A Novel Dorsal Slit Approached Non-Ischemic Partial Nephrectomy Method for a Renal Tissue Regeneration in a Mouse Model

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Abstract

BACKGROUND: Kidney ischemia–reperfusion (IR) via laparotomy is a conventional method for kidney surgery in a mouse model. However, IR, an invasive procedure, can cause serious acute and chronic complications through apoptotic and inflammatory pathways. To avoid these adverse responses, a Non-IR and dorsal slit approach was designed for kidney surgery.

METHODS: Animals were divided into three groups, 1) sham-operated control; 2) IR, Kidney IR via laparotomy; and 3) Non-IR, Non-IR and dorsal slit. The effects of Non-IR method on renal surgery outcomes were verified with respect to animal viability, renal function, apoptosis, inflammation, fibrosis, renal regeneration, and systemic response using histology, immunohistochemistry, real-time polymerase chain reaction, serum chemistry, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and Masson's trichrome staining.

RESULTS: The Non-IR group showed 100% viability with mild elevation of serum blood urea nitrogen and creatinine values at day 1 after surgery, whereas the IR group showed 20% viability and lethal functional abnormality. Histologically, renal tubule epithelial cell injury was evident on day 1 in the IR group, and cellular apoptosis enhanced TUNEL-positive cell number and Fas/caspase-3 and KIM-1/NGAL expression. Inflammation and fibrosis were high in the IR group, with enhanced CD4/CD8-positive T cell infiltration, inflammatory cytokine secretion, and Masson's trichrome stain-positive cell numbers. The Non-IR group showed a suitable microenvironment for renal regeneration with enhanced host cell migration, reduced immune cell influx, and increased expression of renal differentiation-related genes and anti-inflammatory cytokines. The local renal IR influenced distal organ apoptosis and inflammation by releasing circulating pro-inflammatory cytokines.

CONCLUSION: The Non-IR and dorsal slit method for kidney surgery in a mouse model can be an alternative surgical approach for researchers without adverse reactions such as apoptosis, inflammation, fibrosis, functional impairment, and systemic reactions.

Keywords Ischemia–reperfusion · Renal regeneration · Inflammation · Apoptosis · Fibrosis

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1 Introduction

Kidney ischemia–reperfusion during partial nephrectomy is a conventional method in mouse experiments [1]. However, this method can cause serious complications and is closely related to chronic renal dysfunction [2]. Among several renal cell death mechanisms associated with ischemia–reperfusion, apoptosis and inflammation seriously damage the renal tubule epithelium, which plays an important role in maintenance of kidney function [3]. In case of ischemia–reperfusion injury, renal tubule cells express ‘death receptors’ on their surfaces [3], and their downstream caspases cause apoptotic cell death [3, 4]. The key molecules in this pathway are Fas [5] and caspase-3 [5, 6]. The injured tubule cells release specific proteins, such as kidney injury molecule-1 (KIM-1) [5] and neutrophil gelatinase-associated lipocalin (NGAL) [5]. Inflammatory responses follow apoptotic cell death [5, 7], which causes endothelial cell dysfunction and immune cell influx [5]. The infiltrated leukocytes secrete pro-inflammatory cytokines that further amplify inflammatory reactions [5]. Excessive inflammation promotes fibrosis [3], which subsequently worsens kidney function. In addition, the surgical approach for exposing kidneys requires improvements. Kidneys lie in the *retroperitoneal space*, and hence, *dorsal slit* can be an alternative approach to laparotomy. Compared to laparotomy, the *dorsal slit* has several advantages, such as minimal invasiveness, easy approach, and early recovery [8].

In the case of renal experiments such as scaffold implantation for restoring renal damage by partial nephrectomy, renal ischemia–reperfusion might interfere with evaluation of the effects of the treatment. Therefore, a non-apoptotic,—inflammatory and—fibrotic approach would be better for analyzing the effect of the treatment for kidney regeneration. Hence, it is necessary to develop a

surgical procedure that can replace renal ischemia–reperfusion. The newly designed renal approach for mouse experiments consisted of non-ischemia–reperfusion with dorsal slit. To verify the effectiveness of the new method for renal tissue regeneration, apoptosis, inflammation, fibrosis, release of factors from injured tubule cells, renal cell differentiation, and other distant organ responses were compared between the new and conventional methods.

2 Materials and methods

2.1 Animal models and surgical procedures

All procedures were performed in accordance with an animal protocol approved by the Yeungnam University Institutional Animal Care and Use Committee (YUMC-AEC2016-003). Sixty 5-week-old male ICR mice weighing 20 g (Orient, Seongnam, Korea) were randomly divided into three groups (each group $n = 20$): (1) Ctrl, sham-operated control group; (2) IR group, where a ventral incision was made and the renal artery and vein of the left kidney were tightly occluded with a vascular clamp for 20 min during scaffold implantation (Fig. 1A) and kidney reperfusion was allowed after the clamp was removed; and (3) Non-IR group, where the dorsal slit was made (length: 5 mm) and kidneys were exposed (Fig. 1B). The scaffold was implanted into the left kidney without renal artery clamping. The right kidneys of all experimental mice were removed after scaffold implantation. The amount of bleeding was measured by weighing the gauzes used during surgery, and the surviving population was counted for 14 days. Body temperature was maintained with a homoeothermic blanket (37 °C) until the mice were awake. The implanted renal scaffold (size $5 \times 2 \times 2 \text{ mm}^3$, Fig. 1C) was composed of poly(lactic-co-glycolic acid) (PLGA, MW = 40 kDa; Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) and 10% $\text{Mg}(\text{OH})_2$ (Sigma-Aldrich Corporation, St Louis, MO, USA) [9]. Animals were sacrificed 1, 3, 7, and 14 days after the surgery, and kidney, blood, and other organs (spleen, liver, lung, heart, and brain) were collected.

2.2 Assay for serum creatinine and blood urea nitrogen (BUN)

Serum creatinine and BUN assays were performed for analysis of renal function. These values were measured using the serum obtained at each time point, and the creatinine (R&D Systems, Minneapolis, MN, USA) and BUN kits (Roche, Basel, Switzerland) according to manufacturer’s direction.

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Fig. 1 Mouse surgery for partial nephrectomy and scaffold implant. **A** Ischemia–reperfusion with ventral incision surgery (IR). **B** Non-ischemia–reperfusion with dorsal slit surgery (Non-IR). **C** Scaffold

implanted kidney. Incision and scaffold size: $5 \times 2 \times 2 \text{ mm}^3$. Scaffold composition: PLGA (molecular weight = 40 kDa) with 10% $\text{Mg}(\text{OH})_2$

2.3 Histopathology and immunohistochemistry (IHC) for apoptosis, immune cell infiltration, inflammation, and fibrosis

For histopathological and IHC analysis, the renal cortex and scaffold-implanted region were fixed with 4% paraformaldehyde 1, 3, 7, and 14 days after the operation (5 mice were sacrificed each day from each group). The paraffin-em-bedded samples were cut into 5- μm sections for hematoxylin and eosin (H&E) and IHC staining, and analyzed for apoptosis, immune cell detection, inflammation, and fibrosis. The antibody information for IHC is listed in Table 1. The primary antibody was treated overnight at 4 °C followed by incubation with secondary antibody for 1 h (Alexa Fluor 594, Life Technology, Waltham, MA, USA), and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) was used for staining the nuclei. Histopathological findings were performed by a pathology specialist. Renal injury was assessed with respect to the brush-border of epithelial cells, Bowman's capsule, cellularity in glomerulus, luminal debris, immune cell infiltration, and interstitial fibrosis, and at least 20 visual fields per tissue were observed.

2.4 Gene expression related to apoptosis, inflammation, fibrosis, tubule cell injury and renal regeneration

RNA was extracted with Trizol reagent, and cDNA was synthesized from 20 μg total RNA using the Superscript Choice cDNA synthesis kit (Invitrogen, Waltham, MA, USA). The real-time PCR conditions using SYBR green were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 50 s, and 72 °C for 20 s. The $2^{-\Delta\Delta\text{Ct}}$

method was used for analyzing the relative changes in gene expression. The primer sequences for apoptosis-, inflammation-, fibrosis-, injured tubule cell-, and renal regeneration-related genes, and the housekeeping gene *GAPDH* are shown in Table 1.

2.5 Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay for detecting apoptosis

For determining the histological aspects of apoptosis, TUNEL assays were performed with an ApopTag peroxidase in situ apoptosis detection kit (Chemicon, Bedford, MA, USA) following the manufacturer's instructions. Briefly, after deparaffinization and rehydration, the slides were digested with 20 $\mu\text{g}/\text{mL}$ proteinase K and treated with 3.0% hydrogen peroxide for quenching endogenous peroxidase. The slides were then immersed in $1 \times$ TdT equilibration buffer, working strength TdT enzyme was added, and incubated for 1 h at 37 °C. An anti-digoxigenin conjugate was applied on the slides for 30 min at room temperature and color was developed using the peroxidase substrate for 3–6 min at room temperature. Coverslips were mounted on the slides after 1 min incubation with DAPI. Measurements of TUNEL-positive nuclei were performed on 5 images/slide using a microscope (400 $\times$), and each counted area was 700 μm^2 .

2.6 Masson's trichrome staining for detecting fibrosis

The Masson's trichrome staining kit (IHC WORLD, Elliott City, MD, USA) was used following the manufacturer's instructions for detecting collagen fibers in the

Table 1 Details of antibodies and genes used in immunohistochemistry and real-time PCR

Category	Gene	Full name	Company, dilution	Primer sequences
Apoptosis	Fas	Fas/APO-1/CD95	Abcam, 1:100	5'-tccgagagttaaagctgag-3' 5'-ttgacagcaaaatggcctc-3'
	Caspase-3	Caspase-3	Cell Signaling, 1:1000	5'-gagcagctttgtgtgtga-3' 5'-acaggccattgtccata-3'
Immune cell	CD4	Cluster of differentiation 4	Abcam, 1:200	–
	CD8	Cluster of differentiation 8	Abcam, 1:200	–
Inflammation	IL-1β	Interleukin-1 beta	Abcam, 1:200	5'- gcccatcctctgagactcat-3' 5'- aggccacaggtatttgcg-3'
	IL-6	Interleukin-6	Abcam, 1:400	5'- agttgcctcttgggactga-3' 5'- tccacgattcccagagaac-3'
	TNF-α	Tumor necrosis factor alpha	Abcam, 1:200	5'- agccccagctctgtatcct-3' 5'- ctcccttgcagaactcagg-3'
Injury tubule cell	KIM-1	Kidney injury molecule 1	–	5'-tggagggtgcttcagtgt-3' 5'-tggagggtgcttcagtgt-3'
	NGAL	Neutrophil gelatinase-associated lipocalin	–	5'-ctacaaccagttccatgg-3' 5'- gacagctccttggttctcc-3'
Renal regeneration	Pax2	Paired box 2	Abcam, 1:1000	5'- aaatctctatgcaaatgac-3' 5'- gagagatgcaggcgatga-3'
	Wt1	Wilms tumor 1	Santacruz, 1:200	5'- ggtccgcatcacacatg-3' 5'- ctttcctgctgggatgct-3'
	Emx2	Empty spiracles homeobox 2	Novusbio, 1:100	5'- tggccagaagccaaagc-3' 5'- tccgtcccaccagtaac-3'
	vWF	von Willebrand factor	Santacruz, 1:200	5'- gctgtgcggtgatttaacat-3' 5'- ccgtttacaccgctgttct-3'
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	–	5'- tgtgtccgtcgtggatctga-3' 5'- cctgcttcaccaccttctga-3'

kidney. Briefly, after deparaffinization and rehydration, the slides were re-fixed in Bouin's solution for 1 h at 56 °C to improve staining quality. After rinsing, tissues were stained in Weigert's iron hematoxylin working solution for 10 min. After washing, the slide was stained in Biebrich scarlet-acid fuchsin solution for 10 min, and then put in phosphomolybdic-phosphotungstic acid solution until collagen was stained red. The slide was transferred into aniline blue solution for 5–10 min and then put in 1% acetic acid solution for 2–5 min. After washing, the slide was dehydrated in ethanol series, cleared in xylene, and mounted.

2.7 Statistical analysis

All experiments were performed at least in triplicate on separate days. A *t* test and one-way analysis of variance (ANOVA) with Tukey's post hoc test were used for statistical analysis. All values are expressed as mean ± standard deviation (SD). Results are representative of at least three experiments.

3 Results

3.1 Animal viability and renal function

Viability of the IR group was 20%, whereas that of the Non-IR group was 100% for 14 days. In the IR group, 80 out of 100 mice were dead within 1.5 days after the operation, and the remaining mice from this time point survived. To determine the reason of low viability in the IR group, serum creatinine and BUN values were matched, because renal functional abnormalities directly affect animal mortality.

The IR group showed significantly increased serum creatinine and BUN concentrations at day 1 ($p < 0.05$), and from day 3, levels were restored to normal (Fig. 2A). The mean values of serum creatinine of the IR group on 1, 3, 7, and 14 days were 1.29 ± 0.30 , 0.33 ± 0.02 , 0.32 ± 0.03 , and 0.27 ± 0.01 , respectively, and those of the Non-IR group were 0.33 ± 0.15 , 0.21 ± 0.06 , 0.22 ± 0.04 , and 0.22 ± 0.01 mg/dL, respectively. The value of the control group was 0.205 ± 0.002 mg/dL. The mean BUN values of the IR group on 1, 3, 7, and 14 days

Fig. 2 Analysis for renal function and apoptosis. **A** Creatinine and BUN values for renal function. **B** Gross images of scaffold-implanted kidney. **C** Histological analysis with H&E stain. **D** Apoptotic cell identification using TUNEL assay (brown color) and positive nuclei quantification. **E** Immunohistochemistry and real-time PCR for

detection of apoptotic markers (Fas and caspase-3). **F** Renal tubule injury markers (KIM-1 and NGAL) expression with real-time PCR. Magnification: $\times 400$, $*p < 0.05$ and $**p < 0.01$, Ctrl: sham operated normal ICR mouse, IR: ischemia–reperfusion with laparotomy, Non-IR: non-ischemia–reperfusion with dorsal slit

were 167.65 ± 25.75 , 27.45 ± 6.58 , 25.20 ± 3.96 , and 27.60 ± 0.57 , respectively, and those of the Non-IR group were 62.54 ± 11.09 , 24.93 ± 3.33 , 23.93 ± 4.68 and 25.05 ± 2.83 mg/dL, respectively. The value of the control was 22.75 ± 0.825 mg/dL. These serological test results indicated that the IR method caused serious acute renal functional deterioration.

3.2 Gross morphology and histology

In terms of gross morphology (Fig. 2B), the ischemic kidneys appeared pale immediately after the scaffold implantation, and their original color returned after reperfusion. The non-clamped kidneys showed bleeding, and blood clotted gradually after scaffold implantation. The amount of bleeding was 78.20 ± 24.90 μ g for the IR group

Fig. 2 continued

Fig. 2 continued

and $193.8 \pm 110.3 \mu\text{g}$ for the Non-IR group. The implanted scaffold showed swelling at day 3 and gradually degraded for 14 days.

In H&E staining (Fig. 2C), histopathological features of tubular epithelial cells were readily visible on day 1 in the IR group. The IR group showed histological abnormalities, such as luminal debris, fewer nuclei, collapsed luminal space, loss of brush-border on the proximal tubular epithelial cell, thinned cellular monolayer, interstitial infiltrates of neutrophils, reduced Bowman's space and increased cellularity in the glomerulus. Whereas, the Non-IR group showed nearly normal cellular morphology similar to control group. These histological acute cellular responses recovered from day 3.

3.3 Apoptosis in renal tubules

Cellular apoptosis was assessed by detecting fragmented chromosomal DNA by the TUNEL assay (Fig. 2D). In the IR group, TUNEL-positive nuclei were detected at the tubular epithelial cells, but the glomeruli were devoid of the stain. The apoptotic bodies extruded into the tubular lumen and were also observed in the extracellular region. Day 1 and 3 samples showed high TUNEL-positive nuclei, and the positive values gradually reduced with time. The Non-IR group showed negligible TUNEL-positive cells, and the histological features were similar to those of a normal kidney. The number of TUNEL-positive cells was quantified. At each time point, the IR group showed

Fig. 3 Analysis for inflammation, fibrosis and renal regeneration. **A** Inflammatory reaction with T cell infiltration, and immune cell released cytokines. **B** Fibrosis in renal interstitial (*) and glomerulosclerosis (arrow) with Masson's trichrome staining. **C** Histological identification of nephrogenic aggregate formation. **D** Immunohistochemistry and real-time PCR to determine the expression of renal

differentiation-related genes. **E** Anti-inflammatory cytokine expression. Magnification: $\times 400$, * $p < 0.05$ and ** $p < 0.01$, Ctrl: sham operated normal ICR mouse, IR: ischemia–reperfusion with laparotomy, Non-IR: non-ischemia–reperfusion with dorsal slit

128 ± 10.25 , 116 ± 5.24 , 65 ± 3.5 , and 37 ± 2.25 positive cells, respectively, whereas, the Non-IR group had no positive cells.

To determine the progression of the apoptotic pathway, Fas and caspase-3 levels were determined by IHC and real-time PCR. In the IR group (Fig. 2E), these proteins were highly expressed on days 1 and 3 and their levels gradually decreased with time. The surface of the tubular epithelial cells showed condensed and expended positive response in IHC. This result was coincident with the gene expression value at each time point. The expression of these genes was significantly higher in the IR group than in the Non-IR group.

Tubular epithelial cell injury was confirmed with KIM-1 and NGAL expression (Fig. 2F), proteins that are released after renal tubule cell injury. The expression levels of KIM-1 and NGAL were highly identified in the IR group,

whereas, they were absent or negligible in the Non-IR group.

3.4 Inflammation and fibrosis

T cell infiltration (CD4+ and CD8+ T cells) and pro-inflammatory cytokine release (IL-1 β , IL-6, and TNF- α) (Fig. 3A) were used to identify apoptosis-induced inflammation. Helper T cells and cytotoxic T cells were identified using CD4 and CD8 antibodies, respectively, and their expression levels in the IR group were high on days 1 and 3, which decreased gradually but was still expressed till day 14. The levels of inflammatory cytokines, secreted by immune cells, were analyzed using IHC and real-time PCR. At day 1, the IR group showed significantly high levels of IL-1 β , IL-6, and TNF- α , whereas the Non-IR group showed relatively weak release of these cytokines.

Fig. 3 continued

Fig. 3 continued

Fibrosis due to excessive inflammation was identified using Masson's trichrome stain (Fig. 3B). Renal interstitial fibrosis and glomerulosclerosis were elevated in the IR group, and the degree of fibrosis increased with time. The Non-IR group showed negligible deposition of collagen in the renal interstitial region.

3.5 Renal regeneration ability

To demonstrate the benefit of the Non-IR method for kidney regeneration, the scaffold was implanted into a partially nephrectomized kidney. The regenerated glomerular and renal tubules were identified by histology,

Fig. 4 Systemic responses by ischemia–reperfusion. Apoptotic genes and pro-inflammatory genes expression in distant organs. * $p < 0.05$ and ** $p < 0.01$, Ctrl: sham operated normal ICR mouse, IR:

ischemia–reperfusion with laparotomy, Non-IR: non-ischemia–reperfusion with dorsal slit

IHC, and expression analysis of renal differentiation-related genes.

Histopathological analysis (Fig. 3C) showed that the neutrophils were the earliest leukocytes to accumulate in the scaffold implanted area, followed by macrophages over time. Migrated cells from host tissue were identified on day 3. Cell number was more frequent in the Non-IR group than in the IR group, and the incidence of glomerular precursor formation at day 14 was frequent in the Non-IR group. Basophilic nephrogenic aggregates were identified as cells with dark-purple tone.

Renal differentiation-related genes were analyzed by real-time PCR (Fig. 3D). The renal progenitor marker Pax2 was expressed early in the Non-IR group on day 1, whereas Pax2 expression was delayed in the IR group. Other renal differentiation-related genes showed relatively higher expression in the Non-IR group at all time points. IHC also showed relatively enhanced expression of renal

differentiation proteins in the Non-IR group (only showed at day 14 data).

Among the cells that migrate from the host, mesenchymal stem cells are known to secrete anti-inflammatory factors. IL-10 and TGF- β 1 play important roles in tissue regeneration by decreasing inflammatory response and enhancing angiogenesis and matrix synthesis. Compared to the IR group, in the Non-IR group, IL-10 and TGF- β 1 levels were significantly increased at the early period (Fig. 3E). This enhanced anti-inflammatory effect observed in the Non-IR group could provide a suitable microenvironment for intrinsic cell migration, attachment, proliferation, and renal differentiation.

3.6 Systemic responses for ischemia–reperfusion

Local renal ischemia–reperfusion can exert systemic influences. During the evaluation of apoptotic factors, the IR group showed enhanced Fas and caspase-3 expression in

the spleen, liver, lung, heart, and brain (Fig. 4). Increased expression of these genes was observed at the early time points, which decreased gradually with time. The mean density of apoptotic factors was significantly higher in the IR group than in the Non-IR group ($p < 0.05$). Pro-inflammatory factor analysis showed that IL-1 β expression showed similar pattern in both groups; however, the expression levels of IL-6 and TNF- α were significantly higher in the IR group than in the Non-IR group (Fig. 4). This indicated that renal ischemia–reperfusion causes apoptotic and inflammatory responses in remote organs.

4 Discussion

The primary objective of this study was to evaluate the effectiveness of kidney regeneration without renal ischemia–reperfusion. This is the first study to assess the efficacy of non-ischemia–reperfusion with dorsal slit for renal tissue regeneration in a partially nephrectomized mouse.

In viability analysis, 80% of the animals in the IR group died 1.5 days after surgery, which might be because of impaired renal function and invasive surgery. Renal function is represented by serum BUN and creatinine level. BUN is a protein metabolite and creatinine is a product of muscle degradation, both of which are filtered from blood by the glomeruli. At day 1, serum BUN and creatinine values of the IR group were at life-threatening levels [10], considering that normal BUN range is 6.0–23.0 mg/dL, and that of creatinine is 0.2–0.9 mg/dL in mice [11, 12]. At day 3, the values returned to normal range, and the mice survived. Thus, even though 20 min ischemia is classified as mild injury in mice [10], ischemia–reperfusion and laparotomy results in lethal functional abnormality.

Ischemia–reperfusion induces renal apoptotic cell death. Tubular cell apoptosis is a major pathological event among the renal cell responses to ischemia–reperfusion. Ischemic apoptosis is mitochondrial dysfunction following caspase activation, and reperfusion accelerates this apoptotic process [13]. Apoptotic cells with DNA fragmentation was identified by TUNEL staining. In the IR group, brown stains were observed in the nuclei of tubular epithelial cells and debris was present within the tubular lumen. In contrast, negligible number of positive cells was detected in the tubules of the Non-IR group. The numbers of TUNEL-positive cells were highest on day 3, because DNA fragmentation by ischemia–reperfusion occurs 12 h after reperfusion and is more evident from 24 h [14]. Tubule cell apoptosis occurs mainly via the Fas-caspase pathway [15]. Fas is the main factor that initiates the extrinsic apoptotic pathway [15], and its downstream signal-transducing key protease is caspase-3 [14], the endonuclease responsible for DNA degradation [13]. KIM-1 and NGAL levels are

frequently used to determine renal tubule injury. KIM-1 is released predominantly on the apical membranes of proximal tubular epithelial cells, and NGAL is mainly expressed in the Henle loop or the distal tubule [5, 16]. As expected, KIM-1 and NGAL levels were significantly high in the IR group.

Apoptosis-induced inflammation was identified by assessing T cell infiltration and cytokine release. Significant numbers of CD4+ and CD8+ T cells were identified in the IR group. T cell is a major pathogenic factor in ischemic acute renal failure [17] and causes endothelial dysfunction, stimulating immune cell influx [18]. These immune cells secrete kidney-specific pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α [5]. Injured renal endothelial, epithelial, mesangial or tubule cells also synthesize these cytokines [19]. Enhanced pro-inflammatory cytokines accelerate the progression to chronic kidney disease [20]. Kinetically [20], TNF- α is expressed first, followed by IL-1 β and IL-6. TNF- α is a primary cytokine that stimulates other cytokines. IL-6 is expressed mainly in mesangial cells and its high concentration promotes chronic renal disease [20] because of abnormal permeability of glomerular endothelium, mesangial cell expansion, and fibronectin overexpression [19].

The inflammatory microenvironment may trigger an epithelial-to-mesenchymal transition process [19], resulting in extracellular matrix accumulation at interstitial parenchyma and development of renal fibrosis [19]. Furthermore, ischemia-induced hypoxia participates in renal fibrosis [20]. Hypoxia stimulates collagen I production and decreases matrix metalloproteinase-2 levels. Masson's trichrome staining showed that ischemia–reperfusion induced a significant increase in tubulointerstitial collagen deposition in the kidney. The collagen deposition is a harmful condition leading to deterioration of renal function [21]. Thus, ischemia–reperfusion could result in chronic renal failure in the long term.

The benefit of the Non-IR method was verified for kidney regeneration using a biocompatible scaffold. In H&E staining, the intrinsic cells appeared in the scaffold implant region on day 3, numerous mesenchymal clusters and pronephric duct and tubules were observed on day 7, and nephrogenic aggregates were formed on day 14. The sources of the migrated mesenchymal cells are endogenous renal tubule epithelial cells and extrarenal progenitors from the bone marrow [5]. However, the dedifferentiated resident tubule cells appear to be the primary cell source [22]. The appearance of developing nephrons can be a sign of renal regeneration, because new nephrons arise from basophilic cell clusters that enlarge, form lumens, and eventually elongate into eosinophilic tubules as a fully mature nephron [22–24]. This neonephrogenesis was supported by the expression of genes associated with renal

differentiation (*Pax2*, *Wt1*, *Emx2*, and *vWf*), and identification of these markers in the scaffold region indicated that renal regeneration follows the natural differentiation course. The upregulated genes of the Non-IR group were the result of effective glomerular and renal tubule reformation.

The migrated mesenchymal cells also exhibited immunosuppression by expressing IL-10 and TGF- β 1 [25] in the Non-IR group. IL-10 and TGF- β 1 suppress the secretion of pro-inflammatory cytokines [25] and maintain immune tolerance [25]. Since these anti-inflammatory factors protect renal tubule cells, kidney regeneration of the Non-IR group was further improved.

Although renal ischemia–reperfusion is a local event, it is accompanied by the complication of circulatory shock. Renal tubule cell injury enhances circulating pro-inflammatory cytokine and chemokine levels [26]. The cross-talk between injured kidney and lung, heart, liver, spleen, or brain stimulates Fas, Caspase-3, IL-1 β , IL-6, and TNF- α expression. Apoptosis in splenocytes, hepatocytes, pneumocytes, and cardiac cells suggested that renal ischemia–reperfusion initiates biochemical signaling events in distant organs that cause extrarenal apoptosis, inflammation, and fibrosis. The Non-IR method does not adversely affect other organs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement Animal protocol was approved by the Yeungnam University Institutional Animal Care and Use Committee (IACUC, YUMC-AEC2016-003).

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