of the suggested methods (summarized in Figure 2–1). Decell/recell technology is removing cells from an organ (such as a pig’s kidney) and replacing them with human cells. Organoid formation is growing a new organ directly from cells. Xenoembryos can be used to initially grow human organs in a pig prior to transplantation into a patient. Blastocyst complementation can be used to grow a human organ in a pig, then allow the pig to grow to maturity prior to transplanting the organ into a human. Each of these techniques is further discussed below.

2.1 Decellularization/Recellularization techniques

Patient-specific cells combined with tissue-specific scaffolds could provide a plentiful source of bioengineered organs without the risk of immune rejection. Promising results on the reconstruction of whole rat hearts (12), kidneys (13), livers (14), and lungs (15) have been reported in the last five years, but there are still major challenges that need to be solved. In the following sections, the primary studies on the reconstruction of whole kidneys are reviewed.
2.1.1 Decellularization strategies

The main requirements for an appropriate scaffold in decell/recell technology to regenerate an entire kidney are sufficient mechanical strength to resist the vascular pressure, appropriate morphology, especially the nephron structure, to perform the filtration function, and desired composition of kidney-specific cells or stem cells directed toward migration and differentiation (6, 16). Synthetic polymeric scaffolds were implemented for engineering of the dermis (17, 18),
bladders (19), and blood vessels (20, 21), which are thin enough to obtain nutrients and exchange oxygen through simple diffusion. However, polymer scaffolds would not be applicable to create whole solid organs such as kidneys (22), hearts (23), lungs (24), or livers (25) with complex microstructure and vascular networks. Naturally obtained scaffolds composed of kidney-specific extracellular matrices (ECMs) with intact vasculature and ultrastructure should provide a more appropriate platform for reconstruction of the whole kidney (6, 26, 27).

Generated by an organ’s resident cells, the ECM contains a variety of fibrous proteins (including fibronectin, laminin, elastin and various types of collagen,) and glycosaminoglycans (such as heparan sulfate, chondroitin sulfate, keratin sulfate, and hyaluronic acid), which are highly organ-specific (6). It has been demonstrated that tissue-specific ECMs have a higher potential to support tissue-specific cell growth and differentiation by mimicking the natural \textit{in-vivo} environment. For example, kidney-derived scaffolds improved the \textit{in-vivo}-like properties of renal epithelial cells (HK2) (28) and guided differentiation of human embryonic stem cells to the renal phenotype (29-32). Finesilver \textit{et al.} reported upregulation of kidney-specific genes in HK2 cells when cultured on kidney-derived scaffolds compared to when the cells were grown on tissue culture plastic. Cytokine release of these cells was also improved significantly after growth on kidney ECM (28). Growth and metabolism of kidney stem cells can also be spatially regulated by porcine ECMs. O’Neil \textit{et al.} decellularized biopsies of different regions of porcine kidneys including cortex, medulla, and papilla and formed solubilized ECM, hydrogels of ECM and sheets of ECM. They showed that kidney stem cells had significantly higher metabolic activity when cultured on papilla ECM, which is the niche for stem cells in the renal structure, compared to other regions of the kidney (33). This feature of natural ECMs is of critical importance for highly complicated organs such as kidneys that have more than 26 cell types since the hypothesis is that
the chemical and physical components of the ECM dictate the differentiation of pluripotent repair cells.

Perfusion of native kidneys with detergent solutions through the inherent vasculature removes cellular materials leaving a collagenous structure with an intact vasculature (9, 34-38). Efficient cell removal during decellularization is crucial to avoid the induction of an immune response at the time of transplantation; however, enough care should be taken to keep the composition and structure of ECM intact (7, 39). Freezing/thawing, high flow rate, and high pressure might physically destroy microcapillaries and the ultrastructure of the organ (40). Additionally, detergent solutions generally denature proteins and solubilize crucial components involved in cell growth and differentiation (41). Faulk et al. reported that ionic detergents such as sodium dodecyl sulfate (SDS) and sodium deoxycholate contain negatively charged head groups that can denature proteins, damage collagen fibers, and result in less cell adhesion and proliferation (41).

Due to highly conserved protein sequences across different mammalian species (5, 42), minimal immune response is expected after xenogeneic ECM engraftments (43). Consequently, rat (13, 44-47), monkey (30, 48, 49), and pig (8, 33, 50-53) kidneys are frequently used as representative models for whole renal tissue regeneration studies. In 2009, Ross et al. pioneered the decellularization of whole rat kidneys with 1% Triton X-100, 5mM MgSO₄, 1 M NaCl solutions, and 0.0025-deoxyribonuclease perfusion under constant pressure (approximately 100 mmHg) to achieve an ECM with intact vasculature and collagenous structure. They also showed partial preservation of laminin and collagen IV after complete cell removal (54). In 2012, this group described the decellularization of whole rat kidneys using a combination of SDS and Triton X-100 perfusion. They perfused the ECMS with mouse stem cells and after 14 days, endothelial
cell markers were observed throughout the structure, which was further evidence of ECM potential to support cell growth and differentiation (55). Bonandrini et al. perfused rat kidneys with 1% SDS solution for 17 h to achieve acellular scaffolds with partially preserved collagen IV, fibronectin, and laminin as well as an intact vascular network. They also repopulated their ECMs with murine embryonic stem cells and kidney-specific markers were expressed after three days of cell culture (47). In 2013, Song et al. reduced the decellularization process time to 12 h by 1% SDS perfusion under constant pressure (40 mmHg). They showed that the intact ECM had potential to support proliferation of epithelial and endothelial cells, which consequently resulted in urine-producing kidneys (13).

In 2014, Yu et al. decellularized rat kidneys in 7 h with a combination of 0.1% Triton X-100 and 0.8% SDS. They showed that grafted sections of decellularized ECMs into the partially nephrectomized kidneys improved the healing process and reduced scar tissue formation compared to control nephrectomized kidneys without any scaffold repair. This study is another significant evidence for the regenerative potential of renal scaffolds (45). In another study, Peloso et al. implemented a combination of 1% Triton X-100 and 1% SDS to decellularize rat kidneys under constant perfusion rate. They demonstrated that their fabricated scaffold was able to maintain blood perfusion after in-vivo transplantation (56). Caralt et al. decellularized rat kidneys with three different perfusion patterns including 1% Triton X-100, 1% Triton X-100/0.1% SDS, and 0.02% Trypsin-0.05% EGTA/ 1% Triton X-100. They found that 1% Triton X-100 combined with 0.1% SDS was the best method for both cell removal and preservation of ECM. They also demonstrated that their fabricated ECMs had the capacity to maintain blood perfusion in-vivo with an intact vascular network (44).
In 2012, several groups reported decellularization of whole porcine kidneys using SDS perfusion (8, 50, 57). Sullivan et al. perfused whole porcine kidneys with 0.25% SDS, 0.5% SDS, and 1% Triton X-100 for 36 h. They designed a high-throughput system to automatically decellularize porcine kidneys with intact vasculature and partially preserved components, which supported human cell adhesion and growth. Considering efficient cell removal and retaining intactness of the ECM, they suggested 0.5% SDS perfusion as the best method for decellularization of whole porcine kidneys (8). Orlando et al. also decellularized porcine kidneys with 0.5% SDS in 48 h. They implanted their ECM and showed satisfactory mechanical properties and retained flow rate. However, after 60 min, blood flow was obstructed because of massive thrombi formation (50). In both reported methods, long SDS exposure caused enormous damage to collagen and reduced the amount of intrinsic growth factors in the ECM (41). Discarded human kidneys were also decellularized using 0.5% SDS solution in 48 h (3, 58). Intact microcapillary and ultrastructure retained arterial pressure, and partial preservation of essential components were the critical characteristics of human renal ECMS. A summary of common decellularization methods for whole kidneys is presented in Table 2–1.

### 2.1.2 Recellularization strategies

After producing a scaffold by decellularization, the subsequent step is decontamination of the scaffold before cell seeding. Perfusion with autoclaved DI water or 1X PBS containing high concentrations of penicillin G, streptomycin, and amphotericin B (13, 47, 59), as well as a solution of peracetic acid in 4% ethanol (60), were frequently applied to sterilize intact rat renal ECMS. Decontamination of porcine kidneys is challenging due to their large size compared to rat kidneys and non-aseptic harvesting conditions. Orlando et al. applied 25 kGy gamma irradiation to sterilize
whole porcine kidney ECMs at room temperature (50), while Sullivan et al. suggested 10 kGy gamma irradiation as an efficient method to sterilize the entire porcine kidney ECM (8).

Recellularization of entire intact renal ECMs has been attempted several times (summarized in Table 2–1). In 2009, Ross et al. repopulated whole rat kidneys with GFP-labeled murine embryonic stem cells for the first time. (54). They were able to keep cells proliferating on scaffolds and detected the up-regulation of Pax-2 and Ksp-cadherin after 14 days of cell culture, which was strong evidence for the capacity of ECM to direct differentiation of stem cells. Four years later, in 2013, Song et al. reported a breakthrough in renal tissue regeneration of the bioengineering and transplantation of partially functional rat kidneys. They seeded the rat kidney

Table 2-1. Whole kidney decellularization

<table>
<thead>
<tr>
<th>Species</th>
<th>Predecellularization reagents</th>
<th>Decellularization solutions</th>
<th>Flow condition</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Saline solution containing nitroprusside</td>
<td>Triton X-100, solution of SmM CaCl₂ and SmM MgSO₄, 1M NaCl, DI water, 0.0025% deoxyribonuclease, 0.05% sodium azide</td>
<td>100 mmHg</td>
<td>(54)</td>
</tr>
<tr>
<td>Rat</td>
<td>Heparinized PBS</td>
<td>12 h 1% SDS, 15 min DI water, 30 min 1% Triton X-100</td>
<td>30 mmHg</td>
<td>(13)</td>
</tr>
<tr>
<td>Rat</td>
<td>Saline solution containing nitroprusside</td>
<td>17 h 1% SDS and DI water</td>
<td>0.4 ml/min</td>
<td>(47)</td>
</tr>
<tr>
<td>Rat</td>
<td>50 U/ml heparin in PBS</td>
<td>3 h 0.1% triton X-100, 30 min DI water, 3 h 0.8% SDS, and 1 day DI water</td>
<td>8 ml/min</td>
<td>(45)</td>
</tr>
<tr>
<td>Rat</td>
<td>...</td>
<td>1 h and 40 min DI water, 20 h 1% Triton X-100, 3 h and 20 min 0.1% SDS, and 1 h and 40 min DI water</td>
<td>5 ml/min</td>
<td>(44)</td>
</tr>
<tr>
<td>Rat</td>
<td>...</td>
<td>30 min DI water, 30 min 0.66% SDS, 30 min DI water, 30 min 0.66% SDS, and 1 h DI water</td>
<td>100 mmHg</td>
<td>(59)</td>
</tr>
<tr>
<td>Rat</td>
<td>Cold heparinized saline</td>
<td>1% Triton X-100, PBS, 1% SDS, and PBS</td>
<td>1.2 ml/min</td>
<td>(56)</td>
</tr>
<tr>
<td>Pig</td>
<td>0.9% isotonic solution</td>
<td>12 h DI water, 48 h SDS, and 5 days PBS</td>
<td>12 ml/min</td>
<td>(50)</td>
</tr>
<tr>
<td>Pig</td>
<td>Heparinized PBS</td>
<td>12 h 1% SDS and 1 day PBS</td>
<td>100 ml/min</td>
<td>(51)</td>
</tr>
<tr>
<td>Pig</td>
<td>10 USP units/ml sodium heparin</td>
<td>36 h 0.5% SDS in PBS, 2 days PBS, overnight DNase solution</td>
<td>...</td>
<td>(8, 53)</td>
</tr>
<tr>
<td>Pig</td>
<td>NaCl 8.3g/l, KCl 0.5 g/l, HEPD 2.4 g/l, EGTA 0.95 g/l</td>
<td>3 h Distilled water, 18 h 1% SDS, and 3 h PBS</td>
<td>15 ml/min</td>
<td>(61)</td>
</tr>
<tr>
<td>Pig</td>
<td>Heparinized 1X PBS</td>
<td>7 h 0.5% SDS and 2 days DI water</td>
<td>Increasing flow rate</td>
<td>(40)</td>
</tr>
<tr>
<td>Pig</td>
<td>...</td>
<td>2 h DI water, 28 h 1% SDS, 2 h Triton X-100, and 4 days PBS</td>
<td>10 ml/min</td>
<td>(62)</td>
</tr>
<tr>
<td>Human</td>
<td>...</td>
<td>12 h DI water, 48 h 0.5% SDS, and 5 days DI water</td>
<td>12 ml/min</td>
<td>(3)</td>
</tr>
<tr>
<td>Human</td>
<td>Cold saline</td>
<td>12 h PBS, 48 h 0.5% SDS, 6 h DNase, and 5 days PBS</td>
<td>12 ml/min</td>
<td>(58)</td>
</tr>
</tbody>
</table>
ECMs with human umbilical vein endothelial cells through the artery and rat neonatal cells through the ureter to obtain a urine-producing kidney with improved creatinine clearance (13). In 2014, Bonandrini et al. perfused rat acellular renal ECMs with murine embryonic stem cells through the artery. The cells were metabolically active and proliferative throughout the ECM with the most density in glomeruli structure and peri-tubular capillaries. After 72 h of cell culture, the cells showed expression of kidney-specific proteins as a further proof of ECM effect on directed differentiation of stem cells (47). In an effort to achieve high cell coverage on the tubule side of renal tissue (re-epithelialization), Caralt et al. perfused rat kidney ECMs with renal epithelial cells through the renal artery. They achieved more than 50% cell coverage, and the cells were proliferative with high metabolic activity (44, 60). Regarding human-sized kidney recellularization, there is only one report on re-endothelialization of porcine kidneys. Ko et al., in 2014 enhanced murine endothelial cell adhesion to porcine renal ECM by conjugating a specific antibody to the vascular network, which resulted in reduced blood clot formation after implantation. They also showed that platelet adhesion was significantly decreased by improved endothelial cell adhesion (53).

Perfusing the organs with endothelial cells through the vasculature is a common method for re-endothelializing organs (13, 63-65). However, re-epithelialization is not as straightforward, especially for kidneys with complicated tubular structures. Song et al. introduced epithelial cells through the ureter and used either positive pressure on the ureter or negative pressure on the whole kidney chamber. They suggested that vacuum pressure on the chamber is the only technique to draw the cells to the cortex region (13). In a creative approach, Uzarski et al. perfused the vasculature of rat kidneys with RCTE cells with high pressure in an attempt to push the cells
through the vessel’s basement membrane and repopulate the tubule side. They reported more surface coverage with cells compared to the results of Song et al. (44, 46, 60).

<table>
<thead>
<tr>
<th>Species</th>
<th>Type and number of cells</th>
<th>Method of introducing cells to kidney</th>
<th>Perfusion condition through cell adhesion and growth</th>
<th>Oxygenation of media</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>2×10^6 GFP-labeled murine embryonic stem cells</td>
<td>Manual injection</td>
<td>Controlled pressure in the range of 120/80 mmHg</td>
<td>Tube oxygenator</td>
<td>(54, 55)</td>
</tr>
<tr>
<td>Rat</td>
<td>50×10^6 HUVECs through artery and 60×10^6 rat neonatal kidney cells through ureter</td>
<td>Perfusion of the cells with flow rate of 1 ml/min (vacuum was applied for kidney cells)</td>
<td>Cell adhesion overnight then resuming the perfusion of media at flow of 1.5 ml/min</td>
<td>Tube oxygenator</td>
<td>(13)</td>
</tr>
<tr>
<td>Rat</td>
<td>12×10^6 murine embryonic stem cells</td>
<td>Infusion via syringe pump</td>
<td>24-72 h perfusion at 0.3 ml/min</td>
<td></td>
<td>(47)</td>
</tr>
<tr>
<td>Rat</td>
<td>40×10^6 RCTEs</td>
<td>Perfusion of cells with high flow rate (25 ml/min) and high pressure (232 mmHg)</td>
<td>4 ml/min media perfusion without any adhesion time</td>
<td>Faltered cap on bioreactor</td>
<td>(44, 60)</td>
</tr>
<tr>
<td>Rat</td>
<td>10×10^6 primary human osteoblast</td>
<td>Two step injection through a catheterized artery</td>
<td>No perfusion for 4 h for cell- 24 h perfusion (50 mmHg)-</td>
<td></td>
<td>(59)</td>
</tr>
<tr>
<td>Pig</td>
<td>100×10^6 GFP-labeled endothelial cells (MS-1)</td>
<td>Static seeding followed by ramping perfusion of cells</td>
<td>Perfusion rate at 2 ml/min and gradually increased to 5, 10, and 20 ml/min at 10-12 intervals</td>
<td></td>
<td>(53)</td>
</tr>
</tbody>
</table>

It has been suggested that 3D bioprinting of renal tissue, which includes printing of hydrogels combined with cells to construct the complex structure, could eliminate the complicated procedure for recellularization. However, no study has been reported on this approach for regeneration of whole kidneys (66).

To summarize, the recellularization process of entire intact kidneys is an enormous challenge and despite significant achievements, there are many parameters still requiring consideration and optimization. Optimum cell concentration, cell type, seeding method, perfusion rate, and homogenous cell distribution are some of the unsolved issues in whole kidney regeneration.
2.2 De-novo kidney regeneration through organoid formation

In 1998, Thomson et al. isolated the first human pluripotent stem cell line from the blastocyst, which initiated the excitement about potential uses of stem cells for therapeutic and regenerative medicine (67). Embryonic stem cells (ESCs) are able to differentiate into all cell types of the body; however, ethical issues, non-specificity, and teratoma formation are associated drawbacks (6, 68, 69). Ten years later, in 2006 and 2007, Takashi and Yamanaka successfully created induced pluripotent stem cells (iPSCs) from fibroblast cells (70, 71), which have a majority of the embryonic stem cells’ characteristics without related ethical issues (72, 73). Since then, human iPSCs have been obtained from cardiac cells (74), renal tubular cells present in urine (75), human blood (76), human kidney mesangial cells (77), and adult human adipose stem cells (78). While conquering ethical and political issues, teratoma formation is still one risk of iPSCs (79). Targeted differentiation of pluripotent cells and purification of fully differentiated cells could reduce the risk of teratoma formation (80). Pluripotent cells can theoretically be differentiated into any mature somatic cell types by reconstructing the spatial and temporal conditions of embryonic development. Consequently, detailed information on embryonic development is advantageous in the interest of mimicking the natural milieu (81, 82).

Through embryonic development, three pairs of excretory organs (pronephros, mesonephros, and metanephros) form, but only metanephroi survive for postnatal duration and shape adult kidneys (83, 84). These organs are all from intermediate mesodermal tissue derived from the mesoderm germ layer (85). Adult kidney forms by the reciprocal interaction of two embryonic renal progenitor populations, ureteric bud (UB) and metanephric mesenchyme (MM), procured from intermediate mesoderm (86, 87). UB induces MM differentiation (mesenchymal to epithelial transition) (82) into constructing epithelium of glomeruli and convoluted tubules as well
as vascular and glomerular endothelium (86). Prompted by MM signaling, UB gives rise to the branching structure of collecting ducts and the lower urinary tract system (84, 88, 89). Considering this temporal embryonic pathway and involved transcriptional factors/regulatory modulators, researchers have attempted differentiation of pluripotent stem cells to renal lineage several times (Figure 2–2).

In 2010, Ren et al. cultured murine ESCs on 1% agar-coated plates to induce embryoid body (EB) formation in 2 days. EBs differentiated to mesoderm cells in 7 days in the presence of retinoic acid and activin. Then, renal progenitor cells were obtained by subsequent culturing in conditioned media of UB cells for 10 days (80). Nishikawa et al. also applied stepwise differentiation of mouse ESCs to mesoderm and then renal progenitors using retinoic acid plus A4L and 1:1 mixture of conditioned media of CMUB-1 and MK3 cells (mouse UB and MM cell lines). They also found CD24 and MM specific cell markers useful for purification of differentiated cells (90).

In 2012, Song et al. described the first report on the differentiation of human iPSCs to functional renal podocyte cells. They cultured iPSCs in the presence of Activin A, BMP7, and retinoic acid on 0.1% gelatin-coated plates in 10 days. They showed that the differentiated cells had a morphology similar to mature kidney podocytes with cytoplasmic extensions and tight junction-like structures between adjacent cells. Those iPSCs-derived podocytes had also related functionality such as contractile response to angiotensin II addition and albumin uptake, which were comparable with contractility and permeability of mature human podocytes. They also reaggregated labeled iPS podocytes with embryonic mouse kidneys and cultured the explants for 4 days to confirm successful incorporation of these cells into the correct regions in developing kidneys (91).
In 2013, Lam et al. induced mesoderm phenotype derivation from human iPSCs or ESCs using WNT3A, Activin A, and CHIR. They subsequently obtained the intermediate mesoderm (IM) phenotype using a combination of FGF9 and retinoic acid in 2 days. Tubule marker expressing cells were finally formed using a combination of FGF9 and Activin A in 5 additional days. They suggested that timing and duration of signaling factors were of critical importance to obtain the desired cell fate in their differentiation system (92).
Narayanan et al. examined differentiation of human ESCs on laminin, fibronectin, collagen IV, and matrigel to find the optimum coating for the most efficient differentiation. They suggested matrigel as the optimum platform for the differentiation procedure and, subsequently, induced functional renal proximal tubule cell differentiation from human ESCs by culturing of stem cells in renal epithelial growth medium supplemented with 0.05% FBS, 10 ng/ml BMP2, and 2.5 ng/ml BMP7 on matrigel-coated plates for 20 days. They showed that their differentiated cells had functional characteristics comparable to mature human primary proximal tubule cells (PTCs), such as increased cAMP concentration in response to parathyroid hormone (PTH), γ-glutamyl transferase (GGT) activity, ammonia production, water transport, and integration into the tubule niche of a neonatal mouse kidney in an ex-vivo experiment (93).

Differentiation of polycystic kidney disease patient’s iPSCs into a renal lineage was reported in 2013. Xia et al. optimized the differentiation procedure of human pluripotent stem cells to a renal lineage by 2 days of cell growth in the presence of FGF2 and BMP4 followed by an additional 2 days of exposure in retinoic acid, activin A, and BMP2. They implemented this process for patient specific cells and demonstrated that the differentiated cells could be successfully incorporated into ureteric bud tips in organ culture compared to random localization of undifferentiated patient iPSCs (94).

Reprogramming implies the possibility of direct conversion of one mature cell type to another cellular state. This approach would eliminate the need for dedifferentiation to pluripotency and consequent differentiation to a final phenotype in separate stages (82). Direct reprogramming of fibroblasts to neural cells (95-98) and hepatocytes (99, 100) has already been reported. In 2013, Hendry et al. demonstrated the feasibility of direct adult kidney cell (HK2) reprogramming to a nephron progenitor population. They found six genes through a combinatorial screen that activate
a network of genes representing cap mesenchyme/nephron progenitor cells. However, they reported that expression of these genes was not sufficient to induce epithelial-to-mesenchymal transition, suggesting more required factors (101).

After successful induction of renal lineage from pluripotent stem cells, the next step is to generate a functional tissue capable of blood filtration and urine production. Culturing the cells on a 3D pre-patterned scaffold or mimicking the embryonic environment to induce self-organization of cells would be the two feasible options to create functional tissue. The first evidence of self-organization of pluripotent stem cells was reported as the observation of neural regeneration from pluripotent stem cells (102-105). Self-formation of endoderm-derived organs such as intestine (106), stomach (107), and liver (108) has also been reported in the literature.

The first evidence of potential self-organizing kidneys was described in 1960. Wiess and Taylor isolated single cells of mesonephric kidneys procured from chick embryos and revealed that those cells were able to reform a well-organized renal structure (109). Recently, it was also shown that stem cell-derived renal cells could be successfully integrated with disaggregated embryonic kidney explants and incorporated at the right location (91-93, 110).

Furthermore, stem cell-derived kidney cells were able to form renal structures by small organoid formation without the presence of kidney explants or any pre-patterned scaffolds. Morizane et al. differentiated mouse ESCs to kidney progenitor cells that formed tubule-like structures upon culturing on matrigel. They suggested that this self-organization would be promoted by co-culturing of NIH3T3-Wnt4 cells and differentiated cells for 24 h with significantly increased branched tubular structure (111). In another effort, Taguchi et al. reported differentiation of mouse ESCs and human iPSCs to renal cells capable of reconstructing 3D embryoid bodies including glomeruli with podocytes and renal proximal and distal tubules, which could be
vascularized upon transplantation (112). In an effort to vascularize renal organoids, Rogers et al. and Deker et al. subcapsularly implanted embryonic metanephroi in rat and mice models and obtained partially functional renal tissue (113, 114).

Despite significant improvements in renal organoid formation, these structures remain immature and limited to 2-3 mm in diameter. They are also devoid of vascular networks and passages for urine excretion (86). Consequently, this technology is far from ready for creating a transplantable organ. Kidney organoids also have limited applications in disease modeling, drug screening and regenerative treatment (89).

2.3 De-novo organ regeneration using xenoembryos

The use of mesenchymal stem cells (MSCs) in a xenoembryonic environment is another proposed method for kidney regeneration. Theoretically, this method consists of borrowing the developing xenoembryo of another species and its natural process of stem cell direction and differentiation to grow a new organ using human mesenchymal stem cells injected into the xenoembryo. Similar to using the growth factors and proteins in decellularized ECM, this method of de-novo regeneration borrows the natural signals and growth factors present in a developing xenoembryo. The embryo from a donor animal is used to grow a kidney using the host’s MSCs and then the developing organ is transplanted into the host, where it can grow to maturity. This method is relatively new, and only a few successful experiments have been performed, mainly involving rat or chick xenoembryos and MSCs.

Mesenchymal stem cells, found primarily in bone marrow, but also in kidney and adipose cells, are ideal for renal organogenesis because of their potential to self-renew and their multipotency in differentiation (115). In various studies, human MSCs have shown the ability to
develop into a wide variety of tissues under the proper differentiation conditions, such as adipocytes, cardiomyocytes, and thymic stroma (116). When this process of de-novo renal organogenesis was tested in a whole-organ culture with glial cell-derived neurotrophic factor (GDNF)-expressing human MSCs injected into the early-stage metanephros (the most mature form of the developing kidney), the cells naturally differentiated into various parts of the kidney (tubular and glomerular epithelial cells and interstitial cells) (117). After some time for cell growth and differentiation, this more developed metanephros was then inserted into the omentum of the host species to be able to connect to the vascular system of the recipient. The resulting “neokidney”, composed of a human nephron within a host vascular system, performed several of the functions of a normal kidney, such as producing urine with high concentrations of urea nitrogen and creatinine (suggesting that the urine was produced by hemofiltration, and the neokidney was capable of some level of blood filtration). Additionally, when the host animal showed signs of anemia, the neokidney secreted human erythropoietin (Epo) in response, showing the neokidney also had some capacity for hormone release, and that the hormones were of host origin (118).

The neokidney formed with the GDNF-expressing human MSCs, however, did not form a ureter or ureteric bud (UB). Another experiment was performed using chick xenoembryos to see if human MSCs would form the UB under influence from the proper xenosignals. The cells migrated down the Wolffian duct (WD) and then expressed the LIM-class homeobox gene, LIM1, that is required for the extension of the WD, showing that these cells are able to form the UB when placed in the correct environment (119). This leads to the conclusion that using human MSCs inserted at the right time and in the right place during xenoembryonic development can be used to construct a whole kidney with both the mesenchymal membrane and the ureteric bud. This method
is very promising using renal lineage human MSCs, but it has yet to undergo testing with human iPSCs.

Further experimentation with xenotransplanted metanephroi showed that the metanephros transplanted from rat to mouse, and from pig to cat, expressed EPO of host animal origin (the animal into which the metanephros had been transplanted). These EPO-producing cells were revealed to be derived from circulating host cells, and not from integrated host vessels (from the integrated vasculature) since they did not express endothelial markers, as did the other integrated host cells. These findings led to a possible conclusion that migration and differentiation of the donor cells in a xenotransplanted organ were similar and consistent across species since host cells circulating around the transplanted metanephros were able to be incorporated into the developing organ, even though the organ itself contained xenotissue from another species. With this knowledge, the xenotissue could be eliminated using suicide-inducible donor animals once the developing metanephros is transplanted into the host, leaving only the EPO-producing tissue of host origin. Hence, the metanephros can be used as a niche to differentiate MSCs into EPO-producing tissue, and with a suicide-inducible gene, this tissue can be engineered to consist solely of host cells (115).

The current research in this area is being directed toward attempting this method with larger animals (specifically pigs), because the porcine kidney and the human kidney are extremely similar in volume and functions (119).

2.4 De-novo organ regeneration by blastocyst complementation

Among the many methods for whole organ regeneration, blastocyst complementation has shown a great deal of potential for growing organs in a host culture. This method uses a developing
blastocyst in a host species (that has been compromised so that it cannot grow the organ itself) to
grow a new organ using donor stem cells, with the ultimate purpose of transplanting the organ into
the donor. Since this is a relatively new method, there are a limited number of examples on this

Blastocyst complementation was originally accomplished using embryonic stem cells
(ESCs). For renal regeneration, wild-type mouse ESCs were injected into the blastocysts of Sall-
1-null mice (lacking kidneys), resulting in a metanephros composed entirely of tissue differentiated
from the wild-type ESCs (120). In light of the ethical difficulties surrounding ESCs, and with
iPSCs reducing the likelihood of rejection, a new potential for the blastocyst complementation
method of organ regeneration has emerged. Induced pluripotent stem cells are biologically similar
to ESCs and are derived from the patient’s own cells (117). Using rat pluripotent stem cells (PSCs)
Injected into the blastocyst of a Pdx1⁻/⁻ (pancreatogenesis-disabled) mouse, a rat-mouse chimera
with a functioning rat pancreas was created (121). A similar study in mice with
fumarylacetoacetate hydrolase deficiency succeeded in using iPSCs to grow hepatocytes
developed entirely from the iPSCs. Further analysis showed that these iPSCs were able to produce
mature hepatocytes that would act as a fully functioning liver (122). These examples show that an
unoccupied developmental niche in a developing blastocyst can be used to differentiate pluripotent
stem cells and grow the missing organ, even if the iPSCs are from a different species.

This method was attempted in renal organogenesis with mouse iPS cells in Sall-1-null
(lacking protein necessary to form kidney) mice. This resulted in newborn mice whose kidneys
were composed almost entirely of donor iPSCs. However, after careful analysis of the regenerated
kidney, it was discovered that the renal vascular system with its arterioles was a chimeric structure
demonstrating a mixture of both host and donor cells (118). The chimeric vascular structure leads
to the conclusion that in order to create a kidney composed entirely of donor iPSCs, the host animal would have to be lacking in all of the strains that contribute to the kidney, such as vascular and nervous systems (115). At present, there are no functional methods to create these systems (119).

Another more recent study examined the functionality of blastocyst complementation in pigs. A generation of pancreatogenesis-disabled pigs were created, and their offspring, when given wild-type iPSCs, were able to generate the pancreas. These chimeric pigs had normal levels of glucose at all stages of testing (every several months) and grew and developed normally (123). These chimeric pigs provide another example of the utility of this method in larger animals, more on the scale of humans.

With kidney organogenesis, the chimeric vasculature is the biggest challenge in using blastocyst complementation. There are also many ethical issues surrounding the use of iPSCs in host blastocysts. Still, this method shows a great deal of potential for renal organogenesis.

### 2.5 Comparison of different approaches

The pros and cons associated with different approaches are summarized in Table 2–3. Promising results have been reported in decell/recell technology throughout the last decade. The risk of immune rejection is very low since the collagenous structure is essentially devoid of potential immunogens and iPSCs are patient specific. However, all previous efforts for recellularization of a whole kidney have been limited to rat kidneys, which are far smaller than human-sized kidneys. Repopulation of a human-sized kidney ECM with all kidney cell types to achieve healthy functional kidneys seems relatively impossible, especially considering the complex structure and the number of required cells. That is why the end goal is to use stem cells and have them differentiate to the proper phenotype.
Creation of healthy kidneys through organoid formation is the approach that is currently attracting research funding attention. Since the kidney will be constructed through the embryonic developmental procedure, all crucial cell types will form and locate in the correct niches within the 3D structure of the organ. However, the present kidneys created by this technology are limited to 2-3 mm in diameter without a vascular network or passages for urine excretion. Although several groups have tried to vascularize these structures and increase the size of grown kidneys by providing nutrition through the vasculature, these structures are immature and far from being ready for clinical use.

Building a kidney through xenoembryo formation or blastocyst complementation also has the advantage of creation through embryonic development with all cellular phenotypes localized in the right place. These kidneys are also vascularized and, therefore, are not limited in size. However, there is no guarantee that the final construct is completely composed of patient-specific cells, and the risk of immune rejection is a substantial issue. In addition, ethical issues are also associated with these last two approaches.

Table 2-3. Comparison of different approach in cell-based kidney regeneration

<table>
<thead>
<tr>
<th>Approach</th>
<th>Positive Potential(s)</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decell/Recell</td>
<td>• The use of pre-patterned scaffolds with inherent vasculature</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Easy to connect to vascular system of the host</td>
<td>• Even distribution of cells throughout the whole scaffold and localizing the cells to the right niches is challenging</td>
</tr>
<tr>
<td></td>
<td>• Having the cells grow to full confluence and form corresponding tight junctions is unfeasible</td>
<td></td>
</tr>
<tr>
<td>Organoid formation</td>
<td>• No need to recellularize a whole scaffold</td>
<td>• Limited in size (2 to 3 mm) compared to human sized kidney</td>
</tr>
<tr>
<td></td>
<td>• No risk of immune rejection</td>
<td>• Not vascularized</td>
</tr>
<tr>
<td>Xenoembryo</td>
<td>• No need to recellularize a whole scaffold</td>
<td>• Related ethical issues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hard to connect to vascular system</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• High risk of immune rejection</td>
</tr>
<tr>
<td>Blastocyst complementation</td>
<td>• No need to recellularize a whole scaffold</td>
<td>• Related ethical issues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hard to connect to vascular system</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• High risk of immune rejection</td>
</tr>
</tbody>
</table>
3 OBJECTIVES

The goal of this research was to improve and scale up previous technology for rat kidneys to a whole human-sized kidney using porcine kidneys. To achieve this, we pursued the following specific aims.

Specific aim 1. Characterization of renal tissue scaffold after treatment with various cell-lysing agents to find the best process for decellularization. A variety of cell lysing solutions were studied to remove cellular materials from native scaffolds. The end goal in this study was to apply different solutions and figure out the least damaging cell lysing agents with sufficient cell removal capacity (Chapter 5).

Specific aim 2. Development of improved procedures for decellularizing whole porcine kidney scaffolds. After finding the best process, we designed and constructed an automated bioreactor to efficiently remove all potential immunogens from renal scaffolds (Chapter 6).

Specific aim 3. Development of improved preservation methods for native and acellular renal scaffolds. We characterized native and acellular renal tissue after various freezing/thawing cycles to achieve the best preservation procedure and evaluated protein disturbance during various preservation procedures (Chapter 7).

Specific aim 4. Development of decontamination procedures for whole renal tissue scaffolds. Decontamination is a critical step before recellularization, but it could cause damage to
the collagenous structure of the scaffold. We applied various decontamination procedures and characterized the resulting scaffolds to discover the least damaging approach while effectively removing microbial entities (Chapter 8).

Specific aim 5. Recellularization of the entire porcine renal tissue scaffold with canine renal cells and analysis of the 3D structures of tubules and glomeruli (Chapter 9).
4 MATERIALS AND METHODS

4.1 Materials

Sodium dodecyl sulfate (SDS), Triton X-100, Peracetic acid, ethanol 200-proof, formaldehyde, Tris, EDTA, guanidine-HCl, bovine serum albumin (BSA) and 0.05% Trypsin-EDTA were purchased from Sigma-Aldrich, St. Louis, MO. Proteinase-K was purchased from Qiagen Inc., Valencia, CA. Spectra/Por® 1 RC dialysis membrane was purchased from Spectrum® Laboratory, Rancho Dominguez, CA.

Specific materials for cell culture and cell labeling were purchased from different sources, including Fetal Bovine Serum (FBS) from Hyclone, Logan, UT; 1X Dulbecco’s Modified Eagle Media (DMEM), and 1% Pen/Strep from Gibco by Life Technologies, ThermoFisher Scientific, Waltham, MA; TrypLE Express, and the lipophilic tracer DiI (#V-22889) from Life Technologies, Grand Island, NY.

DNeasy Blood and Tissue Kit was purchased from Qiagen Inc., Valencia, CA. Quant-iT PicoGreen dsDNA assay kit was purchased from Invitrogen Corp., Carlsbad, CA. Sircol Soluble Collagen Assay Kit was purchased from Biocolor Ltd., Newtownabbey, UK. The Blyscan sGAGs Assay Kit was purchased from Biocolor, Ltd. Fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) OmniKine ELISA kits were purchased from AssayBiotech, CA. The resazurin-based PrestoBlue™ reagent was purchased from Molecular Probes, Eugene, OR.
4.2 Equipment

In every set of experiments, the tissue samples were prepared as explained later (in the methods section) and mounted on aluminum stands. A Phillips®/FEI XL30 ESEM™ FEG Environmental Scanning Electron Microscope (Hillsboro, OR) was used to acquire images with a field emission gun (FEG) electronic beam at high vacuum.

Tissue samples were fixed in formaldehyde and mounted on microscope slides with coverslips using SlowFade® Gold Antifade mountant (Molecular Probes™) and imaged using an Olympus™ FluoView FV1000 (Center Valley, PA) laser scanning confocal microscope. Imaging of all stained samples was accomplished using a standard light microscope equipped with a digital camera (Olympus America Inc., Center Valley, PA).

MR images were acquired on a 3 Tesla Siemens Trio MR scanner (Erlangen, Germany) with a 32-channel head matrix coil. The multi-slice two-dimensional gradient echo (GRE) sequence was employed to generate the T2* weighted images. The field of view (FOV) of the scan was 120 mm (readout) x 120 mm (phase) x 1 mm (slice) and the acquisition matrix was 512 (readout) x 512 (phase encode) and 35 in the slices direction which yielded the voxel size equal to 0.23 x 0.23 x 1 mm. Eight averages and phase stabilization was applied and the total acquisition time was about 75 minutes. The ratio of repetition time to echo time (TR/TE) was 1090/10 ms and the flip angle was 25 degrees. Other acquisition parameters were readout bandwidth = 260 Hz/pixel, and the slice distance was 0.2mm.
4.3 Decellularization procedure and equipment

4.3.1 Kidney retrieval

Porcine kidneys were harvested from six-month-old slaughter weight swine at a local abattoir, shortly after exsanguination. The kidneys were removed with special care to ensure that a sufficient length of renal artery was preserved. Heparinized phosphate buffered saline (PBS) solution was perfused into the kidneys through a catheter to prevent thrombosis. The harvested kidneys were then preserved at -20°C until decellularization. After thawing overnight at 4°C, fat was stripped from the renal capsule, excess arterial tissue was excised, and the kidneys were cannulated via the renal artery with white nylon tubing (Value Plastics MTLS210-1, male Luer slip to 200 series barbed coupler, 1/16" tube ID).

4.3.2 Decellularization of slices of renal cortex

Solutions of 0.1 N NaOH (pH 11.8-12) (124), 1% (w/v) peracetic acid (pH 2.6) (125), 3% (v/v) Triton X-100 (pH 7.2), 1% (w/v) sodium dodecyl sulfate (pH 8.1) (41), and 0.05% Trypsin/ethylenediaminetetraacetic acid (EDTA) (126) were made on the day of experiments. Several slices of washed renal tissue (n=10 for each solution, all experiments were repeated 5 times) were submerged in 300 ml of the various solutions for 24 h on a bench top shaker (70-80 rpm) at room temperature except trypsin/EDTA solution, which was placed in a shaker incubator at 37°C to get the optimum activity of enzyme. After 24 h, the tissues were immersed in DI water under mild shaking for 15 min and then the water was changed. This procedure was repeated 10 times to remove residual agents that might be toxic for cell growth and make the treated tissues ready for further experimentation.
4.3.3 Bioreactor and decellularization apparatus for whole porcine kidneys

The bioreactor was a custom-made 2.2 liter jacketed glass vessel (Figure 4–1). The solutions were pumped via a DRIVE MFLEX L/S 1.6-100RPM115V peristaltic pump through 1/8 ID, 3/16 OD, 1/32 wall (NALGENE 8000-0010) tubing into the organs in the bioreactor. The tubing was connected to the kidney through the renal artery for continuous antegrade flow. Bubble traps were employed between the pump and kidneys to avoid introducing any air bubbles. Three solenoid valves controlled by LabVIEW software (National Instruments Corp, Austin, TX) were used to automatically control switching between solutions. A pressure control loop was used to maintain flow pressure through the kidneys to less than 80 mmHg during perfusion. A schematic view of the apparatus is shown in Figure 4–2.

Figure 4–1. Apparatus used for decellularization; (A) bioreactor, (B) bubble traps, (C) lab view program used for controlling sequence of solutions and pressure of the flow rate, (D) peristaltic pump and solenoid valves.
Figure 4–2. Schematic diagram of the decellularization apparatus for improved (left) and control method (right): (1) containers for solutions, (2) solenoid valves to automatically alternate between solutions every 0.5h, (3) peristaltic pump, (4) bubble trap to avoid introducing air bubbles to the renal vasculature, (5) decellularization bioreactor, and (6) LabView program to control perfusion rate and solenoid valves.

4.3.4 Decellularization procedure for whole porcine kidneys

For the control process, kidneys were perfused with a 0.5% solution of SDS for 7 hours for complete decellularization. The process began with low flow rates and the flow was gradually increased during the perfusion while keeping the arterial pressure below the natural diastolic pressure of the body (80 mmHg). After decellularization, the kidneys were perfused with deionized water for approximately 2 days to remove SDS detergent from the ECM. This procedure was regularly applied in our lab to obtain decellularized whole porcine kidneys.

In the improved decellularization method, hypertonic solution (0.5 M NaCl in H₂O) was pumped into the kidneys (n=10) for 30 min to induce crenation and remove remaining thrombus. An SDS solution was then pumped into the organs for 30 min to disrupt cell membranes and begin
the decellularization process. After the initial anti-thrombogenic wash, the process followed a strict repetitive cycle of solutions until a completely white kidney was achieved. The cycle was as follows: 0.5 M NaCl solution (hypertonic solution) for 30 min, then 0.5% w/w SDS solution for 30 min, followed by deionized (DI) water (hypotonic solution) for 30 min. This sequence caused the largest osmotic gradient and lysed cells most effectively. The perfusion flow rate began at 10 ml/min and was incrementally increased every 30 min by 1.5 ml/min to approximately 40-50 ml/min while the flow pressure was controlled with a pressure control loop to make sure that arterial pressure did not exceed the physiological pressure of the kidney vasculature (80 mmHg corresponding to a flow rate of 240-300 ml/min). The kidneys were submerged in DI water throughout the entire procedure.

4.4 Freezing/thawing cycle for preservation of renal tissue

To evaluate the freezing/thawing effect on native and decellularized ECM, several native and decellularized whole kidneys (n=10) were subjected to freezing/thawing cycles as follows: the kidneys were frozen at -20°C for 24 h, then at -80°C for 12 h, then for 24 h at -20°C and finally, they were thawed at +4°C.

4.5 Decontamination methods of slices of renal tissue

Slices of renal cortex (7 mm in diameter and 2 mm in depth) were subjected to four decontamination treatments (n=60 for each method), including γ-irradiation and three decontaminating solutions. These solutions were: 70% (v/v) ethanol in deionized (DI) water, pH: 7.4; 0.2% (v/v) peracetic acid (peracetic acid solution 32% (w/v) in dilute acetic acid, Sigma Aldrich, St Louis, MO) in 1M NaCl aqueous solution, pH: 3.1, which was freshly made just before each experiment; and 0.2% (v/v) peracetic acid in 4% (v/v) ethanol in DI water, pH: 3.0. Slices of
tissues were soaked in 50 ml of the decontaminating solutions under mild shaking for various times (0.5, 1, 2, 3, 4, and 5 h) at room temperature. For γ-irradiation, samples were submerged in DI water in plastic tubes and irradiated at room temperature with intensities ranging from 1 kGy to 10 kGy with a J.L. Shepherd Mark I model 22 self-shielded irradiator. To decontaminate whole porcine dECMs, three whole kidney dECMs that were cannulated through the renal artery were submerged in 3 L of each type of solution. Then, the solutions were perfused through the kidneys with flow rates of 10 ml/min for 1, 2, and 3 h. The treated dECMs were then perfused 5 times with fresh autoclaved DI water to remove any remaining agent.

Samples were aseptically excised from the renal cortex and incubated in cell culture media to check for contamination. The decontaminated samples as well as non-decontaminated controls were washed with autoclaved DI water and were incubated in Dulbecco's Modified Eagle Medium (DMEM, Gibco®) supplied with 10% (v/v) fetal bovine serum (GeneMate FBS, Bioexpress, UT) at 37°C for at least 14 days. The presence of contamination was checked visually under the microscope. The shortest duration of decontamination time or irradiation intensity that produced acceptable samples was used for the remaining tests and comparisons.

4.6 Physical characterization tests

4.6.1 Compression test

To compare the effects of decellularization procedures (control and improved method) as well as freezing on the structural integrity of the extracellular matrix, an Instron 3342 Single Column Universal Testing System and Instron Model 1321 were used to perform compression tests. Samples of the renal cortex (10 mm x 10 mm with a height of 7 mm) were taken from both
the native and decellularized kidneys for compression measurements. The control samples were compressed at a rate of 0.07 mm/sec until a compression force of 45 N was reached. The samples subjected to freezing cycles were frozen and thawed in the process described above and then subjected to the same compression tests. Using the compression data, the elastic moduli were then calculated for each sample at low stress values.

### 4.6.2 Arterial pressure measurement

A simple apparatus was configured to measure the arterial pressure of the native and decellularized kidneys. The kidneys were cannulated through the renal artery and then connected to a peristaltic pump, which drew 1X PBS from a 1000 mL graduated cylinder. The pump was then set to the lower limit of 10 rpm. The pressure observed was recorded manually, and the flow rate was calculated by determining the time required to drain 10 mL from the graduated cylinder. The peristaltic pump speed was then incrementally increased by 5 rpm and the process was repeated. The pump speed was increased until it reached 70 rpm while maintaining less than the maximum pressure (< 80 mmHg).

### 4.6.3 Swelling test

Water absorption was measured to determine the structural integrity and swelling properties of the ECM after decontamination with various agents. Lyophilized samples treated via the four decontamination methods were weighed and submerged in 1X PBS. The amount of absorbed water was measured every hour for 6 h and the swelling ratio was calculated as \( \frac{w-w_{\text{initial}}}{w_{\text{initial}}} \).
4.6.4 Fourier Transform Infrared Radiation (FTIR) Spectroscopy

Decellularized renal cortex samples either after decellularization or after decontamination with different agents were excised, pulverized while submerged in liquid nitrogen, and lyophilized. Background spectra were collected using a Smart Orbit single-reflection, diamond ATR accessory (Thermo Scientific) in a Nicolet 6700 FTIR Spectrometer (Thermo Scientific). Then 100 mg of lyophilized tissue powder was applied to the crystal surface. A consistent maximum pressure was applied to the tissue sample using an adjustable pressure tower. The resulting IR spectra were a composite of 20 scans imaged from 4000 cm\(^{-1}\) to 400 cm\(^{-1}\) wavenumbers. The absorptions represented a tissue penetration depth of 2.0 micrometers at 1000 cm\(^{-1}\). An ATR correction was performed using OMNIC software. Peak assignments were made using integrated library searches within the OMNIC software.

4.6.5 Scanning electron microscopy (SEM)

Five cubic mm samples were taken from the cortex tissue of native kidneys and treated tissues in each set of experiments. Subsequently, the samples were soaked in 2% glutaraldehyde in Millonig's phosphate buffer (MPB) at pH 7.3 for 24 h at 4°C. Samples were then exposed to a buffer wash process involving six steps of 15 min washes in MPB (pH 7.3) on a lab shaker. Samples were then placed in a cryoprotectant consisting of 25% sucrose and 10% glycerol in a 0.5 M PBS solution for 2 h. After having replaced the solution with fresh cryoprotectant, samples were flash frozen and fractured under liquid nitrogen. The buffer wash procedure was repeated, followed by 1.5 h in a 1% OsO\(_4\), MPB (pH 7.3) solution. Samples were then dehydrated using graded (10, 30, 50, 70, 95, 100 %) ethanol baths. Each sample was submerged for 15 min up to the 70% concentration ethanol solution and left overnight at 4°C. The dehydration process was completed inside critical dryer baskets with 70%, 95% and three steps of 100% ethanol solution baths.
Samples were then placed in 100% ethanol in a CO₂ critical point dryer and subsequently mounted on aluminum stands. Samples were sputter-coated with 15 nm of a Gold-Palladium (Au-Pd) alloy before imaging with a scanning electron microscope (Phillips/FEI XL30ESEMSEG, Hillsburrow, OR). Fibers in 40,000X magnified images were characterized using DiameterJ (NIH) software (127). Briefly, 5 random images of each sample were chosen and segmented (the image was partitioned into multiple sets of pixels to make it easier to analyze) using Segment Mixed plugin to delineate the boundaries of the fibers. The most appropriate segmented images were selected and characterized by DiameterJ 1.011 plugin and the final results of fiber diameter and intersection densities were averaged for each sample.

4.6.6 Magnetic Resonance Imaging (MRI) of native and decellularized porcine kidneys

MR images were acquired on a 3 Tesla Siemens Trio MR scanner (Erlangen, Germany) with a 32-channel head matrix coil. The multi-slice two-dimensional gradient echo (GRE) sequence was employed to generate the T2* weighted images. The field-of-view (FOV) of the scan was 120 mm (readout) x 120 mm (phase) x 1 mm (slice) and the acquisition matrix was 512 (readout) x 512 (phase encode) and 35 in slices direction which yielded a voxel size equal to 0.23 x 0.23 x 1 mm. Eight averages and phase stabilization were applied and the total acquisition time was about 75 minutes. The ratio of repetition time to echo time (TR/TE) was 1090/10 ms and the flip angle was 25 degrees. The readout bandwidth was 260 Hz/pixel, and the slice distance was 0.2 mm.
4.7 Chemical characterization of ECM

4.7.1 DNA isolation and quantification

Residual DNA was extracted from native, decellularized, or recellularized tissues using the high salt method or Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). In high salt method, approximate 4-5 mg of lyophilized native and treated renal tissues were incubated in a solution of 600 µl TNES buffer and 35 µl Proteinase-K (Qiagen Inc., Valencia, CA) at 50ºC overnight. TNES buffer contained 50 mM Tris (Sigma-Aldrich, Saint Louis, MO), 0.4 M NaCl, 100 mM EDTA (Sigma-Aldrich, Saint Louis, MO), and 0.5% SDS with the pH adjusted to 7.5. Subsequently, 166.7 µl of 6 M NaCl were added to each sample and after 20 seconds of vigorous shaking, the samples were centrifuged at 12,000 rpm for 5-10 min. The supernatants were collected for DNA quantification.

Quantification of extracted DNA was performed using Quant-iT PicoGreen dsDNA assay kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s instructions. Briefly, PicoGreen reagent was added to each sample, and the fluorescence of all samples was determined using a Synergy 2 Multi-Mode Microplate Reader (Biotek Instruments, Winooski, VT). Gel electrophoresis for DNA extract loaded on 1% agarose gel was performed to characterize the residual DNA in the structure of ECM.

4.7.2 Collagen and GAGs isolation and quantification

Collagen and sulfated glycosaminoglycans (sGAGs) levels of renal tissue after various treatments were examined using the Sircol Soluble Collagen Assay Kit (Biocolor Ltd., Newtownabbey, UK) and the Blyscan sGAGs Assay Kit (Biocolor, Ltd), respectively. For
collagen determination, approximately 3 mg of dry samples were solubilized for 3 days in acid/pepsin solution at 4°C. Then, Sircol Dye Reagent was added to each sample to form a collagen-dye complex. After solubilizing the dye-reagent complex with Alkali reagent, the absorbance of samples was measured at 555 nm. For sGAGs measurements, each sample was incubated at 65°C in papain extraction solution for 3 h. After achieving a clear solution, Blyscan dye reagent was added to all samples to precipitate the sGAGs-dye complex. Then, dissociation reagent was added to dissolve the complex and absorbance was measured at 659 nm using a Synergy MX (Biotek Instruments, Winooski, VT) microplate reader.

4.7.3 Growth factor extraction and quantification

Total growth factors were extracted from native and any kind of treated tissue according to a previously established protocol (128). A total of 20 mg of lyophilized fine powder of different samples were placed in microcentrifuge tubes, and 1.5 ml of extraction solution was added to each tube. Spectra/Por® 1 RC dialysis membrane (Spectrum® Laboratory, Rancho Dominguez, CA 90220) was then placed over the tube openings and secured with melted out tube caps as previously described (129). Then the extraction was performed by passive dialysis against distilled water at 4°C for 24 h under mild shaking. Consequently, the tubes were centrifuged at 12,000 rpm and supernatant was used for growth factor quantification tests. The extraction solution included 0.05 mol/L EDTA (Sigma-Aldrich, Saint Louis, MO), 4M guanidine-HCl (Sigma-Aldrich, Saint Louis, MO), 30 mM Tris (Sigma-Aldrich, Saint Louis, MO), and 1 mg/ml bovine serum albumin (Sigma-Aldrich, Saint Louis, MO). Several protease inhibitors were also added to extraction solution including 5 mM benzamidine-HCl (Sigma-Aldrich, Saint Louis, MO), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, Saint Louis, MO), and 0.1 M aminocaproic acid (Sigma-Aldrich, Saint Louis, MO).
Human FGF and HGF were quantified and normalized to dry weight of lyophilized samples using OmniKine ELISA kit (AssayBiotech, CA) according to the manufacturer’s instructions. Briefly, 100 µl of samples and standard solutions were incubated at room temperature in 96 well plates coated with growth factor antibodies. Then, the unbound substances were removed and enzyme coupled secondary antibodies were added. After further washing and incubation with a color developing solution, the color reactions were stopped and optical density was recorded at the proper wavelength (450 nm and 540 nm). All experiments were performed in triplicate, and the reported results are Average±SD.

4.7.4 Histology and immunohistochemistry

Renal cortex biopsies of native and decellularized kidneys after various treatments were excised and fixed in 4% paraformaldehyde overnight and then partially dehydrated in 30%, 50%, and 70% ethanol prior to complete dehydration in a graded alcohol series. Afterward, samples were immersed in xylene and embedded in paraffin. Samples in paraffin blocks were sectioned with a microtome into 5 μm slices and placed on slides. The sectioned samples were dehydrated and deparaffinized, then stained either with standard hematoxylin and eosin (H&E, Thermo Scientific, Pittsburgh, PA), Orcein (O7380; Sigma, St. Louis, MO), Picro-Sirius Red stain (Direct Red 80, Sigma-Aldrich, Saint Louis, MO, #365548), or IHC WORLD NovaUltra Alcian Blue/PAS Staining Kit (IHC WORLD Inc. Woodstock, MD) according to the manufacturer’s instructions. The aforementioned stains were used to detect cellular material as well as other proteins, elastin, and general collagen in the structure of the ECM. Staining of sectioned native and decellularized samples with Safranin O and Fast Green was also performed to detect the presence and distribution of proteoglycans.
For immunohistochemistry of collagen IV, the sectioned samples were dehydrated and deparaffinized and then antigen retrieval was performed via a citrate buffer method (130, 131). The slides were incubated with the appropriate primary and secondary antibodies that use HRP conjugation using a Vector Elite Kit (Vector Laboratories; Burlingame, CA). The applied antibody was anti-collagen IV (1:500, Abcam, Cambridge, MA, ab6586). Imaging of all stained samples was accomplished using a standard light microscope equipped with a digital camera (Olympus America Inc., Center Valley, PA) at magnifications of 20X and 40X.

4.8 Cell culture

Madin-Darby Canine Kidney (MDCK) epithelial cells and mouse endothelial cells (MS-1) (provided by Feinberg school of Medicine, Northwestern University, Chicago, IL) were used for recellularization purposes. These cells were grown in T-75 flasks under regular cell culture conditions in 1X DMEM (Dulbecco’s Modified Eagle Media, Gibco by Life Technologies) containing 10% Fetal Bovine Serum (FBS; Hyclone, Logan, UT) and 1% Pen/Strep (Gibco by Life Technologies) at 37°C in 5% CO2 environment and were detached for passaging using TrypLE Express (Life Technology, Grand Island, NY).

RAW cell line (TIB-71™; American Type Culture Collection (ATCC), Manassas, VA) was also grown to perform macrophage-stimulating assay. This test was performed to examine immunogenicity of ECM. These cells were cultured in six-well plates in DMEM 1X (Dulbecco’s Modified Eagle Media, Gibco by Life Technologies) containing 10% FBS (Hyclone, Logan, UT).
4.9 Macrophage-stimulating assay

To evaluate the immunogenic potential of decellularized ECM, we stimulated macrophage cells with different samples and determined the activation of macrophages by nitric oxide concentration in cell culture media. The vacuum-dried acellular matrices and native kidneys were crushed with a mortar and pestle while they were submerged in liquid nitrogen to achieve a fine powder. RAW cell line, which is a neutral mixture of different macrophage cells, were cultured in six-well plates and when 70% confluence was achieved, 20 mg of powdered sample were added to each well. Vacuum-dried bacterial extract was used as a positive control and a well with no powder as a negative control. After 20 h incubation at 37°C, the supernatants were centrifuged and nitric oxide content was measured using a Griess Reagent Kit (Life Technology, Grand Island, NY) according to the manufacturer’s instructions.

4.10 Various tests to examine cultured cell behavior

4.10.1 Labeling renal epithelial cells (MDCK) with fluorescent color

Renal epithelial cells (MDCK) were grown to full confluence in T-75 flasks and were detached on the day of experiment using TrypLE Express (Life Technology, Grand Island, NY). The suspended cells were labeled with the lipophilic tracer Dil (#V-22889, Life Technologies, Grand Island, NY). The detached cells were suspended in 1X DMEM containing 5 μM SP-DilC18 and incubated at 37°C for 20 min. Subsequently, the cells were washed three times prior to seeding onto the scaffold.
4.10.2 Labeling renal epithelial cells (MDCK) with iron oxide particles

The cells were exposed at 70-80% confluence to cell culture media containing 20, 40, and 60 µg/ml magnetic nanoparticles of iron oxide (II, III) (#725358, Sigma-Aldrich, St. Louis, MO) for 4 and 24 h. Subsequently, the media was removed, and the cells were washed with sterile saline solution. The optimum concentration and time exposure was applied for further cell labeling and MRI imaging.

4.10.3 Live/Dead assay to check cytotoxicity of ECM

Small pieces of decellularized tissue were sterilized and incubated in DMEM with 10% FBS and 1% Pen-Strep (Gibco®) at 37°C and 5% CO₂ in 24 well plates. The growth medium was renewed after 2 days and MS-1 cells (1×10⁵) were added to each well. The growth medium was changed every 2 days and after 3 days of cell culture, tissue samples were taken out of the wells and Biotium® Viability/Cytotoxicity Assay (Hayward, CA) was performed for cells adhered to culture plates. The viability of the cells was imaged using a FLoid® Cell Imaging Station (Grand Island, NY).

4.10.4 Cell culture on decellularized tissues

Small pieces of treated tissues (7 mm in diameter and 2 mm thick) were excised using a biopunch tool (TED PELLA, Inc. Redding, CA) and incubated in 70% ethanol solution containing 1% Pen-Strep (Gibco by Life Technologies, ThermoFisher Scientific, Waltham, MA) and 2.5 µg/ml Amphotericin B (Sigma-Aldrich, Saint Louis, MO) at room temperature for an hour. The tissues were then washed with autoclaved DI water to remove residual ethanol and rehydrated in DMEM supplemented with 10% FBS and 1% Pen-Strep for at least 2 h before cell seeding in a 96 well plate.
Subsequently, the rehydration media was removed and approximately $2.8 \times 10^4$ labeled canine renal epithelial cells suspended in culture medium were added to each sample ($n=6$ for each treated tissue). Cell adhesion and growth were continued for 4 days at 37°C in a 5% CO$_2$ environment. Representative fluorescence images were obtained after 4 days using an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, NJ).

### 4.10.5 Resazurin reduction assay

Resazurin reduction assay was performed every day for cells adhered to treated tissues to examine the viability and metabolic activity of MDCK cells on porcine treated ECM. The culture medium was removed from each well, which contained pieces of treated tissue with cultured cells and 100 µl of media containing the resazurin-based PrestoBlue$^\text{TM}$ reagent (Molecular Probes, Eugene, OR) was added to wells at a resazurin/media ratio of 1:20 (v/v) and the samples were incubated at 37°C for 1 h. Fluorescence of resazurin-containing media was recorded using a Synergy MX (Biotek Instruments, Winooski, VT) microplate reader at 560/590 nm excitation/emission and sensitivity of 50.

### 4.10.6 Gene expression study using quantitative real-time PCR

For studying gene expression, total RNA was extracted from reseeded tissues and cells cultured on tissue culture plastic according to the Sambrook method (132). cDNA was synthesized using SuperScript$^\text{TM}$ IV Reverse Transcriptase (Life Technologies) according to the manufacturer’s instructions, and the quality and quantity of products were measured by a Nanodrop spectrophotometer (BioTek's Synergy$^\text{TM}$ Mx microplate reader). qRT-PCR was performed in Roche LightCycler® 480 using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA) and gene-specific primers (Table 4–1). Results were normalized
based on the housekeeping gene elongation factor 1-alpha (EF1-a) and calculations were performed using the relative quantification method) to compare gene expression levels among different samples.

Table 4-1. Primer sequences

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<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
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<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-CATCCAGGTCATACTCGGT G-3'</td>
</tr>
<tr>
<td>CDH16</td>
<td>Forward</td>
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<tr>
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<td>5'-CAAAATCGAGAGCCACA-3'</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TGTGGCAATCAAATACGAGG-3'</td>
</tr>
</tbody>
</table>

4.11 Whole porcine kidney recellularization

4.11.1 Recellularization bioreactor design and construction

Two Nalgene wide-mouth straight-sided polymethylpentene (PMP) jars (500 ml and 1000 ml, Thermo Scientific, Waltham, MA) were connected through cannulations on sidewall. Three white polypropylene adaptors (Value Plastics MTLS210*1, male Luer slip to 200 series barbed coupler, 1/16” tube ID) were inserted in the cap of the 1000 ml jar (main container) to provide connections for artery, ureter, and vacuum line. Four filtered caps were also attached to the 500 ml jar to oxygenate the cell culture medium (media container). The main container was equipped with a handheld digital manometer (HHP-100G, OMEGA, Stamford, CT) and connected to a vacuum pump. Two Luer-lock valves were also inserted on the artery and ureter lines of the main
container for injection of cells into the renal ECM. Silicon rubber tubing was used to connect the containers to the peristaltic pumps. The adaptors were sealed in place with STIK’N SEAL® outdoor adhesive and Epoxy instant mix™ (LOCTITE, Hankel Corporation, Westlake, OH). Various parts of the system were designed to be sterilized via autoclave and fit in an incubator. A schematic diagram of the recellularization bioreactor is shown in Figure 4–3.

4.11.2 Whole kidney ECM decontamination and preparation for recellularization

After two days washing with DI water, the renal ECMs (n=60) were perfused with solution of 70% ethanol in autoclaved DI water containing 1% Pen-Strep (Gibco by Life Technology) and 2.5 µg/ml Amphotericin B (Sigma-Aldrich) for 2 h in a laminar flow hood, and washed with autoclaved DI water containing antimicrobial agents. Subsequently, the sterile ECM was aseptically moved to the autoclaved bioreactor and perfused with cell culture medium at 37ºC at least for 2 h prior to introducing cells to the ECM.

4.11.3 Renal kidney cell growth and recellularization of whole renal ECM

Madin-Darby Canine Kidney (MDCK) epithelial cells (provided by Feinberg School of Medicine, Northwestern University, Chicago, IL) were grown in multi-layer flasks (Nest Scientific, Rahway, NJ) in 1X DMEM (Dulbecco’s Modified Eagle Media, Gibco by Life Technology) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone, Logan, UT) and 1% Pen-Strep (Gibco by Life Technology) at 37ºC and 5% CO₂ environment. On the day of the experiment the MDCKs were detached using TrypLE Express (Life Technology, Grand Island, NY) and 600×10⁶±50×10⁶ cells were suspended in 120 ml, 60 ml, and 6 ml of cell culture medium depending on the desired concentration of cells for recellularization.
Figure 4–3. Schematic diagram of recellularization apparatus (A) and whole renal ECM reseeding strategies (B-D). Injection of cells through artery with high flow rate and pressure (B), Injection of cells through ureter with high positive pressure (C), Injection of cells through ureter while the kidney chamber was under vacuum (D).

*Arterial reseeding*: the cell solution was injected through arterial Luer valve via a Luer-lock syringe and media perfusion was resumed immediately with 70-80 ml/min flow rate and
continued for 30 min. Subsequently, the media perfusion was continued for 7 days with 5 ml/min flow rate in an incubator at 37°C and 5% CO₂ environment.

**Ureteral reseeding with high positive pressure:** the cell solution was injected through ureteral Luer Valve via a Luer-lock syringe and media was perfused through ureter with 40 ml/min flow rate for 30 min. Subsequently, arterial perfusion of cell culture medium was resumed with 5 ml/min and was continued for 7 days.

**Ureteral reseeding under vacuum pressure:** the cell solution was injected through the ureter while the kidney chamber (main bioreactor) was under 140, 70, and 40 mmHg vacuum depending on the desired vacuum pressure for the ongoing experiment. After cell injection, the vacuum was released and arterial medial perfusion was resumed at 5 ml/min and continued for 7 days.

During 7 days of cell growth the cell culture medium was changed every other day. At day 7, the ECM was taken out and random samples of medulla and cortex regions were excised and prepared for H&E staining as described above.

### 4.11.4 Cell labeling and 3D imaging of recellularized ECM

The optimized reseeding method was applied using fluorescently labeled MDCKs to track cells in ECM structure using 3D imaging. On the day of recellularization the detached cells were suspended in 1X DMEM and labeled with SP-DiOC18 (Life Technologies). The recellularization of whole porcine ECM with labeled cells was performed via ureteral injection while 40 mmHg vacuum was applied to the kidney chamber. After 7 days of cell growth, random slices of renal cortex and medulla were excised and fixed in 4% PFA overnight. Images of the 3D structure of
recellularized ECM were obtained using an Olympus™ FluoView FV1000 (Center Valley, PA) laser scanning confocal microscope.
5 THE IMPACT OF DECELLULARIZATION AGENTS ON RENAL TISSUE EXTRACELLULAR MATRIX

5.1 Introduction

This chapter is an adaptation of the article entitled “The impact of decellularization agents on renal tissue extracellular matrix” published in October 2016 in the *Journal of Biomaterials Applications*. This work was developed and led by myself with the help of Lara B Schaumann, Evan M Buckmiller, Nima Momtahan, Jason R Gassman, Ho Hin Ma, Dr. Beverly L Roeder, Dr. Paul R Reynolds, and Dr. Alonzo D. Cook.

Various physical (*e.g.* snap-freezing, mechanical agitation, sonication (6)) and chemical methods can be employed to lyse cells. Alkaline (133, 134), acidic (135, 136), ionic (137, 138) and non-ionic (10, 139) detergents combined with protease enzymes have been widely used to decellularize a variety of tissues and organs. To our knowledge, prior to this work there was no complete investigation of the impact of different decellularization agents on renal tissue ECM. Furthermore, there was no comparison study on a broad range of solution’s effects on basement membrane of natural matrices.

In this study, alkaline and acidic solutions, ionic and non-ionic detergents as well as protease enzymes were employed to decellularize slices of the porcine kidney cortex. Cell removal efficiency, physical and chemical intactness, surface chemistry, and the decellularized ECM’s
potential to support human cell growth were evaluated after treatments to achieve the optimum
decellularization with the least damage to the extracellular matrix of the renal tissue scaffold.

5.2 Experimental design

Slices of renal cortex were excised and decellularized as described in detail in section 4.3.2. Decellularized tissues obtained from different methods were characterized to investigate the impact of various cell lysing agents on renal tissue. Standard H&E staining, Sirius Red, and Orcein elastin staining were performed to check the structural integrity and cell removal as described in section 4.7.4. Residual DNA (section 4.7.1), Collagen (section 4.7.2), GAGs (section 4.7.2), and growth factor (section 4.7.3) contents of acellular samples were quantified to compare the various agents in terms of cell removal and intactness of the ECM. Scanning Election Microscopy (SEM) of native and acellular samples were obtained to compare the ultrastructure of scaffolds as described in section 4.6.5. Surface chemistry of various samples were examined using FTIR according to the protocol in section 4.6.4. Finally, the renal epithelial cells were labeled with fluorescent colors (section 4.10.1) and cultured on slices of renal tissue (section 4.10.4). The metabolic activity of seeded cells was measured using resazurin reduction assay (section 4.10.5).

5.3 Results

Decellularization of slices of porcine renal cortex with a broad range of lysing agents (including basic/acidic solutions, ionic/non-ionic detergent solutions, and protease enzyme) was performed to examine cell removal efficiency as well as the ECM’s structure and potential to support cell growth. The conditions of experiments were chosen according to frequently used protocols, and all comparisons were performed for the tissues excised from the same kidney to eliminate the inherent variability between different kidneys.
5.3.1 Characterization of decellularized slices of renal cortex

Gross anatomy of slices of tissue before and after decellularization are shown in Figure 5–1-A. All solutions turned the kidney ECM scaffold completely white except the trypsin/EDTA solutions. The trypsin-treated tissue was yellowish and became soft, showing evidence of disruption of structural integrity and loss of mechanical strength. H&E staining of treated tissues demonstrated complete removal of cellular remnants in NaOH- and SDS-treated samples with preserved collagenous structure. However, a few cell nuclei could be observed in Triton X-100-treated samples. Peracetic acid treatment led to preserved collagenous constructs with partial damage to the cells, but the cellular remnants were mostly retained in the structure. Trypsin-treated samples also retained cellular material with significant damage to the collagenous structure of the tissue.

Remaining residual DNA, which is one of the principle components to induce immune rejection (7, 35), was quantified in all treated tissues. Residual DNA was isolated from treated and untreated tissues by proteinase K solution and quantified using PicoGreen quantification assay. DNA content of native kidneys was approximately 3,900 ng/mg of dry tissue (140), which was reduced to less than 700 ng/mg for all of the decellularization procedures, with NaOH reaching 98% removal (Figure 5–1-B). Peracetic acid and trypsin were not as efficient in removing residual DNA from the native tissue. The 0.1N NaOH solution was the only effective method to reduce the residual DNA close to the immunogenic threshold suggested for residual DNA (50 ng/mg of dry tissue) (35).

A histologist, blind to the identity of the samples, examined H&E stained samples to evaluate the structural integrity of the tissue after treatment with the various solutions. The averages of the structural scores assigned to each type of sample are reported in Figure 5–1-C.
Scores were assigned with 5 for the least damage to 1 for the most damage to the general structure, tubule, and glomeruli of the ECM. NaOH, detergents, and enzyme decellularization caused similar disruption to the structure of the tissue; while peracetic acid treatment resulted in the least damage to the structural integrity of the tissue.

Figure 5–1. Decellularization of slices of renal tissue with various cell-lysing agents (n=10). (A) Gross anatomy and H&E staining of native and decellularized renal cortex showed complete cell removal only for NaOH and SDS treated tissues; (B) DNA content of decellularized ECMs (n=3), (C) The scoring system was based on preservation of the microscopic architecture of decellularized ECMs examined by a histologist blinded to the applied treatments (n=6). Score 5 is for the least damage and score 1 for the most: 5) No structural damage, 4) Outline visible/minimal damage, 3) Outline visible/moderate damage, 2) Outline visible/marked damage, and 1) No outline visible marked damage. All reported results are Ave ± SD. SDS: Sodium dodecyl sulfate, PAA: Peracetic acid.
5.3.2 Physicochemical preservation of the ECM

One of the end goals in native tissue decellularization is intactness of structure and crucial components to enhance the most potential of cell function in the recellularization process. Sirius Red stain was used to detect general distribution and preservation of collagen throughout the ECM (Figure 5–2). Collagens are the major proteins of the ECM that should be retained in their natural conformation with exposed RGD (Arg-Gly-Asp) sequences to facilitate cell adhesion and growth. NaOH- and detergent-treated samples showed the partially preserved collagenous structure with increased void fraction due to cell removal. Peracetic acid-treated tissues had an almost intact collagenous structure as could be observed in native tissue with residual cellular materials (yellow stain). Trypsin-treated ECM had the most disrupted collagen structure due to protease properties of trypsin.

Elastin is another fibrous protein that provides essential mechanical properties of tissues. The dark brown color from Orcein staining of treated tissues demonstrated partial preservation of elastin fibers. However, a darker color could be observed in peracetic acid-treated samples compared to native tissues that might be due to partial cell removal and more exposed elastin fibers for staining. Additionally, elastin fibers in the glomerulus that contribute to stability and strength of capillaries could not be observed in native kidneys because of cell coverage. These elastins were visible, however, in peracetic acid-treated tissue.

5.3.3 Ultrastructure and fiber characterization

The ultrastructure is the crucial physical characteristic required for the filtration function of renal tissue. Thousands of nephrons, small filtration units in the renal cortex and medulla comprised of glomeruli, Bowman’s capsules, distal and convoluted tubules, perform this primary
function. These structures were retained through the decellularization step as can be observed in all stained samples.

Figure 5–2. Distribution of essential components and collagen fibers throughout the decellularized ECMs. EDTA: ethylenediaminetetraacetic acid, SDS: Sodium dodecyl sulfate, SEM: scanning electron microscopy.

Surface fiber arrangement is also essential for cell adhesion and growth (141). SEM imaging of treated samples was performed to examine fiber topography on the surface of ECM. Peracetic acid and Triton X-100 treatment preserved fiber topography in its natural form as in native tissue; however, exposure to NaOH and SDS resulted in more tightly compacted and amorphous fibers on the ECM’s surface. The average fiber diameters were found to be in the range of 23-28 nm, with no significant difference among various treatments, which were close to native tissue fibers (28-30 nm).
5.3.4 Essential components of ECM

Soluble collagen, GAGs, and FGF were extracted and quantified via corresponding quantification assays. All decellularization methods resulted in more than 90% collagen preservation except SDS and trypsin solutions (Figure 5–3-A), which caused substantial damage to the ECM structure by 50% and 64% collagen elimination, respectively.

Glycosaminoglycans (GAGs) are crucial components of ECMS that bind growth factors and keep water inside the ECM resulting in gel-like properties and biocompatibility of the ECM. Alkaline and trypsin treatments caused the most GAGs removal amongst all treatment methods by removing more than 75% and 58% of intrinsic GAGs of native tissue, respectively (Figure 5–3-B). Ionic and non-ionic detergents retained more GAGs compared to other treatment methods by preserving more than 65% of inherent GAGs.

FGF is one of the major growth factors required for cell growth and metabolism. The Triton X-100 solution retained more than 86% of native tissue FGF (Figure 5–3-C). Among all treatments, SDS exposure removed the majority of FGF, only preserving 16% of endogenous FGF. NaOH and peracetic acid solutions were the next two most detrimental methods, preserving 33% and 53% FGF respectively.

5.3.5 Surface chemistry of treated tissues

Fine powder of treated tissues was obtained by submersion in liquid nitrogen, milling, and lyophilization of samples. Surface chemistry of these lyophilized powders was examined via ATR-FTIR (Figure 5–4). All samples showed relatively similar surface chemistry with the same band for different functional groups. Amide I, II, and III bands are the major functional groups for cell adhesion to the ECM (142). No detectable shift in these bands was observed for any of the
treatment methods except Trypsin/EDTA, confirming no further denaturation as a result of exposure to decellularization solutions, which is in complete agreement with previous studies (143). The Amide III shift observed for trypsin-treated tissue was most likely due to collagen denaturation that happened as a result of extended exposure to trypsin at 37 °C.

Figure 5–3. Essential components of native and treated tissues. (A) Soluble collagen content (n=3); (B) Glycosaminoglycan (GAGs) content (n=3); (C) Fibroblast growth factor (FGF) content (n=3). All reported results are Ave ± SD. NS = no significant difference (p-value>0.05) and ** means significantly different (p-value<0.05) from native tissue.
5.3.6 Surface chemistry of treated tissues

Fine powder of treated tissues was obtained by submersion in liquid nitrogen, milling, and lyophilization of samples. Surface chemistry of these lyophilized powders was examined via ATR-FTIR (Figure 5–4). All samples showed relatively similar surface chemistry with the same band for different functional groups. Amide I, II, and III bands are the major functional groups for cell adhesion to the ECM (142). No detectable shift in these bands was observed for any of the treatment methods except Trypsin/EDTA, confirming no further denaturation as a result of exposure to decellularization solutions, which is in complete agreement with previous studies (143). The Amide III shift observed for trypsin-treated tissue was most likely due to collagen denaturation that happened as a result of extended exposure to trypsin at 37 °C.

5.3.7 Growth and metabolic activity of MDCKs cultured on treated ECMs

Small pieces of treated tissues (7 mm in diameter) were seeded with labeled canine renal cells. After 4 days of cell growth in culture conditions, confocal microscopy was implemented to examine cells adhered to the ECM (Figure 5–5-A). Renal cells were attached to all treated ECMs and grew almost to full confluence. These results were further evidence for the potential of porcine ECM to support renal cell adhesion and growth.

Cells must remain metabolically active to perform their primary function. Resazurin-based reagents were used to track the metabolic activity of cultured cells on ECM (65). These reagents can rapidly diffuse through the cell membrane, are metabolized by active cells, and result in a high fluorescence compound that diffuses back to the surrounding environment. Fluorescence of cell culture medium after resazurin metabolism is indicative of live and active cells. Metabolic activity of cells over four days in culture conditions on treated ECMs was measured and is reported in
Figure 5–5-B. The ECM showed potential to support cell adhesion and growth over time regardless of applied treatment method. However, NaOH-treated ECMs showed significantly higher potential to produce healthy cell growth. Peracetic acid treated tissue also resulted in more cell growth compared to other treated samples.

Figure 5–4. Surface chemistry of treated tissues examined by FTIR spectra. No significant shift in surface bands responsible for cell adhesion (Amide I, II, III) was observed after treatment with cell-lysing agents.
Fluorescence intensity in the depth of reseeded treated tissues was determined as an indication of cell penetration through the ECM (Figure 5–6). No cells were observed deeper than 250 microns for NaOH-treated tissue possibly due to fused fibers. Peracetic acid and detergent-treated ECMs showed the same amount of penetration (300 microns). However, deep penetration of cells in trypsin-treated tissue was another demonstration of structural disruption and fiber disintegration.

Figure 5–5. Recellularization of treated tissues with canine renal epithelial cells (MDCK). (A) Fluorescently-labeled cells after 4 days growth on treated tissues; (B) Resazurin reduction assay results over 4 days of cell growth (n=6). All reported data are Ave ± SD.

5.4 Discussion

Tissue regeneration using naturally obtained scaffolds combined with patient-specific cells is a promising approach in tissue engineering and regenerative medicine. Scaffold preparation by
decellularization is the first step in this technology, and involves removing all cellular materials from the native tissue while retaining ECM structure and crucial components. Elimination of cellular remnants is critical; otherwise inflammation, fibrosis, and scar tissue formation can occur after transplantation (7, 144, 145). A variety of materials have been used for decellularization of native tissues including alkaline solutions, acidic solutions, ionic detergents, non-ionic detergents, and protease enzymes. Alkaline and acidic solutions solubilize cytoplasmic components through phospholipid hydrolysis (146) and disrupt nucleic acids (9). Detergents dissolve cellular membranes and extract DNA from proteins resulting in efficient cell removal (35). Protease enzymes such as trypsin cleave peptide bonds, but in the case of prolonged exposure can lead to the disintegration of the ECM (9, 35). All of these decellularization agents have damaging side effects leading to scaffolds with less cell support potential and weak mechanical properties. There is always a trade-off between cell removal and retaining the intactness of the ECM. The optimum procedure varies for different types of tissue, and should be selected exclusively for any native tissue from which the scaffold is harvested.

In this study, we examined the impact of a broad range of chemistries (as suggested by Crapo et al. (35)) on renal ECM and evaluated ECM’s physicochemical properties after various treatments. Applied solutions were chosen due to their proven efficiency for animal tissue decellularization (NaOH (124), peracetic acid (135, 144), Triton X-100 (41), and SDS (41)) as well as to almost cover all variety of decellularization agents. A summary of the comparison of the various agents is in Table 5–1.
Figure 5–6. Penetration of MDCK cells through treated ECMs. The y-axis is the fluorescence intensity that was measured in the tissue and the x-axis is the depth in the cultured tissue (µm).
Table 5-1. Summary evaluation of decellularization methods.

<table>
<thead>
<tr>
<th>Decision criteria</th>
<th>0.1 N NaOH</th>
<th>1% Peracetic acid</th>
<th>3% Triton X-100</th>
<th>1% SDS</th>
<th>0.05% Trypsin/EDTA</th>
</tr>
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<tbody>
<tr>
<td>Cell removal</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>DNA removal</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Cell adhesion and growth</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tubular and glomerular structure</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
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<tr>
<td>ECM intactness</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Surface chemistry</td>
<td>+</td>
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<td>Cost</td>
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<tr>
<td>Waste management</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>Process feasibility</td>
<td>-</td>
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</table>

Every treatment has inherent advantages and disadvantages and the optimum method should be selected by considering the corresponding effect and application of the ECM. For example, if cell removal is important and the ECM is used as a scaffold to deliver the cells, then the NaOH-treated tissue is the best platform. Otherwise, if the structural integrity is desired, detergent treatment would work well.

Alkaline decellularization of whole rat lungs and human muscles has been previously reported for CHAPS (147, 148) and Triton X-100 (133), demonstrating that alkaline solutions with pH around 10-12 can significantly damage collagen fibers, elastin, fibronectin, and GAGs (134). In this work, we also observed more than 70% GAGs and growth factor removal after NaOH treatment of renal tissue (Figure 5–3). Collagen fibers were fused together after NaOH treatment leading to an amorphous surface on the ECM instead of a network of separate visible fibers (SEM images in Figure 5–2). However, alkaline solutions are highly efficient in removal of cellular
remnants since DNA denatures to single strands at pH higher than 11 which leads to reduced viscosity and more effective removal (149). In spite of more efficient cell removal, lung ECM decellularized at pH 10-12 elicited a more severe host response, fibrotic tissue formation, and cell infiltration compared to treated tissue at pH 8 after subcutaneous implantation (134). These observations suggest that decreased residual DNA content is not enough to predict immunogenicity of the ECM but that degradation residues after decellularization also can contribute to immune response induction.

In this study, the metabolic activity of renal cells cultured on NaOH-treated tissues was significantly higher than other applied methods, possibly due to the flattened surface of tissue as a result of fiber fusion. Since the cell-ECM interactions are the most important characteristics of fabricated ECM, the results produced by NaOH treatment are preferred for whole porcine kidney decellularization. We perfused the porcine kidneys with 0.1 N NaOH through the renal artery and after 7 h, completely white ECMs were obtained. We recommend further examination of ECM properties to achieve a comprehensive understanding of NaOH impact on whole renal ECM.

The peracetic acid solution in different concentrations has been previously used to decellularize small intestinal submucosa (136, 144) and porcine tendon (150). We found peracetic acid as the best method for preservation of tissue structure and collagen fibers (Figure 5–2), but its acidic pH removed more growth factors compared to detergent solutions (Figure 5–3). Metabolic activity of cultured cells on PAA-treated ECMs was significantly higher than detergent-treated tissues and increased over time mostly because of preserved collagen fibers (Figure 5–5). More active metabolism of cultured cells on PAA-treated ECM was previously demonstrated compared to Triton X-100 and SDS-treated SIS-ECM (136). However, with PAA treatment, the majority of
cellular components were retained in the structure of ECM and immunogen removal was insufficient (Figure 5–1), which was in complete agreement with previous studies (136, 144).

Consequently, peracetic acid should be combined with low concentration detergent solutions to result in efficient decellularization (135, 151). The major application of peracetic acid is as a disinfectant for collagenous ECM rather than as a decellularization agent, especially in high ionic solutions (140) or with different concentrations of ethanol solutions (60, 152, 153).

Detergent decellularization of whole organs has been the preferred method in previous studies (8, 37, 40). We also have shown that SDS-treated tissue was completely devoid of cellular materials with significantly reduced DNA content while Triton X-100-treated ECM still contained cellular remnants with more residual DNA. However, SDS caused significant damage to GAGs, growth factors, and collagen fibers - leading to fused fibers (Figure 5–2). Sulfated GAGs quantification showed the same GAGs preservation in SDS and Triton-treated samples, but it was mostly due to residual SDS in treated tissue which bound Blyscan reagent (1,9-dimethylmethylene blue) instead of sulfated GAGs.

Cultured cells on Triton-treated ECMs showed more active metabolism growth compared to SDS-treated tissue; however, it was still much lower than PAA and NaOH-treated ECM. Since SDS, which is an ionic detergent, was proven to be more disruptive to ECM structures (41), it has been advised to use the combination of SDS and Triton X-100 for decellularization of whole organs with reduced exposure time to SDS (13, 64). We have previously shown that SDS combined with hypo/hypertonic solutions would result in efficient cell removal and produce perfectly intact whole porcine kidneys (154). Protease enzymes are usually used for 1-2 h before decellularization with detergent to enhance cell removal efficiency since they cleave peptide bonds and result in major damage to collagenous fibers. The results of the present study provide practical
information for a rational choice of a decellularization protocol for whole organs, especially kidneys. Further experimentation is required to compare mechanical properties of treated tissues as one of the crucial characteristics of decellularized scaffolds.
6 EFFICIENT DECELLULARIZATION OF WHOLE PORCINE KIDNEYS IMPROVES RESEEDED CELL BEHAVIOR

6.1 Introduction

This chapter is an adaptation of the article entitled “Efficient decellularization of whole porcine kidneys improves reseeded cell behavior” published in March 2016 in the Journal of Biomedical Materials. This work was developed and led by myself with the help of Nima Momtahan, Amin SM Salehi, Daniel R Scott, Cory A Fronk, Dr. Beverly L Roeder, Dr. Paul R Reynolds, Dr. Bradley C Bundy, and Dr. Alonzo D. Cook.

Despite reports of the damaging effects of sodium dodecyl sulfate (SDS) on protein structure (41) and the need for limited SDS exposure, there is no reported robust and reproducible procedure for efficient decellularization of whole porcine kidneys with reduced SDS exposure time. To our knowledge, there is no previously published research on the impact of detergent exposure time of ECM on in vivo-like properties of cultured cells, which is the prominent factor to consider for evaluation of ECM characteristics. To improve upon the previously published methods and devise an efficient method for whole kidney decellularization, we combined physical and chemical steps to minimize SDS exposure time. In this section, we show that achieving less than 5 h SDS exposure during the decellularization procedure preserves essential components of the ECM and improves tissue-specific cell adhesion, growth, and gene expression patterns. We examined cell-
ECM interactions to assess the influence of SDS exposure and herein propose an improved, automated decellularization method that greatly reduces detergent exposure time.

6.2 Experimental design

Porcine kidneys were harvested from a local slaughterhouse according to the protocol described in section 4.3.1. We designed and constructed two bioreactors for decellularization (section 4.3.3). Whole porcine kidneys were perfused with different solutions to achieve decellularized scaffolds (section 4.3.4). Then, the slices of renal cortex were excised for further experiments. A variety of histology and immunohistochemistry stainings were performed to examine the structure of the decellularized scaffolds (section 4.7.4). Residual DNA (section 4.7.1), GAGs (section 4.7.2), collagen (section 4.7.2), and growth factors (4.7.3) content of native and decellularized ECM were quantified according to protocols described in chapter 4. Mechanical properties of native and decellularized renal tissue including compression properties (4.6.1) and arterial pressure (section 4.6.2) were determined. Scanning Electron Microscopy of native and decellularized renal tissue was performed to determine changes in the ultrastructure of ECM (section 4.6.5). To further investigate the immunogenicity of the ECM, a macrophage stimulating assay was applied to acellular and native tissue as well as bacterial cell lysate as a positive control (section 4.6.2). Finally, slices of renal tissue were cultured with renal epithelial cells and a gene expression study was performed to study how different ECM treatments resulted in different gene expression patterns in renal cells (section 4.10.6).
6.3 Results

6.3.1 Whole kidney decellularization

Perfusion decellularization of whole porcine kidneys (n=20) was performed using a combination of physical and chemical steps. The control method required 9 to 10 h to achieve a completely white scaffold (depending on initial kidney weight). The time for the improved method was 11 to 12 h; however, SDS exposure was only 4 to 5 h (approximately 5 h less than the control method). The completely white kidneys obtained from both methods can be seen in Figure 6–1 (c and e). In the improved method, the SDS efficiency of cell removal was increased by applying osmotic shock to the cell membranes. The H&E staining also demonstrated that the cytoplasmic and nuclear materials were removed (Figure 6–1 d and f).

DNA quantification was performed for decellularized and native kidney samples. After 48 h incubation in cell culture media, the DNA content of decellularized samples was less than 50 ng/mg of dry weight, which is less than the expected threshold for immune rejection (35). The total DNA was reduced from 3,949 ng/mg dry tissue to 7.6 ± 2.0 ng/mg and 12.5 ± 2.3 ng/mg dry tissue for the control and improved methods, respectively (the reported results are Avg ± SD for n=6). More than 99% of the DNA was removed as a result of decellularization (Figure 6–2).

6.3.2 Macrophage-stimulating assay

In addition to the nuclear materials, other proteins from outside the nucleus, such as cell surface proteins (antigens) may cause immune response at the time of transplantation. To determine the immunogenicity of the acellular ECM due to cell surface antigens, macrophage cells were incubated with samples of native and decellularized kidneys obtained after treatment with
the improved and control methods. Nitric oxide is produced through macrophage stimulation and converted to nitrite. Griess reagent reaction was used to measure the nitrite production after 20 h. The results are shown in Figure 6–3. Stimulation capacities of acellular samples (obtained with both methods) were like negative controls, demonstrating the removal of the clear majority of different antigens from the decellularized matrix.

Figure 6–1. Decellularization of whole porcine kidney. Visual inspection (a,c,e) and H&E staining (b,d,f) demonstrated complete cell removal from the renal tissue (n=10 for each method).
Figure 6–2. Left: DNA quantification results for native kidney, control ECM, and improved ECM. DNA content of acellular samples via both methods was less than the threshold for immune rejection (50 ng/mg of dry tissue). Right: Electrophoresis gel image of residual DNA in decellularized kidneys shows no DNA more than 200 bp in length (Ave ± SD, n=6).

Figure 6–3. Macrophage stimulating assay results. The nitrite concentration in the media after culturing macrophage cells in the presence of various samples is an indication of the amount of macrophage stimulation by antigens that existed in the samples. Nitrite concentration of samples obtained from tonic cycle (improved method) and SDS only (control method) were similar to negative control (no stimulation of macrophage cells) while this value for native kidneys and positive controls (bacterial cell lysate) were significantly higher (Ave ± SD, n=4, **p-value<0.05)
6.3.3 Preservation of essential components and microstructure of the ECM

One of the most important advantages of naturally obtained scaffolds is that they contain essential components as well as critical structures that provide chemical and physical clues for cell growth and differentiation. Harsh decellularization methods can damage the structure of the ECM and denature the proteins that are required for cell adhesion (e.g. fibronectin and collagen (39)). Collagen is the most abundant component of the mammalian ECM that contributes to mechanical properties of the ECM and plays a significant role in cell adhesion and signaling. Glycosaminoglycans (GAGs) are elements of the ECM that, although they are present in tiny amounts, have a considerable effect on cell growth and differentiation since they bind growth factors. GAGs are also necessary to retain water in the ECM and maintain its gel properties (5). The importance of these two components for the recellularization step led us to quantify the content of collagen and GAGs. The results are reported in Figure 6–4-A, B normalized to the dry weight of the native kidneys. The collagen preservation improvement is an additional proof verifying previous findings that SDS can remove essential components and denature the major proteins of the ECM.

Growth factors are also of significant importance to change cellular fate and growth rate. Human fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) were quantified in native, control, and improved ECMs and the results are reported in Figure 6–4-C, D. The FGF content of ECM was significantly reduced after decellularization that was partially due to the removal of cell-bound growth factors. The content of FGF and HGF were the same for each type of ECM, signifying similar preservation of growth factors from the different methods.

In addition to the amount of essential components, the distribution of components also plays an important role in cell homing and differentiation. Stained samples with Safranin O, Sirius,
and Orcein staining showed the same partial preservation of GAGs, general collagen, and elastin fibers for both decellularization methods (Figure 6–5). There are different types of collagen in the extracellular structure of mammalian organs, but collagen IV particularly plays an essential role in the adherence of epithelial cells and vascular endothelial cells to the ECM (5). Immunohistochemistry was performed with native and decellularized samples in order to evaluate the spatial distribution of collagen IV (Figure 6–5). Accumulation of collagen IV in the capillary structure of glomeruli was partially preserved equally in both methods of decellularization compared to native kidneys.

Figure 6–4. GAGs (A), Collagen (B), FGF (C), and HGF (D) contents of native, control, and improved ECMs. The GAGs and collagen data are averaged over 5 repetitions and growth factors contents are averaged over 3 repetitions. All contents are normalized to dry weight of initial tissue without cells. Reported data are Avg±SD.
Glomeruli and renal tubules are prominent structures that are prone to damage because of high perfusion pressure. Scanning electron microscopy (SEM) of native kidneys, improved and control ECM samples are also shown in Figure 6–5. Glomeruli, Bowman’s capsule, macula densa, and renal microtubules were all detectable by SEM in all decellularized samples, confirming minimal damage to the microstructure of the kidney after decellularization.

Fiber characterization was performed using SEM imaging (three measurements per image and 3 images per sample). Fiber diameter was 22.1 ± 0.9 nm and 23.9 ± 3.4 nm for improved and control ECM, respectively. Intersection density was (3.5 ± 0.7)×10⁻³ and (3.2 ± 0.3)×10⁻³ intersections per nm² for improved and control ECM. No significant difference was detected in fiber characteristics by either method (reported data are Avg±SD, n=6).

6.3.4 Mechanical Properties of ECM

The stiffness of the ECM is a key factor guiding cell differentiation during recellularization with stem cells. It has been shown, for example, that large elastic moduli of the ECM causes mesenchymal stem cells to differentiate into osteoblasts while a low elastic moduli leads them to become brain cells (155-158). To compare the mechanical strength of dECM obtained from both methods of decellularization, compression tests of biopsies of various ECMs were performed. Elastic moduli in low and high strain (ECM resistance to deformation) were calculated as the slopes of stress/strain curves at low and high strains. There was no statistically significant difference between the elasticity of the ECMs obtained from the two different decellularization methods (p>0.05); however, significant decrease was observed compared to native tissue, which was predictable because all cellular material was removed and the remaining structure was like a sponge with huge void fraction (Figure 6–6-A).
Figure 6–5. Microstructural characterization of native and decellularized renal tissue. Picro Sirus Red stain, Orcein Elastin stain, Safranin O stain, and immunostaining representative of general collagen, elastin fiber, proteoglycan, and collagen IV, respectively, for native, control method, and improved method. Distribution of all non-cellular components for decellularized samples was the same as native kidneys. SEM images of the native kidney, control, and improved methods show that the glomeruli, macula densa, Bowman’s capsules, and tubules were intact in all decellularized samples (the arrows indicate the intact glomerular structures).
Figure 6–6. Mechanical properties of decellularized kidneys. Nominal stress/strain curves of control ECM (A) and improved ECM (B) are similar in shape. Elastic modulus of samples in low and high strain regions shows similar mechanical strengths of acellular ECM by both methods.

In another experiment, the renal arterial pressure (representative of vasculature resistance) was measured for decellularized kidneys prepared from both methods and native kidneys. The kidneys were perfused with 1X PBS and flow rates versus pressure were recorded (Figure 6–7). Native kidney and acellular ECMS showed the same trend of an increase in arterial pressure with an increase in flow rate. Since there are no cells in decellularized ECMS, there would be less resistance and, consequently, less arterial pressure. Improved ECM has more resistance compared to control ECM, indicative of more structural integrity.

6.3.5 Cytotoxicity of ECM

The most important characteristic of ECM is its support for cell adhesion and proliferation. Since we used SDS for decellularization we desired to prove that there was no cytotoxicity caused
by ECMs produced by either method. Cytotoxicity of the ECMs was analyzed by live/dead assay for MS-1 cells growing in growth media in the presence of biopsies of decellularized ECM from both methods. After 3 days of cell culture, the cells grew to 100% confluence and no dead cells were detected, demonstrating that no toxic extracts were released by the decellularized tissue samples (Figure 6–8).

Figure 6–7. Renal arterial pressure. Renal vasculature resistance was measured for decellularized kidneys obtained via both methods and native kidney controls. All showed approximately linear trends with increasing flow rate. However, tonic cycle decellularized ECM had more resistance compared to SDS-only decellularized ECM, demonstrating that the integrity of the ECM was better preserved in the tonic cycle method (Ave ± SD, n = 4).
To demonstrate that our acellular ECMs were supporting cell adhesion and proliferation, we took biopsies after 3 days and performed H&E staining with sectioned samples. Cell culture on ECM was performed in 96-well plates, assuring that only the upper surface was repopulated (Figure 6–8). After 3 days the cells were adhered to the ECM and approached 100% confluence, signifying that the acellular ECMs from both methods possessed the capacity for supporting cell growth.

Figure 6–8. Cytotoxicity of acellular ECMs. Live/dead assay of culture cells in 96 well-plates contained control ECM (A and D), improved ECM (B and E), no ECM (C and F) after 1 day and 3 days. Control ECM (G) and improved ECM (H) repopulated with MS1 cells after 3 days of cell culture. No toxicity was observed for acellular ECMs and the cell proliferation was the same as samples with no ECM.
6.3.6 Renal cell growth on decellularized ECMs

Pieces of tissues obtained from both methods of decellularization were repopulated with MDCK cells for 7 days (Figure 6–9-a and b). H&E staining results demonstrated that the porcine ECM was able to support the attachment and growth of canine renal cells. No significant visual difference in cell adhesion could be observed for the two decellularization procedures. During cell growth on decellularized ECMs, viability and number of cells were tracked using the resazurin reduction assay (Figure 6–9-c). Resazurin-based PrestoBlue reagent can diffuse through the cell membrane and be metabolized, resulting in a high fluorescence compound able to diffuse back into the cell culture medium (65). Fluorescence of medium after 1 h resazurin metabolism is representative of viable cells on the ECM. The number of live and active cells on the improved decellularized ECM was higher than the control ECM. Since the cells cultured on ECMs and tissue culture plastic form a monolayer and do not diffuse into the ECM within 7 days, the cells initiate programmed apoptosis in 3-5 days after reaching confluence.

The relative expression patterns of five different genes of MDCKs cultured on decellularized ECMs were determined, normalized to expression of a housekeeping gene (EF1-α), and compared to cells grown on tissue culture plastic. In addition to the observed increase in cell growth, gene expression patterns of MDCK cells with less SDS exposure were improved (Figure 6–9-d). AQP1 (this gene encodes an aquaporin which functions as a molecular water channel protein) and CDH16 (this gene is exclusively expressed in kidney to produce cadherin-16 protein, which functions as the principal mediator of homotypic cellular recognition) were downregulated on the control ECM compared to cells grown on tissue culture plastic.
**Figure 6–9.** Canine MDCK cell growth on porcine ECM; (a) Control ECM and (b) Improved ECM. MDCK cells were used to repopulate slices of decellularized tissues. (c) Resazurin reduction assay results for cultured cells on ECMs and tissue culture plastic. (d) Gene expression pattern of MDCK cells cultured on ECMs and tissue culture plastic. Data are normalized to expression of housekeeping gene (EF1-α) and compared with cultured cells (the value of 1 on the y-axis is equivalent to cells on tissue culture plastic), data are Ave ± SD, n=6; **Represents significant difference with p-value<0.05, NS represents no significant difference with p-value>0.05). In panel a and b, dark purple and pink represent cells and collagenous structure, respectively.

### 6.4 Discussion

Creation of whole intact acellular renal ECM, as an appropriate platform for whole renal tissue regeneration, has been reported in previous studies that are summarized in Table 6–1. Sullivan *et al.* decellularized whole porcine kidneys with 0.5% SDS, 0.25% SDS, and 1% Triton X-100 and suggested 0.5% SDS as the optimum solution to keep the ECM intact and remove cellular materials (8). Wang *et al.* also reported 1% SDS as the most efficient method to create
an intact and acellular ECM compared to 1% Triton X-100, 1% peracetic acid, and 1% sodium deoxycholate (61).

Table 6-1. Summary of applied decellularization methods for whole porcine kidneys

<table>
<thead>
<tr>
<th>Decellularization procedure</th>
<th>Flow Rate</th>
<th>GAGs content</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h 0.5% SDS, and 5 days wash with PBS</td>
<td>12 ml/min</td>
<td>...</td>
<td>(50)</td>
</tr>
<tr>
<td>12 h 1% SDS and 1 day wash with PBS</td>
<td>100 ml/min</td>
<td>...</td>
<td>(51)</td>
</tr>
<tr>
<td>36 h 0.5% SDS in PBS, 2 days wash with PBS</td>
<td>...</td>
<td>~10 µg/mg</td>
<td>(8, 53)</td>
</tr>
<tr>
<td>18 h 1% SDS, and 3 h wash with PBS</td>
<td>15 ml/min</td>
<td>~12 µg/mg</td>
<td>(61)</td>
</tr>
<tr>
<td>28 h 1% SDS, 2 h Triton X-100, and 4 days wash with PBS</td>
<td>10 ml/min</td>
<td>~14 µg/mg</td>
<td>(62)</td>
</tr>
</tbody>
</table>

Although SDS perfusion through the innate vasculature of whole native kidneys has been the most preferred decellularization method, SDS in concentrations more than 0.04% can denature the collagenous structure of the ECM, damage other proteins, and remove key components needed for cell growth (159, 160).

In this study, we succeeded in efficiently reducing the decellularization SDS exposure time of porcine kidneys to less than 5 h by using a combination of freezing/thawing, automated pressure control, osmotic shock, and SDS washes. Utilization of hypo/hypertonic solutions to exert osmotic shock to the cells in order to decellularize tissues has been previously performed (161-163); still no previous attempts have been made to combine osmotic shock with SDS wash for whole organ decellularization. To avoid spoilage and also induce partial damage to cellular membranes, the kidneys were subjected to freezing at -20°C before the decellularization procedure. We previously
evaluated the freezing/thawing effect on the structure of the kidneys (40). We observed that freezing can damage the cellular material and reduce arterial pressure, which leads to enhanced perfusion decellularization; however, it minimally damages ECM protein structures. Less SDS exposure in the improved decellularization method is not only due to using osmotic shock but also due to controlling the pressure during the process to avoid damaging the ECM by high pressure. As the cells were removed during the decellularization procedure, a reduction in arterial pressure was overcome with increased flow rate, which led to more homogenous and faster decellularization without damaging the ECM structure. The decellularization process was initiated at a low flow rate (10 ml/min). Once the cells began to be removed, the resistance decreased and the flow rate increased, forcing the perfusate through the intact tissue. This method facilitated cell removal and resulted in more homogeneous decellularization.

Collagen is the most abundant component of the ECM and was better preserved by the improved method with less SDS exposure. GAGs are the other essential components of the ECM for growth factor adhesion and function. The GAGs content of decellularized ECM obtained by the improved method was 65 μg/mg of dry tissue compared to less than 14 μg/mg of dry tissue for decellularized porcine kidneys after decellularization with any other previously reported protocols (Table 6–1).

These factors maintain cells in a proliferative state and control cell fate and gene expression patterns (30, 39). It has previously been reported that insulin-like growth factor (IGF), HGF (111), and FGF9 (94, 110, 164) growth factors are crucial for kidney-specific gene expression (especially aquaporin AQP1). We quantified FGF and HGF in control and improved ECMs and observed no significant difference in the 2 methods of decellularization.
Expression levels of several essential genes involved in kidney function were measured to examine *in vivo*-like properties of cultured cells. The LRP2 gene produces megalin protein that is an endocytic receptor on the apical surface of renal epithelial cells responsible for reabsorption of a variety of ligands such as nutrients and hormones (165). The AQP1 gene regulates the aquaporin water channels in the epithelium of distal and proximal tubules of the kidney (166). The CDH16 gene encodes kidney-specific cadherin, a calcium-dependent, trans-membrane glycoprotein required for cell adhesion, migration, and polarization (167). NPHS2 is a podocin-producing gene that is mostly active in the epithelium of glomeruli (168). All of these genes are required to be expressed to produce functional and healthy renal tissue. In general, the presence of extracellular matrix improved gene expression patterns compared to tissue culture plastic due to endogenous growth and signaling factors that exist in the renal tissue. The enhanced *in vivo*-like gene expression patterns of cultured cells on improved ECM was mostly due to preserved conformation of collagen and GAGs, which bind to other endogenous growth factors.
7 FREEZING/THAWING WITHOUT CRYOPROTECTANT DAMAGES NATIVE BUT NOT DECELLULARIZED PORCINE RENAL TISSUE

7.1 Introduction

This chapter is an adaptation of the article entitled “Freezing/thawing without cryoprotectant damages native but not decellularized porcine renal tissue” published in January 2015 in Organogenesis Journal. This work was developed and led by myself with the help of Timothy S Frost, Daniel R Scott, Brinden B Elton, Dr. Paul R Reynolds, Dr. Beverly L Roeder, and Dr. Alonzo D. Cook.

To our knowledge, there was no previous study for evaluation of freezing/thawing cycle effects on decellularized kidneys compared to native renal tissue. Therefore, the purpose of this study was to determine the extent of damage due to cycles of freezing and thawing to the structure of decellularized renal tissue. In this study, freezing/thawing cycles were used without any cryoprotectant, freezing at -20°C for at least 24 h, cooling to -80°C for 12 h, slow warming at -20°C for another 24 h, and finally thawing slowly at 4°C for 12 h. Compression and arterial pressure measurements were used to evaluate structural integrity of the ECM. Microstructures of renal tissue were examined through histological staining and scanning electron microscopy (SEM). Frozen/thawed and non-frozen decellularized ECM samples were also recellularized with canine renal epithelial cells (MDCK) to compare the capacity of ECM to support cell attachment and proliferation.
7.2 Experimental design

Whole porcine kidneys were decellularized as described in section 4.3.4. The native and decellularized kidneys were subjected to a freezing/thawing cycle as described in section 4.4. Then, the frozen and non-frozen kidneys were examined using measurements of arterial pressure (section 4.6.2) and compression tests (4.6.1). SEM imaging and various staining was performed to determine ultrastructure and porosity of ECM before and after freezing/thawing according to the protocol described in sections 4.6.5 and 4.7.4. Finally, renal epithelial cells were cultured on frozen and non-frozen decellularized ECM to see the effect of a freezing/thawing cycle on the capacity of ECM to support cell growth (section 4.10.4).

7.3 Results

In this study, the microstructure and mechanical properties of native porcine kidneys (n=10) and decellularized porcine ECM (n=10) were examined to investigate the effect of freezing/thawing on the tissue. The 20 porcine kidneys were analyzed as 4 groups: native non-frozen, native frozen/thawed, decellularized non-frozen, and decellularized frozen/thawed (n=5 per group). Some of the kidneys in the decellularized groups were stored at -20°C before decellularization.

7.3.1 Compression test

To evaluate the mechanical properties of native and decellularized tissues, samples (n=15-22 per group) of renal cortex were excised and subjected to compression. The stress/strain curves representing the upper and lower bounds of the measurements are shown in Figure 7–1. The elastic modulus representing the elasticity of renal tissue was calculated as the slope of the linear section of the stress/strain curves at low strain. The results of calculating the elastic moduli are shown in
The average elastic modulus of decellularized kidneys was 6.4 ± 2.7 kPa before and 4.6 ± 3.0 kPa after freezing/thawing. The average elastic modulus of non-frozen native kidneys was 41.6 ± 22.4 kPa, which is in the range of previously reported averages (169). The elastic modulus of native kidneys was reduced 22 times (with 95% confidence interval of 12.4 - 32.9), to 5.6 ± 2.0 kPa, after being subjected to one freeze/thaw cycle (p-value<0.0001). The data obtained after freezing/thawing are in complete agreement with Ternifi et al.’s observations of a decrease in renal cortex elastic modulus after storage at -18°C, -34°C, and -80°C (170).

The average elastic modulus for decellularized ECM was approximately the same before and after being frozen/thawed (p = 0.0636); however, it was slightly smaller for frozen/thawed ECMs, which can be attributed to elastin and collagen fiber damage as demonstrated by elastin and collagen staining. Similarly, Nonaka et al. reported no significant changes in mechanical properties of decellularized lungs after several freeze/thaw cycles (171). Both decellularization and freezing/thawing resulted in a decrease in stiffness, but the two processes were not additive in their damage to the mechanical properties of the porcine kidneys.

Figure 7–1. Stress/strain curves representing the upper and lower bounds for frozen/thawed and non-frozen samples (n=20). Freezing/thawing significantly reduced the modulus of elasticity of native kidneys (p value<0.0001); however, there was not an additive effect of freezing/thawing for decellularized renal ECM (p value = 0.0636).
Figure 7–2. Elastic moduli for frozen/thawed and non-frozen samples (n=15-22 per group) were calculated as the slope of the linear part of the stress/strain curves at low strains. Freezing/thawing caused a significant reduction in elastic modulus of native kidneys (NK), while no significant reduction was observed for decellularized kidneys (DKS). Data are reported in log-transformed format for a clearer comparison and reliable statistical analysis.

### 7.3.2 Arterial pressure measurement

All frozen/thawed and non-frozen kidneys (n=3 per group) were perfused with 1X phosphate buffered saline to measure arterial pressure. PBS perfusion was performed over a range of flow rates to determine the preservation and integrity of renal capillaries in native kidneys (NK) and ECM from decellularized kidneys (DK) after being subjected to freezing/thawing cycles (see Figure 7–3). The approximately linear trend of arterial pressure versus flow rate was preserved during freezing/thawing for both native and decellularized kidneys. The average vasculature pressure for non-frozen native kidneys (range: 40 – 70 mmHg) was significantly larger than for frozen/thawed native kidneys (range: 1 – 15 mmHg, p-value<0.0001); however, this difference was not significant for decellularized ECMs (non-frozen range 3-30; frozen/thawed range: 2-29;
p-value = 0.18). The average reduction in pressure after decellularization was $42.6 \pm 2.2$ mmHg (p<0.0001), compared to the average reduction in pressure after freezing/thawing of $52.4 \pm 3.5$ mmHg (p<0.0001) for native kidneys. In contrast, the effect of freezing/thawing on decellularized kidneys was only $4.2 \pm 1.5$ mmHg (p<0.0001). These results are in agreement with Orlando et al.’s observations for non-frozen native and decellularized kidneys (3, 50).

7.3.3 Microstructure of frozen/thawed and non-frozen samples

Scanning electron microscopy of frozen/thawed and non-frozen kidney samples (n=4 per group) was performed and the results are shown in Figure 7–4. No detectable damage, in terms of dilation or increased porosity, was observed at the scale of these images. Renal glomeruli, distal and proximal convoluted tubules, and Bowman’s capsules (arrows) all were preserved during the freezing/thawing cycle.

Figure 7–3. Arterial pressure measurements in frozen/thawed and non-frozen native kidneys (NK) and decellularized kidneys (DK) (n=12) were indicative of renal vasculature integrity. Arterial pressure was highly reduced for NK after freezing/thawing (p<0.0001), while it was essentially unchanged for DK (p=0.18).
7.3.4 Histological evaluation

Renal cortex biopsies of all frozen/thawed and non-frozen samples (n=8 per group) were fixed in 4% paraformaldehyde solution (PFA), sectioned and stained with Orcein elastin and Sirius Red specific to elastic and general collagen tissue, respectively. Some damage to elastin occurred from the freezing/thawing process in native kidneys, as shown in Figure 7–5 at 10X and 20X magnification. Figure 7–6 shows elastin staining for frozen/thawed and non-frozen decellularized ECM with all imaging performed under the same conditions in terms of focus and light intensity. As a result of freezing/thawing, previously frozen samples stained a lighter color compared to non-frozen samples, which was attributed to a reduction in total elastin. However, the lighter color might also be due to ice formation and elastin fiber damage that occurred during freezing. As was also shown by the elastic modulus data (Figure 7–2), the damage caused by freezing/thawing was more pronounced for native kidneys, and produced a greater color difference than was found with frozen/thawed and non-frozen decellularized samples.

General collagen distribution of frozen/thawed and non-frozen native kidneys is shown in Figure 7–7 and the collagen distribution for decellularized kidneys can be seen in Figure 7–8. The red color represents general collagen, and was less detectable for previously frozen native or decellularized ECM compared to non-frozen samples. It is suspected that collagen fibers were damaged during the freezing/thawing cycle because of ice formation, leading to reduced stiffness of the ECM and reduced arterial pressure. In all the histology images, the arrows depict preserved renal corpuscles.
Figure 7–4. Scanning electron microscopy images of frozen/thawed and non-frozen kidney samples at 250X (A1-D1) and 500X (A2-D2) magnifications. No microstructural damage was detected after freezing/thawing. Renal corpuscles, distal and proximal tubules all were preserved during freezing/thawing cycles.
7.3.5 Renal cell viability on the ECM

Repopulation of both frozen/thawed and non-frozen decellularized ECM (n=3 per group) was performed using MDCKs. After 12 days, the repopulated ECM samples were stained with H&E for cell detection and the results are presented in Figure 7–9. As shown in this figure, there was no detectable difference between frozen/thawed and non-frozen ECM in terms of supporting cell attachment and growth. Cells proliferated and covered the whole surface in both cases.

Figure 7–5. Orcein stain imaging for frozen/thawed and non-frozen native kidney samples at magnifications of 10X (A-1 & B-1) and 20X (A-2 & B-2). Generally, the elastin fibers were damaged as a result of freezing/thawing (affinity for Orcein stain color was lower for frozen/thawed samples which resulted in lighter color in images). Because of fibril damage the structure was more porous and had less integrity as indicated by the white spaces that were more frequent in frozen/thawed samples (arrows show renal corpuscles).
Figure 7–6. Orcein stain imaging for frozen/thawed and non-frozen decellularized kidney samples at magnifications of 10X (A-1 & B-1) and 20X (A-2 & B-2). Generally, the elastin fibers were damaged as a result of freezing/thawing (affinity for Orcein stain color was lower for frozen/thawed samples which resulted in lighter color in images). Also it appeared that because of fibril damage the structure was more porous and had less integrity as white spaces were more frequent in frozen/thawed samples (arrows show renal corpuscles).

Figure 7–7. Sirius red stain imaging representative of general collagen for frozen/thawed and non-frozen native kidney samples at 10X (A-1 & B-1) and 20X (A-2 & B-2) magnifications. General collagen appeared to be partially damaged through freezing/thawing as evidenced by decreased stain color in frozen/thawed samples, which was detected as lighter colors. Void spaces were more detectable in frozen/thawed samples, which was again indicative of fibril damage and less integrity (arrows show renal corpuscles).
Figure 7–8. Sirius red stain imaging representative of general collagen for frozen/thawed and non-frozen decellularized kidney samples at 10X (A-1 & B-1) and 20X (A-2 & B-2) magnifications. General collagen seemed to be partially damaged through freezing/thawing since stain color was lighter for frozen/thawed samples; however, the difference was not as stark as for native kidneys after freezing/thawing. Also, the porosity was greater in frozen/thawed decellularized kidney samples, which is again indicative of fibril damage and diminished integrity (arrows show renal corpuscles).

Figure 7–9. H&E staining of recellularized non-frozen (A) and frozen/thawed (B) decellularized ECM with MDCK cells after 12 days. Both non-frozen and frozen/thawed decellularized ECM showed the potential to support proliferation of renal cells.

7.4 Discussion

Freezing/thawing effects were studied for both native and decellularized porcine kidneys. Whole native and decellularized kidneys were sequentially frozen at -20°C for at least 24 h, then
deep-frozen at -80°C for 12 h, followed by slow warming (without phase transition) at -20°C for 24 h, and finally thawed at 4°C for 12 h. The mechanical properties of all frozen/thawed and non-frozen, native and decellularized kidney samples were examined to assess the effect of ice crystal formation in the extracellular matrix. Previous research had demonstrated the detrimental effects of freezing/thawing on the biomechanical properties of native kidneys (170). Some loss of mechanical strength was observed for decellularized kidneys in preliminary experiments in our laboratory. One of the key motivations for this work was to determine if decellularized ECM would be further damaged by freezing to -80°C followed by thawing.

Mechanical properties of renal tissue were the most important characteristic for this evaluation, since the mechanical properties of biological tissue are essential for normal function and stability of an organ during recellularization. Previous researchers have focused on measurements of the biomechanical properties of abdominal organs (169, 172) but not kidneys. Another reason for these experiments, which is critical for decellularized tissues, is that the stiffness of the ECM can dictate cell differentiation during repopulation with stem cells (155, 173). Engler et al. showed that mesenchymal stem cells could be differentiated to brain cells on soft matrices, to muscle cells on stiffer matrices, and to bone cells on comparatively rigid matrices (155). Therefore, it is desired to preserve the decellularized ECM stiffness and elasticity as close as possible to natural tissue.

Cell survival is essential for both whole organ transplantation and regenerative medicine. There are several previous papers that have described methods for safe preservation of organs before transplantation (174-176). In most of these methods high concentrations of cryoprotectants were used to avoid phase transition and crystal formation at low temperature (referred to as vitrification instead of freezing) while preserving cells. However, it has been shown that hearts
and kidneys do not function properly after freezing and thawing if they are kept at temperatures lower than -20°C (177, 178). In this study, the damage that occurs to native tissue from freezing/thawing without any cryoprotectants was assessed. It was found that frozen/thawed kidneys demonstrated significantly lower stiffness than non-frozen native kidneys, indicating that intact cells contribute significantly to mechanical properties. Elastic modulus reduction has also been observed for several other tissues after treatment with freezing/thawing such as porcine aortic tissue (179), decellularized lung (171), porcine kidney (170), rabbit tendons (180), and bovine liver (181).

In addition to the cellular disruption caused by freezing and thawing, ice formation can damage both elastin and collagen fibers. Giannini et al. used TEM imaging to characterize collagen fibers of human posterior tibial tendons after being frozen at -80°C (182). They reported an increase in the mean of collagen fibrils’ diameter, while the mean number of fibrils was also shown to be decreased. In another study, Chen et al. used H&E staining to assess the freezing/thawing cycle effect on Achilles tendons of rabbits (180). They observed more disordered collagen fibrils, and after several freezing/thawing cycles, gaps were apparent between tendon bundles because of ice crystal formation. However, they did not report any change in elastic modulus of their samples. All of these results are in complete agreement with what was observed in the present studies by histological staining representative of collagen and elastin.

In addition to collagen and elastin damage, increased porosity was detected after freezing and thawing. Several previous researchers have also noted this kind of dilation after freezing/thawing (179, 181-185). For example, O’Leary et al. investigated the effect of long term freezing on the mechanical properties of porcine aortic tissue (179). They reported increased porosity and reduced density of the aortic tissue after being frozen and thawed. Chow et al. also
reported such dilation for aortic tissue (184). They quantified the amount of total collagen and soluble collagen in their frozen and non-frozen samples, and reported a significant reduction or denaturation of collagen fibers in their frozen samples. Similarly, increased porosity has also been observed for frozen/thawed heart tissue (186).

The arterial pressure experiments demonstrated a loss of integrity of the ECM structure, leading to decreased arterial pressures during perfusion. This was most likely due to the denaturation of collagen fibers; however, ice formation can also damage microcapillaries, and that might account for the highest pressure drop during perfusion. Another reason for reduced pressure drop is cellular plasma membrane disruption. This effect is intense enough that some researchers have used freezing/thawing for decellularization, or at least partial decellularization, of tissues and organs (139).

Overall, cell and protein damage leading to increased porosity can account for the observed alterations in elastic modulus and arterial pressure that occurred after freezing/thawing. Porosity contributes more to loss of mechanical properties in native kidneys since the tissue is more densely packed prior to freezing/thawing. These effects were less important for decellularized ECM since porosity was intentionally created as a consequence of decellularization.

In summary, for decellularized organs, results from the current experiments predict that freezing and thawing without cryoprotectants will be acceptable both prior to and after decellularization. When organs are preserved for transplant, cryoprotectants are required to preserve the living cells and prevent proteins from denaturing. In the case of decellularized whole organs, it is not required to maintain cell viability, and there is minimal impact of freezing/thawing on the ECM proteins. Freezing may even prevent denaturation of the proteins during long-term storage prior to recellularization, and the reduced arterial pressure achieved by freezing/thawing
kidneys before decellularization may improve the process by reducing SDS exposure time. The freeze/thaw process described herein is therefore expected to be satisfactory for whole porcine kidney decellularization. In support of these conclusions, recellularization studies with MDCK cells on slices of frozen/thawed decellularized ECM demonstrated substantial cell growth. Future work should include investigating the recellularization of whole porcine kidneys after freezing and thawing.
8 COMPARISON OF FOUR DECONTAMINATION TREATMENTS ON PORCINE RENAL ECM STRUCTURE, COMPOSITION, AND SUPPORT OF MDCK CELLS

8.1 Introduction

This chapter is an adaptation of the article entitled “Comparison of four decontamination treatments on porcine renal ECM structure, composition, and support of human renal cortical tubular epithelium cells” published in November 2015 in the *Journal of Biomaterials Applications*. This work was developed and led by myself with the help of Jeffery J Nielsen, Ryan J Morris, Jason R Gassman, Dr. Paul R Reynolds, Dr. Beverly L Roeder, and Dr. Alonzo D Cook.

Decontamination is essential to prepare decellularized scaffolds for recellularization and transplantation; however, the decontamination process may damage the structure and chemical composition of the dECM. Decontamination is challenging for whole solid organs such as porcine kidneys since care needs to be taken in order to minimize damage to the structure during the treatment. In this study, the conditions for decontamination treatments for whole porcine renal dECM scaffolds were identified.

Porcine kidneys were decellularized using detergent perfusion via the inherent vasculature, and slices of the renal cortex were then exposed to four decontaminating treatments. The minimum required conditions to decontaminate slices were determined to be: 1 h exposure in 70% (v/v) ethanol, 1 h exposure in 0.2% (v/v) peracetic acid in 1 M NaCl solution, 1 h exposure in 0.2% (v/v)
peracetic acid in 4% (v/v) ethanol, and 3 kGy intensity of γ-irradiation (Table 8–1). All of these treatments successfully decontaminated porcine dECM. To determine the overall preferred method, the microstructures and essential components of the renal porcine dECM were compared after decontamination, as well as the potential of the dECM to support cell adhesion and growth (Table 8–2).

### 8.2 Experimental design

Whole porcine kidneys were decellularized (section 4.3.4) and slices of renal cortex were excised for decontamination tests. We did not intentionally contaminate the samples. It was just chance contamination that occurred during the normal kidney retrieval and decellularization process. Four decontamination methods were applied with different conditions to find the best procedure to decontaminate slices of renal tissue (section 4.3.4). Afterward, the acellular renal tissues were decontaminated with developed methods and collected for further experimentation. Collagen and GAGs content of ECMs were quantified (section 4.7.2) along with different stainings (section 4.7.4) to determine the intactness of the ECM after decontamination with different methods. SEM imaging of various samples was performed to examine the microstructure of the ECM according to the protocol described in section 4.6.5. FTIR analysis was used to compare the surface chemistry of different samples (section 4.6.4). Swelling ratios and porosity percentages were measured as an indication of the mechanical properties of the ECM after decontamination with different methods (section 4.6.3). Finally, the cytotoxicity of the decontaminated ECMs was evaluated using renal epithelial cells and a live/dead assay as described in section 4.10.3.
8.3 Results

8.3.1 Decontamination of dECM slices

Slices of renal cortex were decontaminated by four methods: 70% (v/v) ethanol, 0.2% (v/v) peracetic acid in 1M NaCl, 0.2% (v/v) peracetic acid in 4% (v/v) ethanol, and γ-irradiation. Various exposure times and irradiation intensities were studied to achieve minimum required conditions (Table 8–1). It was determined that 1 h of solution exposure was sufficient to completely decontaminate slices of renal tissue. For samples immersed in DI water at room temperature and exposed to γ-irradiation, 3 kGy was the minimum required intensity to eliminate bacterial or fungus growth. The decontaminated tissues along with non-decontaminated control tissues were then incubated in media containing 10% (v/v) FBS at 37°C for at least 14 days and the media was viewed under a microscope to check for the presence of contaminating microorganisms. Since the media contained all required nutrients for viable proliferation of fungi and bacteria and 37°C is the optimum growth temperature, this incubation method was deemed appropriate for confirming decontamination (187, 188). The control sample was contaminated after 12 h, verifying no inhibition of microorganism growth under these culture conditions.

Table 8-1. Determination of decontamination conditions for each applied method

<table>
<thead>
<tr>
<th>Method</th>
<th>Presence of fungus or bacterial growth after 14 days in cell culture conditions at 37°C and 5% CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>0.5 h</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.2% peracetic acid in 1M NaCl</td>
<td>0.5 h</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.2% peracetic acid in 4% ethanol</td>
<td>0.5 h</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gamma irradiation</td>
<td>1 kGy</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) Resulted in microorganism growth, (-) No observed growth
8.3.2 Decontamination of whole decellularized kidneys

Whole kidney decontamination was also performed with the three treatment solutions (n=3 for each method) and the absence of contamination was determined after incubation of randomly selected pieces of tissue in cell culture media for 14 days. Two hours of exposure to the three treatment solutions was determined to be enough for decontaminating whole renal dECM. Since 3kGy \( \gamma \)-irradiation was the most damaging method for slices of kidneys, and we did not intend to pursue this method for whole organ decontamination, \( \gamma \)-irradiation was not tested on whole organs; however, it has been reported elsewhere that 10 kGy (8) to 25 kGy (50) irradiation is required for decontamination of whole decellularized porcine ECM, or human-sized kidneys in wet conditions.

8.3.3 Essential ECM components preservation

To compare the impact of the four decontamination treatments on collagen, the soluble collagen content of decellularized renal cortex samples was determined and the results are shown in Figure 8–1. Since the assay kit quantified the soluble collagen, an increase in collagen content after treatment indicated more exposed and susceptible collagen fibers. However, the collagen content was not an absolute value and an observed increase for some samples might have occurred due to the removal of some other materials (e.g., for peracetic acid these materials could be lipids, nucleic acids, and/or other types of proteins) (189). Among all of the decontamination methods, \( \gamma \)-irradiation reduced the collagen content by more than 50%, and was considered the most damaging method. Ethanol and peracetic acid treatments were equivalent in preservation of general collagen (more than 95%).

Glycosaminoglycans (GAGs) are the other important components of ECM that bind growth factors and retain water due to their hydrophilic structure. Preservation of these components is
required for recellularization to create a whole solid organ. Sulfated GAGs contents of all samples (including non-treated and treated samples obtained from the four decontamination treatments) were determined by the Blyscan sGAGs assay and the results are presented in Figure 8–1. Peracetic acid in 4% (v/v) ethanol resulted in the most removal of sGAGs among all methods. Gamma-irradiated samples also showed significant reduction in sGAGs contents, which is in complete agreement with previous research that demonstrated depolymerization and degradation of GAGs after γ-irradiation (190).

Figure 8–1. Top panel: Collagen quantification of treated and control non-treated samples (n=5). There was no significant (NS) difference between solution-treated and non-treated samples, signifying that no damage to soluble collagen occurred during treatment by ethanol or peracetic acid. However, γ-irradiated samples had significantly (**) less collagen, showing greater damage to collagen fibrils. Bottom panel: GAGs quantification of treated and control non-treated samples (n=5). Treated means 1 h solution treatments and 3 kGy γ-irradiation. All treatment except peracetic acid in 1M NaCl caused significant reduction in GAGs content. (NS) means no significant difference (p-value>0.05) and (**) means significant difference (p-value<0.05).
Figure 8–2. Distribution of essential components of the ECM (n=3). Sirius, Orcein, Immuno, and PAS staining were used to detect general collagen, elastin, collagen IV, and GAGs, respectively. (B) The scoring system is based on preservation of the microscopic architecture of treated dECM examined by a pathologist blinded to the decontamination treatment. Results are reported as Avg±SD (n=5). In the charts, 70% EtOH, PAA-NaCl, PAA-EtOH, and γ-irradiation represent 1 h exposure to 70% ethanol in DI, 0.2% peracetic acid in 1M NaCl, 0.2% peracetic acid in 4% ethanol, and 3 kGy γ-irradiation, respectively. (NS) means no significant difference (p-value>0.05) and (***) means significant difference (p-value<0.05).

Scanning electron microscopy imaging of treated samples is shown in Figure 8–3. As demonstrated in this image, the filtration units of renal tissue (nephrons), which are required for functional kidneys and are considered the most important physical structures for cell migration and differentiation, were well preserved. Fiber diameter (Figure 8–3-E) and intersection density (Figure 8–3-F), the important factors for morphology and phenotype of cells in 3D cell culture, were similar for all decontamination treatments.
8.3.4 Swelling properties of ECM

To determine the possible damage to collagen fibrils of renal tissue during decontamination, the swelling ratio of treated samples was measured and the results are shown in Figure 8–4-A. Gamma-irradiated samples demonstrated the greatest water absorption, which might have been caused by cross-link elimination, increased void fraction, or structural damage to collagen fibrils.
Peracetic acid in 1M NaCl (ionic) solution preserved the integrity of structures more than any other solution. Peracetic acid in 4% ethanol in water caused more damage to structural cross-linking, purportedly due to the acidic pH. Peracetic acid in either ionic solution or 4% ethanol had pH around 3; however, the ionic solution reduced the damaging effect of the acidic component. These results corroborate the collagen quantification values and further confirm the relationship between collagen degradation and swelling ratio. Increased porosity of γ-irradiated samples also confirmed structural disruption that might be caused by irradiation (Figure 8–4-B).

Figure 8–4. (A) 1X PBS absorption of treated samples (n=6) representative of swelling and crosslinking damage during decontamination. (B) Thin sections of treated and non-treated tissue (5 μm) were stained with H&E and used for porosity evaluation using ImageJ software. Treated means 1 h solution treatments and 3 kGy γ-irradiation. DKS represents decellularized kidney scaffold, (NS) represents no significant difference with p-value>0.05 and (**) represents significant difference with p-value <0.05. The reported results are Avg±SD.
8.3.5 Surface chemistry of treated samples

Protein denaturation on the surface of non-treated and treated samples obtained from the four decontamination methods was examined by ATR-FTIR. The resulting spectra are shown in Figure 8–5. Amide I, II, and III bands are the most important determinants of the secondary structure of collagen on the surface of ECM, which has significant effects on cell-ECM interactions (142). No significant shift could be observed in these bands suggesting no further denaturation due to decontamination after decellularization, which is comparable with previous studies (143). The carbonyl group (wavenumber 1710 cm\(^{-1}\)) seemed to be damaged in samples treated with 70% ethanol and PA in 4% ethanol. The damage may have occurred as a result of mild oxidation of the samples as a result of the decontamination step.

![Figure 8–5. FTIR spectra of non-treated (control) and treated dECM samples. No significant differences were observed between the various decontamination methods and spectra showed no shifting in amide bands, signifying that surface proteins were not further denatured during decontamination. Treated means 1 h solution treatments and 3 kGy \(\gamma\)-irradiation.](image)
8.3.6 Cytotoxicity of treated samples and potential for support of cell growth

The various decontamination treatments can produce several by-products that could be toxic to cells and induce necrosis. To remove cytotoxic by-products, treated samples were washed with autoclaved distilled water before adding cell culture media. To compare cytotoxicity, MDCK cells were grown in 6-well plates in the presence of pieces of tissues treated with one of the four decontamination methods. A Live/Dead assay was used to determine the number of viable and dead cells after 3 days and the results are shown in Figure 8–6. Cells grown in media containing treated samples were as viable as cells in control media (no dead cells were detected), which indicates that no toxicity caused by treated samples was observed after 3 days of cell growth.

The most important attribute of dECM that must be preserved during decellularization and decontamination is the capability to support cell adhesion and growth during recellularization. To examine this potential, MDCK cells were used to repopulate treated dECM samples. DNA contents were measured after 1 and 3 days (Figure 8–6), as an indirect method for determination of cell number. Residual DNA after decellularization and incubation in cell culture medium was less than 5 ng/mg of dry tissue; therefore, the quantified DNA after repopulation was attributed to the MDCK cells and representative of cell adhesion and growth. The γ-irradiated samples demonstrated considerably less cell adhesion potential than the three solution decontamination treatments.
Figure 8–6. Cytotoxicity of treated samples (n=3) was compared using canine renal epithelial cells and Live/Dead assay. No dead cells were observed after three days of cell culture in the presence of treated dECM, and cell proliferation was unhindered. DNA content of recellularized samples of treated tissues (n=6) was measured after 1 and 3 days. Cell adhesion and growth of samples treated with ethanol or solution containing peracetic acid in NaCl were significantly higher than the other two samples; especially, γ-irradiated samples showed considerably less capability to support cell adhesion and growth. Treated means 1 h solution treatments and 3 kGy γ-irradiation.

8.4 Discussion

Whole organ regeneration is an active area of research in tissue engineering and regenerative medicine. The hope of this technology is that organs can be built from porcine dECM
and human cells to replace the need for donor organs and reduce the risk of organ rejection by a patient’s immune system (6). Various steps are necessary before a scaffold is ready for recellularization. First, cadaveric or animal organs are harvested and decellularized. Then, a gentle decontamination method is applied to remove contaminants that can cause infection and/or inflammatory response at the time of recellularization and transplantation. Decontamination of complicated solid organs such as kidneys is challenging since common methods, such as ethylene oxide that requires diffusion through solids, are not applicable (191).

In this study, decellularization of whole porcine kidneys with perfusion of 0.5% (w/v) SDS solution through the inherent vasculature was performed using an improved method in less than 7 h. Previous researchers have accomplished the decellularization of whole porcine kidneys using less efficient methods. For example, Orlando et al. perfused kidneys for 48 h with 0.5% (w/v) SDS under a constant flow rate (50) and Sullivan et al. studied whole porcine kidney decellularization using 0.5%, 0.25% (w/v) SDS, and 1% (v/v) Triton X-100 in 36 h. By freezing kidneys at -20°C, as suggested by several previous authors, and increasing flow rate under constant pressure, we were able to reduce the decellularization process time (161, 192). Four different decontamination treatments were compared using slices of kidney tissue. All of the experiments were performed with uniform sizes of tissues, which were obtained from the same kidney for each set of comparison tests. In order to extrapolate these results to whole decellularized organs, and provide a safety margin, it was determined that 2 h perfusion with the decontaminating solutions; or 10-25 kGy γ-irradiation, as suggested by previous researchers, were needed to decontaminate whole kidneys. We now routinely use 2 h perfusion of 0.2% peracetic acid in 1 M NaCl to prepare whole organs for recellularization experiments.
Previous researchers have used sterile PBS containing 10,000 U/ml penicillin G, 10 mg/ml streptomycin and 25 μg/ml amphotericin B to sterilize whole rat kidneys and whole rat hearts (12, 64). However, their method was not effective under the conditions in which kidneys in this study were harvested at an abattoir in a non-aseptic manner. Stronger decontaminants were required to remove all contaminating agents from porcine organs. Other researchers have proposed similar decontamination methods, such as peracetic acid for rat kidneys (44), γ-irradiation for porcine kidneys (8), and the combination of peracetic acid and chloroform gas for rat livers (193).

According to our results, 70% (v/v) ethanol in DI water has a great potential to decontaminate porcine renal tissue. It is also very commonly used to decontaminate equipment and surfaces during cell culture experiments. However, ethanol is not considered strong enough to remove hydrophilic viruses and bacterial spores and therefore is not recommended for clinical use (194). It has also been reported that ethanol can denature proteins, dehydrate ECM, and affect cell-ECM interactions (191). The 70% ethanol solution was included in this study as a comparator.

Gamma-irradiation is another widely used method to sterilize biological tissues, however, substantial damage to the microstructure and composition have been reported (195, 196). The degradation effect of γ-irradiation on different types of collagen has been previously reported for decellularized lung tissue (197), human dermis (198), and porcine pulmonary valves (196). Gamma-irradiation can cause scission of alpha polypeptide bonds or crosslinking in the presence of free radicals (191). In addition, γ-irradiation can induce fragmentation of GAGs by production of hydroxyl, carbonate, and nitrogen dioxide radicals (190). It has also been shown that γ-irradiation intensity greater than 2 kGy could cause ECM instability for dermis tissue and decrease the denaturation temperature of ECM below the temperature of the body (198). In contrast to these results, Uriarte et al. reported that resistance and elasticity of decellularized lungs were improved
and modified after γ-irradiation (197). It has been reported that for the decontamination of an organ as large as a porcine kidney, at least 10 kGy intensity of γ-irradiation would be required. Even with 3 kGy intensity we observed considerable structural damage (Figure 8–1) and a significant reduction in MDCK cell adhesion and proliferation on slices of dECM kidney tissue. We do not recommend the use of γ–irradiation for decontamination of whole organs.

Table 8-2 Summary evaluation of decontamination methods

<table>
<thead>
<tr>
<th>Decision criteria</th>
<th>70% Ethanol in DI water</th>
<th>0.2% Peracetic acid in 1M NaCl</th>
<th>0.2% Peracetic acid in 4% ethanol</th>
<th>3 kGy gamma irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen preservation</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GAGs preservation</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>ECM intactness</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cost</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Waste management</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Process feasibility</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

For each method, the decision criteria were evaluated and samples were assigned one of three scores: good (+), fair (+/-), or poor (-).

Peracetic acid is a strong oxidizing agent that can disrupt the cellular membrane and cause cell death (199). It was observed that peracetic acid in low concentrations was able to inactivate viruses, fungi, and bacteria with the creation of non-toxic by-products such as water, oxygen, and...
carbon dioxide (200). Previous researchers have used peracetic acid to sterilize decellularized lungs (201), human heart valves (202), decellularized anterior cruciate ligaments (203), human spongiosa cuboids (204), and whole rat kidneys (60). It has been suggested that it is better to use peracetic acid in neutral pH solutions or high ionic strength to preserve collagenous structures without any swelling damage. Low concentrations of peracetic acid in ethanol can lead to dissolution of collagen and swelling in collagen fibrils because of low pH.23 Our findings here also confirmed that peracetic acid in ionic solution could preserve microstructure, surface chemistry, and cell adhesion sites more than peracetic acid in ethanol solution or any other applied decontamination treatments.

Different tissues have unique compositions and are used for a variety of purposes. The decontamination process must be selected according to tissue type and the tissue’s specific purpose. A summary of the results of this study is reported in Table 8–2. An aqueous solution of 0.2% (v/v) peracetic acid in 1 M NaCl solution performed the best among the tested decontamination treatments by preserving essential components of the dECM that are necessary to promote increased cell adhesion and proliferation. We suggest that a combination of rapid decellularization with a gentle and reliable decontamination treatment of peracetic acid in aqueous ionic solution is the best method to prepare a whole porcine kidney scaffold for recellularization.
9 RE-EPITHELIALIZATION OF WHOLE PORCINE KIDNEY WITH RENAL EPITHELIAL CELLS

9.1 Introduction

This chapter is an adaptation of the article entitled “Re-epithelialization of whole porcine kidney with renal epithelial cells” submitted for publication in November 2016 to the Journal of Biomaterials Applications. This work was developed and led by myself with the help of Evan M. Buckmiller, Lara B. Schaumann, Haonan Wang, Dr. Jonathan J. Wisco, Dr. Beverly L. Roeder, Dr. Paul R. Reynolds, and Dr. Alonzo D. Cook.

Chronic kidney disease (CKD) and end stage renal failure (ESRF) are among the leading causes of mortality worldwide. According to the National Kidney Foundation, in 2013, more than 47,000 Americans died from kidney failure (205). The only definitive treatment for ESRF is whole kidney transplantation (118, 206). However, many patients will die while waiting for a kidney transplant and those patients who are fortunate enough to receive a kidney must remain on immunosuppressive medication for their whole life (6). Engineering artificial kidneys with patient-specific cells may eliminate the risk of immune response induction and the need for a donor organ. A promising approach for creating artificial organs is decellularization of allogeneic or xenogeneic organs to clear away all potential immunogens followed by recellularization with patient-specific cells to regenerate whole healthy organs (16, 36, 207).
Decellularization of rat (45, 46, 56, 59), porcine (51, 61, 62), and human (3, 207) kidneys has been reported in the previous literature. However, recellularization of whole intact renal scaffolds remains a challenge due to the complex structure of kidneys and the numerous cell types in renal tissue required for healthy function. At least one layer of endothelial cells and one layer of epithelial cells are required to get partial filtration -- the prominent kidney function.

Re-endothelialization of whole hearts (12, 64, 152), livers (208, 209), lungs (210, 211), and kidneys (13, 53) has been previously reported. Although it remains a major challenge to cover the entire organ’s vascular network, re-endothelialization has been more studied than re-epithelialization of the tubules and collecting ducts. In most published studies, endothelial cells were perfused through the organ’s vasculature with controlled flow rate to allow the cells to adhere and grow within the vascular tree. However, re-epithelialization is not as straightforward since there is no specific pathway for perfusion of epithelial cells. Regeneration of cardiac muscle has been achieved by injection of muscle cells directly into the cardiac scaffold (12), although this method would not be applicable for organs such as the lung and kidney with their complicated structures. In 2013, Song et al. reported a novel method for re-epithelialization of whole rat kidneys by creating a trans-renal pressure gradient and introducing cells through the ureter into the intact renal scaffold (13).

In this study, we decellularized whole human-sized porcine kidneys with our previously developed method and investigated vascular preservation by MRI imaging. We also scaled up the previously described re-epithelialization method for rat kidneys, to recellularize porcine kidney scaffolds, and developed the various procedures.
9.2 Experimental design

Whole porcine kidneys were decellularized with the method described in section 4.3.4. Renal epithelial cells were labeled with iron particles and perfused through the renal artery of native and decellularized kidneys (section 4.10.2). Then, MRI imaging of perfused kidneys were obtained to visualize the vascular network of acellular and native kidneys (section 4.6.6).

Special bioreactors were designed and constructed for whole kidney recellularization as described in section 4.11.1. Decellularized kidneys were decontaminated (section 4.11.2) and aseptically hooked up to the sterile bioreactors. Renal epithelial cells were seeded onto the whole kidney ECM as described in section 4.11.3 and after 7 days of culture the samples were taken for standard H&E staining (4.7.4). In another set of experiments, the cells were labeled with fluorescent color and seeded into the renal ECM with the best seeding method. After 7 days of being in culture condition, the samples were taken and 3D imaging was performed as described in section 4.11.4.

9.3 Results

9.3.1 Vasculature investigation of whole porcine renal scaffold using Magnetic Resonance Imaging (MRI)

Native and decellularized porcine kidneys were perfused in either the renal artery or proximal ureter with MDCK cells (1 million cells/ml in a total of 100 ml cell solution for each kidney) that were labeled with iron oxide particles (40 µg/ml). GRE MR images showed the positive location of the cells containing iron oxide. Cells injected into either native (Figure 9–1-a, b) or decellularized (Figure 9–1-c, d) kidneys were confined to the spaces corresponding to the
perfusion vessel, either in the arterial branches or renal spaces. Cells diffused into the cortical vasculature in the decellularized kidney, whereas cells diffused as far as minor calyces in the urinary space in both native and decellularized kidneys. There was no significant difference between cell flow through native and decellularized scaffolds confirming vasculature preservation after decellularization.

Figure 9–1. MRI imaging of native (a, b) and decellularized (c, d) porcine scaffold after arterial (a, c) and ureteral (b, d) injection of MDCK cells labeled with iron oxide particles.
9.3.2 Optimization of cell seeding to the renal scaffold

Introducing the cells back to the renal scaffold was optimized by changing cell solution concentrations and seeding methods. A total number of 500±100 million cells with concentrations 1×10^8 cells/ml, 1×10^7 cells/ml, and 5×10^6 cells/ml were perfused or injected through the renal ureter. We observed that the lower concentrations of cells resulted in more homogeneous distribution of cells throughout the kidney. High concentrations of cells led to cell aggregation formation in the tubules of the medulla region without any cells reaching the cortex region. The best concentration of cells was found to be 4-5 million cells/ml of culture medium for all applied seeding methods.

In the first seeding method, the kidneys were arterially perfused (Figure 4–3-b) with cell solutions at high flow rate (80-100 ml/ml) for 20 min with the hypothesis of forcing the cells into the tubules by pushing them through the vessel’s basement membranes. Subsequently, arterial perfusion with oxygenated cell culture media was resumed at a low flow rate (2 ml/min) to avoid washing the cells out of the ECM while providing nutrients and oxygen for the viable cells. After 24 h the culture media flow rate was increased to 10 ml/min and continued for 7 days. The H&E staining of the recellularized kidneys (Figure 9–2-a, b) demonstrated that a high flow rate and, consequently, high pressure did not push the cells into the tubules and the majority of the cells remained on the vascular side. In addition, the high flow rate led to the cells washing out from the ECM and only a few cells remained in the glomeruli structure. No cells could be found in the medulla region (Figure 9–2-a).

In the second trial, the cells were injected through the ureter with high positive pressure to push the cells all the way to the cortex region and repopulate the tubule side of the kidney (Figure 9–2-c). Even at low concentration, the cells remained in the tubules of the medulla with few cells
reaching the cortex region (Figure 9–2-c, d). Since there was no direct output for the high flow rate solution perfusing through the ureter, the pressure became elevated, which could have damaged the cellular membranes.

In the third trial, the cells were injected through the ureter while the whole kidney chamber was under either 40 or 140 mmHg vacuum for 20 min (Figure 9–2-d). The H&E staining of resultant recellularized kidneys showed that a majority of the cells were in the cortex region with no cells in the medulla region (Figure 9–2-e, f). This result demonstrated that the vacuum pressure was the best option to move the cells to the cortex region. However, vacuum pressure should not be too high, and we found that 40 mmHg was the better negative pressure for porcine kidneys to repopulate the medulla and cortex regions with minimal cell aggregation. As shown in Figure 9–2-e and h, cells lined the tubules in the medulla and cortex regions without cell aggregation.

9.3.3 3D Imaging of recellularized kidneys with best method

Canine MDCKs were labeled with fluorescence color (as described in section 4.10.1) before injection through the ureter under 40 mmHg vacuum. After one week of cell growth, confocal imaging of thin sections of the renal cortex and medulla was performed and the resulting images are shown in Figure 9–3. The thin slices were excised randomly from various regions of renal tissue, and the results showed complete and homogenous distribution of cells throughout the entire structure. In the medulla region, the majority of tubules were repopulated with labeled cells with patent lumens. Imaging of the cortex region demonstrated the presence and growth of renal cells and recellularization of Bowman’s capsules with open lumen without signs of cell aggregation. The intact structure of the convoluted tubules could also be observed in the cortex region.
Figure 9–2. Standard H&E staining of recellularized renal scaffold in medullar (a, c, e, and g) and cortical (b, d, f, and h) regions 7 days after various seeding methods.

Figure 9–3. 3D imaging of recellularized kidney 7 days after ureteral injection of colored MDCK cells with trans-renal pressure gradient (under 40 mmHg vacuum). Bars represent 500 µm (a-1 and b-1), 250 µm (a-2 and b-2), and 200 µm (a-3 and b-3).


9.4 Discussion

Various methods have been proposed to rebuild kidneys (16, 26, 57). The reasons to pursue renal tissue regeneration include the growing waiting list for kidney transplants, poor engraftment of transplanted organs due to immune rejection, and the lifetime need for immunosuppressive medications following transplants. One promising approach to engineer a human-sized kidney without the risk of immune rejection (6) is the decellularization of a porcine kidney to achieve a perfect matrix for healthy cell growth followed by recellularization of the scaffold by patient-specific human cells. In this study, decellularization of whole porcine kidneys was performed by perfusing detergent through the vascular network to remove most of the cellular materials and potential immunogens to avoid immune response induction at transplantation (8, 40, 50, 62). We previously optimized and characterized detergents (212) and processes (154) for whole porcine kidney decellularization and applied the best method in this study. We then recellularized the porcine kidneys with renal epithelial cells and characterized the kidneys using histology, SEM, MRI and confocal microscopy.

One of the fundamental properties of a naturally obtained scaffold is the inherent vascular network, which is critical for healthy organ function (207, 213). Vascular network intactness of porcine kidneys (8) and rat kidneys (44) after decellularization was previously investigated by CT imaging and perfusion of fluorescently labeled cells followed by confocal microscopy, respectively. We decided to investigate imaging of decellularized kidneys by MRI. Gadolinium solution is typically used with MRI to get a high-resolution image of the vascular tree; however, we found that gadolinium diffused out of acellular vessel walls due to the lack of an endothelial layer and the relatively small size of gadolinium molecules. Therefore, in this study, MRI images of the vasculature and tubule structures of native and decellularized porcine kidneys were obtained.
after perfusing the structures with iron oxide-labeled MDCK cells. The labeled cells were injected through the ureter or artery. MDCK cells were not able to diffuse through the vessel walls because of their relatively large size (214, 215). We labeled the MDCK cells with iron oxide particles to achieve high enough resolution in MRI imaging. The intact vasculature and tubule structures of decellularized scaffolds compared to the native kidney is shown in Figure 9–1. Cortical capillaries were not detectable for native kidneys due to the relatively large size of cells and lack of transport through this region.

Human-sized kidney recellularization introduces several challenges due to the relatively enormous size of the scaffold. Previously, researchers recellularized rat kidneys (13, 44, 46, 47, 55), to serve as a smaller model of a human-sized kidney. Recently, Ko et al. reported re-endothelialization of whole piglet kidneys and reconstruction of the vascular tree throughout the renal scaffold (53). In another study, Abolbashari et al. performed re-epithelialization of porcine kidney scaffolds by multiple injections of primary renal cells to the cortical region of renal tissue. They showed partial reconstruction of tubules with limited function of the renal cells compared to a native kidney (216). However, this method would not be applicable for reconstruction of a whole porcine kidney with complicated structures in the cortical and medullar region.

In this study, we have scaled up a previously used method for re-epithelialization of rat kidneys and determined the best cell concentration and seeding procedures to achieve the homogenous distribution of cells throughout the whole porcine kidney scaffold. We tested three methods: perfusion through the renal artery under high pressure, perfusion through the ureter under high pressure, and perfusion through the ureter under moderate vacuum. These methods were based in part on previous literature. For example, in 2014, Caralt et al. reported re-epithelialization of whole rat kidneys with tubular epithelial cells by perfusion of the cells through the vascular
network with high pressure. They showed that high pressure caused the cells to pass through the vessel walls and repopulate the tubule side of the renal structure (44). When we applied this method for whole porcine kidney scaffolds, we found that the majority of cells were washed out from the renal scaffold, and few cells remained in the capillaries of the glomerulus in the cortex region (Figure 9–2-a, b). As another example, in 2013, Song et al. applied different methods of seeding neonatal kidney cells to rat kidneys and found the ureteral injection of cells using a trans-renal pressure gradient under a vacuum of 40 cmH$_2$O (30 mmHg) led to the most homogenous distribution of epithelial cells throughout the entire scaffold (13). We confirm herein that a moderate vacuum (40 mmHg) is the most efficient approach of those we tried for homogeneous distribution of cells through the medullar and cortical regions without resulting in cell blockage (Figure 9–2-g, h).
10 CONCLUSIONS AND RECOMMENDATIONS

10.1 Conclusions

As explained in Chapter 2, decellularization of native porcine kidneys and recellularization with human cells is a promising approach for regenerating a whole human-sized kidney without the risk of immune rejection. In these experiments, we focused on developing this technology and improving some of the steps to achieve this goal.

In the first step, we characterized slices of renal tissue treated with various cell-lysing agents to find the most efficient agent for removing cellular materials while keeping the scaffold as intact as possible. Our results demonstrated that NaOH was the only agent that reduced the residual DNA below established criteria (50 ng/mg). The NaOH-treated scaffold was also the most efficient platform to support growth and metabolic activity of cultured cells (Figure 5–5), which might be due to fused fibers and more preserved collagen (Figures 7 and 8). However, working with NaOH on a large scale would introduce several process challenges such as disposal of the high pH solution after decellularization.

The next reasonable choice was SDS, which is also the most frequently used agent for whole organ decellularization. We decided to use SDS for whole organ decellularization with a lower concentration in a modified manner to reduce SDS exposure of scaffold and therefore, causing minimum damage to the ECM. A solution of 0.5% SDS combined with hypo/hypertonic solutions (tonic cycle method) was used to decellularize the whole porcine kidney scaffold. We
designed and constructed an automated bioreactor equipped with a pressure control loop and bubble trap to provide optimized detergent flow throughout the entire vasculature of porcine kidneys (Figure 4-2). The combination of a pressure control circuit and applying osmotic shock to the cellular membranes resulted in significant improvements in critical properties of renal ECM (Chapter 6). GAGs and collagen were more preserved with optimized decellularization methods (Figure 6–4), which might have been the primary cause of enhanced cell growth and gene expression of cells cultured on the renal scaffold (Figure 6–9).

One of the challenging issues in our lab was preserving native and acellular ECMs. Freezing/thawing with or without cryoprotectants is the most frequently applied method to preserve mammalian tissues. We comprehensively characterized native and acellular ECM after freezing/thawing cycles to find the least damaging way to preserve the collagenous structure. We discovered that elastic modulus (Figure 7–2) and arterial pressure (Figure 7–3) of native kidneys were significantly reduced after freezing/thawing; however, they were the same for decellularized ECM. Our obtained results demonstrated that collagenous fibers remained intact through freezing/thawing for either acellular or native kidneys and the observed reduction in mechanical strength of native kidneys was mostly due to cellular disruption after ice formation.

Decontamination of renal scaffolds before recellularization is a challenging step since sterilizing agents are usually damaging to the protein structure, toxic to human cells, and sometimes not effective in removing fungus contamination. The organ-specific impact of various decontaminating procedures on renal scaffolds was studied to determine the least damaging method for decontamination of whole porcine kidneys. Our obtained results suggest that peracetic acid in a high ionic solution was the least damaging agent to essential components of the ECM
with enough antimicrobial capacity to remove all potential microbes (Figure 8–1). Peracetic acid was also shown to be safe for cell culture with no demonstrated cytotoxicity.

The last part of this work was focused on recellularization of whole renal ECM. We designed and constructed a bioreactor that could be autoclaved and fit in our incubator (Figure 4–3). We improved the seeding procedure to get an homogenous distribution of cells throughout the entire regions of ECM without cells aggregating in the tubules (Figure 9–2). Our results revealed that the only way to draw epithelial cells to the cortex region was to apply vacuum pressure to the kidney chamber. However, the vacuum pressure needed to be adjusted according to kidney size, which for our typical size was 40 mmHg. We also showed that after just 7 days of cell culture the 3D structure of tubules and glomeruli were formed (Figure 9–3), although, for having partially functional kidneys more confluent glomeruli would be needed.

10.2 Recommendations

We studied the impact of cell-lysing agents on slices of renal tissue and then extended our findings to whole organ decellularization; however, it would be advantageous to test whole organ decellularization directly for a more accurate conclusion.

We also developed a procedure for tissue preservation without the addition of cryoprotectants since they are known to be toxic to the cells. We recommend investigating various cryoprotectants and test their cytotoxicity for a more efficient preservation of the collagenous structure.

We used polypropylene materials to construct our recellularization bioreactors. We recommend building the whole system from glass for easier handling and more efficient sealing during autoclave to avoid unwanted contamination. We also suggest the insertion of a more
effective aeration system to the previous design to be able to continue the cell culture process for a longer time, achieve more confluency, and consequently, obtain partially functional kidneys.

We gathered some preliminary data on the functionality of recellularized kidneys by creatinine clearance measurements after perfusing the kidneys with a standard creatinine containing solution. However, more confluent kidneys and more repetition are needed to get consistent results. We recommend multiple injections of cells into renal scaffolds and prolonged incubation of recellularized kidneys under culture conditions as well as more efficient aeration to achieve confluent functional kidneys. We predict the creatinine clearance tests would be more accurate and consistent using confluent kidneys.

One of the main obstacles in re-epithelialization of whole porcine scaffolds was distributing cells through inaccessible areas that might be created during the decellularization procedure. Alternatively, we demonstrated that lyophilizing the naturally obtained scaffolds would result in a more porous structure with more available surface for cells to grow. We recommend modification of the entire renal scaffold by several cycles of lyophilization followed by re-epithelialization of the whole organ to get a more homogeneous distribution of cells throughout the entire scaffold.

We gathered some preliminary data on re-endothelialization of whole porcine kidney scaffolds; although we haven’t tried to find the best seeding condition for these types of cells. We recommend co-culture of endothelial and epithelial cells on whole renal scaffolds and using human blood to measure the functionality of resultant kidneys.

Organ-specific extracellular matrices have been shown to effect the metabolic activity of somatic cells and direct stem cell differentiation toward the proper phenotype. We studied gene
expression patterns of renal cells cultured on various treated tissues and observed significantly
different results due to our characterizations of various ECMs. We recommend testing growth and
differentiation of stem cells on acellular ECMs obtained from various decellularization procedures
as one of the essential characteristics.

It has also been reported that organ-specific cells can produce critical growth and signaling
factors that are able to direct stem cell differentiation to the proper phenotype. We highly
recommend experiments on using conditioned media of MDCK cells to grow and differentiate
stem cells to a renal epithelial lineage.
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