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Effect of Co-culture of mesenchymal stem cell and glomerulus endothelial cell to promote endothelialization under optimized perfusion flow rate in whole renal ECM scaffold

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ABSTRACT

In recent era, many researches on implantable bio-artificial organs has been increased owing to large gap between donors and receivers. Comprehensive organ based researches on perfusion culture for cell injury using different flow rate have not been conducted at the cellular level. The present study investigated the co-culture of rat glomerulus endothelial cell (rGEC) and rat bone marrow mesenchymal stem cells (rBMSC) to develop micro vascularization in the kidney scaffolds culturing by bioreactor system. To obtain kidney scaffold, extracted rat kidneys were decellularized by 1% sodium dodecyl sulfate (SDS), 1% triton X-100, and distilled water. Expanded rGECs were injected through decellularized kidney scaffold artery and cultured using bioreactor system. Vascular endothelial cells adhered and proliferated on the renal ECM scaffold in the bioreactor system for 3, 7 and 14 days. Static, 1 ml/min and 2 ml/min flow rates (FR) were tested and among them, 1 ml/min flow rate was selected based on cell viability, glomerulus character, inflammation/endothelialization proteins expression level. However, the flow injury was still existed on primary cell cultured at vessel in kidney scaffold. Therefore, co-culture of rGEC + rBMSC found suitable to possibly solve this problem and resulted increased cell proliferation and micro-vascularization in the glomerulus, reducing inflammation and cell death which induced by flow injury. The optimized perfusion rate under rGEC + rBMSC co-culture conditions resulted in enhanced endocellularization to make ECM derived implantable renal scaffold and might be useful as a way of treatment of the acute renal failure.

1. Introduction

Fronting the difficulties associated with the limited regenerative ability of the kidneys and the lack of kidneys' donors, dialysis remains the only choice for many patients with end-stage renal disease (ESRD) [1]. Dialysis is only a medium-term solution because the large and protein-bound uremic solutes are not efficiently cleared from the body causing further disease over time [2]. The donor-to-receiver kidneys ratio remains a challenge, therefore an alternative approach is urgently needed to fulfil this clinical gap. Another type of renal failure, acute kidney injury (AKI), is defined as a sudden increase in serum creatinine concentration and decreased urine output [3,4] which often leads to chronic kidney disease. Current developments in the field of tissue engineering and regenerative medicine have provided a variety of alternative cell-based approaches for the treatment of renal failure [5,6]. Regeneration of the entire kidney ECM scaffold through decellularization followed by recellularization is seen as a potential alternative to creating a functional tissue [7]. Despite growing interest in the potential use of decellularized whole kidneys as 3-dimensional (3D) scaffolds to generate ex-vivo renal tissue, several questions remain unanswered [8]. It will be even more challenging to imagine the usefulness of the extracted kidney for decellularization and then recellularization with regenerated function. Research concerning recellularization of whole kidney in conjunction with stem cells has a potential strategy for regenerative medicine [8]. In the case of recellularization of renal organ, the culture flow rate (FR) plays an important role in the vascular system and a high FR might result in over expression of cell death markers on the endothelial cells. The representative example of the body disease, hypertension which keeps body on high blood pressure cause kidney failure. Hypertension makes blood vessel damage and causes of chronal kidney disease due to

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the detrimental symptoms that increased blood pressure has on kidney vasculature. Uncontrolled long term high blood pressure possibly makes damage to glomerular. This symptom leads to abnormal increasing of protein in the urine [9]. Thus, we hypothesized uncontrolled FR might adversely affect the glomerulus morphology and endothelialization. In several published reports, the recellularization of the renal organ was conducted over a wide flow range [10–12]. FR is one of the important factors in the vascular system, and a high level of FR over expresses inflammatory markers on the endothelial cell. It is related to symptoms such as inflammation and edema. Consequently, in the present study, we optimized the FR in rGEC recellularized renal ECM scaffold for their successful growth.

An individual monoculture with rGECs was used for recelleularization of the renal ECM scaffold followed by a co-culture with rBMSCs. rBMSCs are good mediators not only for rGECs survival but also for cell attachment, migration and pro-angiogenic factors such as VEGF, Ang2, Tie2, VEGFR2 and many others, therefore rBMSCs were used as a cell therapy concept for renal vessel recovery *in-vitro*. Chen et al. [13] reported that local MSCs have the potential to differentiate into the endothelial lineage and participate in renal repair through a local paracrine effect, mainly the production of vascular endothelial growth factor to prevent microvascular dropout. It has previously been reported that MSCs derived from bone marrow can differentiate into endothelial cells and that their systemic administration can protect against ischemic renal failure through differentiation-independent mechanisms [14]. Also, local MSCs are useful carriers in cell therapy to produce tissue regeneration after acute renal failure (ARF) [15,16].

The aim of the present study was to develop an artificial renal scaffold using a decellularization system without collapse of the vascular ECM structure. In addition, the formation of blood vessels in the kidneys was confirmed by a bioreactor and the vascular endothelial cell adhesion in the blood vessels. It was hypothesized that rGECs together with rBMSCs could better attach to the scaffold and regenerate towards native kidney vessel cells by immunomodulation.

2. Materials and methods

2.1. Rat whole kidney decellularization and evaluation

Sprague Dawley male Rats (12–13 weeks of age) were sacrificed in this study with the approval of the Animal Ethics Committee (AEC) of Soonchunhyang University (SCH20-0057). Rats were anaesthetized with 3.0% isoflurane (Piramal critical care, USA) and the fur in the abdominal region was shaved. The site was sterilized with 70% alcohol and povidine solution. The blood inside the kidneys was removed by a peristaltic pump with 100 IU of heparin solution diluted in saline to prevent blood clots inside the kidney and stored in cold PBS for further use. Adipose tissue near the kidney was removed for cannulation. The cannulated kidneys were decellularized by an automatic decellularization system in an aseptic space [17]. The kidneys were perfused with 1% of SDS solution for 6 h and the flow rate of liquid was gradually increased from 500 μl/min to 1000 μl/min. An overnight wash with H_2O was performed at a low flow rate to remove all traces of surfactant inside the kidney, the 1% of Triton X-100 was flowed into the kidney for 30 min and washed again with H_2O at 1000 μl/min. In the final step, the decellularized kidney was treated with penicillin (10,000units/ml) - streptomycin (10,000 μg/ml) (Welgene, South Korea) and amphotericin B. The decellularized kidney was then rinsed with PBS to remove any remaining antibiotic traces.

2.2. Evaluation of decellularized kidney scaffold DNA

Qualitative DNA content was evaluated by quantitative analysis. Native kidney and decellularized renal scaffold were subjected to DNA lysis buffer containing 0.5 M EDTA, 5 M NaCl, 1 M Tris-HCl (pH8.0), 20% SDS and 20 mg/ml of proteinase K (Invitrogen, USA) for overnight incubation. After centrifugation at 13,000 rpm, the supernatant was taken and mixed with the same amount of isopropanol. The samples were carefully inverted to precipitate DNA and centrifuged for 15 min at 13,000 rpm. DNA pallets were dispersed in 70% ethanol and centrifuged again for 5 min. The supernatants were discarded again and the DNA pallets were dissolved in diethyl pyrocarbonate (DEPC) and measured with a nano-photometer (Implen, GmbH) (n = 3).

2.3. Structural proteins quantification

Collagen content from native kidney and decellularized renal scaffold was determined using a Sirius Red Collagen Detection kit (Chondrex, USA) measured at a wavelength of 540 nm using an Infinite 50 microplate reader [Reiza]. Sulfated glycosaminoglycans (sGAG) were extracted from the samples using the Blyscan sGAG assay kit (Biocolor, UK) according to the manufacturer's instructions [18].

2.4. Growth factor quantification

Rat kidneys were first cryo grinded to fine powder and then 200 mg was suspended in 2 mL urea–heparin buffer (2 M urea, 5 mg/mL heparin, 50 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 10 mM *N*-ethylmaleimide). The mixtures were shaken at 4 °C overnight and then centrifuged at 10000 rpm for 10 min. The dissolved portion was collected, and the lysate was extracted once more with an additional 2 mL buffer. The supernatants from both extractions were dialyzed at 4 °C against distilled water (total 5 changes) in Slide-A-Lyzer Dialysis Cassettes, 3500 MWCO (Pierce). Dialyzed extracts were aliquoted and frozen in −80° until assayed. ELISA assays were performed with the extracts to quantify basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) using commercial kits (R&D Systems) according to the manufacturer's protocols.

2.5. Preparation of rat glomerulus endothelial cell (rGEC), bone marrow-derived mesenchymal stem cell (rBMSC) and bioreactor for recellularization

The bioreactor design was an updated version of our previously developed protocol by the lab [19]. Each cell type was cultured to 80% of confluency for recellularization. $7.02 \pm 1.60 \times 10^6$/ml rGEC cells were injected via the renal artery. The recellularized renal ECM scaffolds were kept overnight at a static flow of 1 ml/min and 2 ml/min by a peristaltic pump. Throughout the protocol, the recellularized kidneys were kept in the bioreactor at 37 °C with CO_2 incubation.

Rat glomerulus endothelial cells (rGECs, Cell biologics, USA) were cultured with endothelial cell growth medium (Cell biologics, USA) in a 0.2% of pre-gelatin coated plate in a cell culture incubator under standard conditions.

For set up of rBMSCs, it was isolated from 12 weeks rat. Animal was anaesthetized with 3% isoflurane (Piramal critical care, USA) and shaved the fur the part of back limbs. The tibias and femurs were transferred to dish and injected DMEM+10% FBS+1% PS medium pass-through limb bones using. The cell suspension was filtered with cell strainer (100 μm) and centrifuged at 1500 rpm for 5 min. Cell pallet was released and cultured by DMEM+10% FBS+1% PS medium at 37 °C in 5% CO_2 incubator. Cells were cultured in the culture dish and the medium was changed every 2–3 days. 5th passage of expanded cells were used for recellularization.

2.6. rGEC and rBMSC co-culture on 2D

A total $2.6 \times 10^3/cm^2$ number of cells was seeded and co-cultured for 3, 7 and 14 days in the 2D plate. rGECs and rMSCs were cultured at different ratios of 100:0, 75:25, 50:50 and 25:75. The media of all samples were changed every 3days.

2.7. rGEC and rBMSC recellularization in rat renal scaffold using a bioreactor system

All recellularization procedures were performed in an aseptic space. A sterilized recellularization chamber was filled with culture media and circulated inside the recellularization chamber using a closed perfusion system. The decellularized renal scaffold was connected to a sterilized chamber inside the tube and the culture medium was perfused through the renal artery. The scaffold was then transferred to a closed-system bioreactor and perfused at a flow rate of 1 ml/min for 30 min at 37 °C to prepare for recellularization.

The prepared concentration of $5.47 \pm 0.60 \times 10^6$ rGEC cell suspension was injected into the renal scaffold and perfused for 1 min at a flow rate of 1 ml/min. Cells were attached overnight with no flow and medium perfusion was gradually increased to reach the final flow rate. Recellularized renal scaffolds with a flow rate 1 ml/min were cultured for 3 days. After 3 days of rGEC culture, a concentration of $2.15 \pm 0.11 \times 10^6$ rBMSCs suspension was re-injected via the renal artery and cultured for 3 days, 7 days and 14 days. The estimated dynamic pressure was found 115 ± 44 mmHg for our optimized 1 ml/min FR, which will not cause any adverse effect with the effect of blood flow to seeding cells and neovascular networks. Bioengineered renal tissue was cultured in basal medium and ECGM for proliferation in the control group.

2.8. Histological evaluation of recellularized, decellularized renal scaffold

Samples were fixed with 4% paraformaldehyde for 24 h and immersed in flowing water for 1 h. Rinsed samples were run by 50%, 70%, 90% and 100% ethanol dehydration and transferred to xylene 2 times to completely remove the ethanol. To make a tissue paraffin block, the samples were held in 3 paraffin sets namely paraffin 1, paraffin 2 and paraffin 3 each for 1 h, then the paraffin block was embedded and the tissue block was sliced to a thickness of 5 μm. The sliced tissues were attached to a sliding glass and melted at 60 °C in the oven to prepare histological analyzes such as Hematoxylin and eosin stain (H&E) staining, Masson-trichrome staining (MT) and immunohistochemistry.

The expression of vascular cell adhesion protein, VCAM-1(Abcam, UK), ICAM-1(Abcam, UK), eNOS (Cell signaling, USA), Tie1 (Abcam, UK) and Tie2/TEK (Millipore, USA, in a recellularized renal scaffold were analyzed. For immunofluorescence staining, cleaved caspase-3(Asp 175, Cell signaling, USA), and CD73 (Bioss, USA) were incubated overnight as a primary antibody. Secondary antibodies were used with Alexa 594-conjugated goat anti-rabbit, Alexa 488 and Alexa 488-conjugated donkey anti-goat. The nuclei were stained with Hoechst 33,342 and, F-actin was stained with phalloidin 488 for observation under a fluorescence and confocal microscopes.

2.9. Micro - CT imaging of decellularized kidney scaffold

Decellularized kidney scaffold Micro - CT data was obtained by followed process. The vascular contrast medium was mixed with 40% BaSO4 (USA, sigma) and 5% gelatin, dissolved at 42 °C, and administered to the decellularized kidney scaffold. The renal scaffolds directed with the contrast agent scanned using a SKYSCAN 1272 Micro-CT instrument.

2.10. Real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis

The rGECs and rBMSC were seeded on a 2D plate with a cell concentration of $2.63 \times 10^3/mm^2$ for 2 weeks. The vWF, CD31, CD105, Tie1, Tie2, Ang1, Ang2 and VEGF-A genes were analyzed to evaluate the RNA expressions of endothelialization and angiogenesis. The rGEC and rBMSC mixture was seeded on the 2D plate and cultured for 2weeks at different ratios 100:0, 75:25, 50:50 and 25:75 (rGEC: rBMSC). Standard cell culture media were used to maintain the cells in a humidified incubator at 37 °C with 5% CO_2. Total RNA was extracted from the cells for real-time PCR analysis according to the manufacturer's protocol (ReboEX (Geneall, South Korea) and Hybrid R (Geneall, South Korea)). The Biodrop (Biochrom, USA) was used to determine the total RNA concentration. The Maxime RT PreMix kit (LiliF, Korea) was used to convert RNA to cDNA. The sequence of primers is listed in Table 1.

2.11. Statistical analysis

To conduct statistical analyses, GraphPad Prism v8 software was used for making graphs and statistical analysis. Data are presented as means ± SD and analyzed using comparison between groups were made by *t*-test, one way analysis of variance (ANOVA) or 2-way analysis of variance (ANOVA). The significance levels were defined as $p < 0.0001$ ****, $p < 0.001$***, $p < 0.005$**, $p < 0.05$*. For each experiment, at least three samples were considered for statistical analysis.

3. Results

3.1. Kidney decellularization and scaffold characterization

The decellularized kidneys were successfully processed and visually distinguished from native kidneys based on color differences as shown in Fig. 1A. Harvested native kidneys were decellularized using an organ decellularization setup in an aseptic space (Fig. 1B). In the case of decellularized kidney, the hematoxylin dye was injected and found to be evenly spread and flowed into the glomerulus capillaries, therefore the shape of the glomerulus could be clearly observed as presented in Fig. 1C. To assess the integrity and patency of kidney vascular segment, as a result of administering the contrast agent in the decellularized renal scaffold, it was confirmed that no disruption of vasculature was observed in the kidney, as well as the presence of glomeruli in Fig. 1D as obtained from Micro-CT analysis. The presence or absence of cells and remaining proteins were analyzed by histological evaluation such as H&E and Masson's trichrome of the decellularized renal scaffold along with the native kidney. There was no trace of any cell in the decellularized tissue. It was confirmed that the ECM structure was not deformed. Masson's trichrome staining was done to confirm that the remained collagen showed as blue

Table 1
Primer sequence of Genes used for RT-PCR study.

Gene	*Length of]amplicons(bp)*	Primer sequence(F) 5'→3'	Primer sequence(R) 5' →3'
rVWF	93 bp	GTC GGA AGA GGA AGT GGA CAT T	GGG CAC ACG CAT GCG CTC TGT A
rCD31	360 bp	CTT CAC CAT CCA GAA GGA AGA GAC	CAC TGG TAT TCC ATG TCT CTG GTG
rCD105	211 bp	ACT CGG GAG GTG TTT CTG GTC TT	GTG CTG CTA TGG AGG TAA TGG TG
rTie1	81 bp	GCC CTT TTA GCC CTG GTG T	TTC ACC CGA TCC TGA CTG GTA
rTie2	380 bp	GGC GAA AAC TAC ATA GCC AA	CGT TCT TCC AGC ATT CTG TT
rAng1	359 bp	GGA GCA TGT GAT GGA AAA TT	TGT GTT TTC CCT CCA TTT CTA
rAng2	347 bp	GTC TCC CAG CTG ACC AGT GGG	TAC CAC TTG ATA CCG TTG AAC
rVEGF-A	119 bp	CTG TGG ACT TGA GTT GG	CAA ACA GAC TTC GGC CTC TC
GAPDH	119 bp	AAG TTC AAC GGC ACA GTC AA	TAC TCA GCA CCA GCA TCA CC

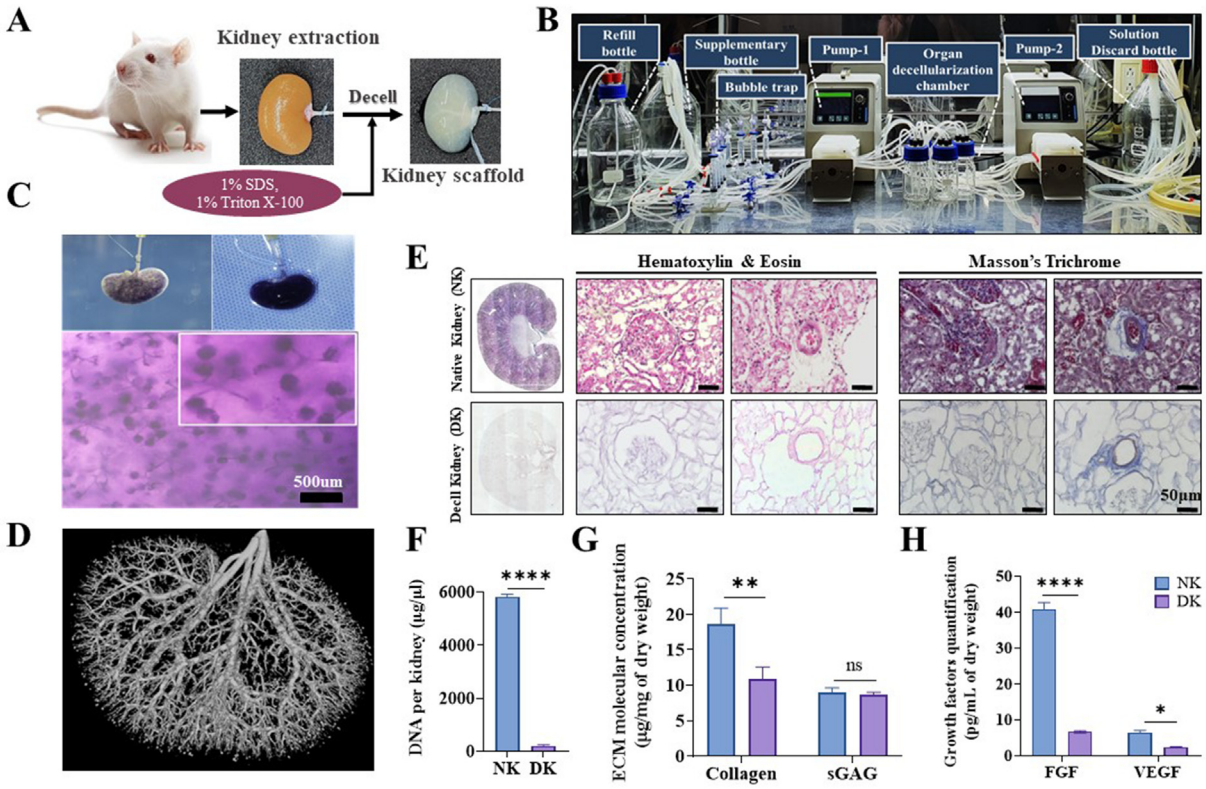

Fig. 1. Decellularization procedure and quantification of decellularized rat kidney scaffold. (A) Photograph of extracted kidney and decellularized rat kidney. (B) Experimental setup for decellularization in the aseptic space. (C) Photograph and glomerulus microvascular observation under microscope using hematoxylin dye. (D) Micro-CT image of decellularized kidney and (E) Hematoxylin and eosin (H&E) staining and Masson Trichrome staining of sliced kidney pre and post decellularization, (F) DNA (G) ECM molecular concentration and (H) Growth factor quantification of native and decellularized kidney for different biomolecules. *($p < 0.0001$ ****, $p < 0.005$**, $p < 0.05$*).*

and the cytoplasm was red in the decellularized kidney (Fig. 1E). In addition, the quantification of DNA, ECM (Collagen, sGAG) and growth factor (FGF, VEGF) was conducted which is presented in Fig. 1E–G. The DNA level in the decellularized renal scaffold was 213.90 ± 38.56 μg/μl, confirming that 96.33% of the DNA was significantly removed ($P < 0.0001$) (Fig. 1F) and that collagen was lessened by approximately 0.5 times. The rate of reduction for sGAG from native kidney 8.96 ± 0.66 μg/mg to decellularized renal scaffold was 8.70 ± 0.30 μg/mg, which was considered as not significant data (n.s, Fig. 1G). FGF concentration was increased by 83.7% from 40.90 ± 1.77 pg/ml on native kidney (NK), to 6.67 ± 0.29 pg/ml for decellularized kidney (DK). VEGF level of native kidney 6.45 ± 0.60 pg/ml and decellularized renal scaffold was 2.35 ± 0.17 pg/ml, the amount gradually decreased (Fig. 1H). Thereafter, the decellularized renal scaffold is referred to as the extracellular renal matrix (ECM) scaffold.

3.2. Renal recellularization procedure with rGEC to optimize bioreactor flow rate

For recellularization, decellularized renal ECM scaffolds were perfused in a cell culture medium at 1 ml/min FR prior to cell injection as shown in Fig. 2A. The pre-detached cell suspension was injected into the scaffold and left overnight for cell adhesion under static conditions. When the decellularized renal scaffold was recellularized using rGECs, the cells were attached to the vessel wall of the renal ECM scaffold in a cell suspension state. The recellularized renal ECM scaffolds with rGECs were cultured under static conditions and perfused at two different flow rates: 1 ml/min and 2 ml/min (Fig. 2A and B). Fig. 2C is a photograph of recelleurarized renal ECM scaffold using rGECs. The cell suspension and the adhesion mechanism by the renewal vessel ECM wall are explained in Fig. 2D.

Based on the above findings, it was possible to confirm the size difference between the static culture, a sample with a 1 ml/min and 2 ml/min of FR. Fig. 2E and F represent a photograph of 3 sets of recellularized renal ECM scaffolds and their quantified values, respectively. Size of recellularized renal ECM scaffolds at 1 ml/min FR, exhibit the highest value of 2.35 ± 0.10, 2.19 ± 0.32 in 2 ml/min FR and 1.23 ± 0.12 in static culture. No significant difference between 1 ml/min FR and 2 ml/min FR was observed. The recellularized renal ECM scaffolds at different perfusion rates were cultured in the bioreactor for 3, 7 and 14 days and observed through H&E staining. From day 3 of observation, it was noted that there was good cell attachment followed by the greatest cell distribution on day 7, which gradually decreased on day 14 (Fig. 2G). Based on the histological results, the number and size of glomeruli in each sample were quantified (Fig. 2H and I). In static culture, the highest number of glomeruli (31.69 ± 3.28) and the lowest size (13.45 ± 4.41) were measured. In the 2 ml/min FR sample, the results showed the widest range of deviation for 9.56 out of all 3 groups (Fig. 2G and H). As an indicator of cell death, cleaved caspase-3 (ccasp3) was observed by fluorescent staining and summarized in Fig. 2J. At 2 ml/min FR, cell death was noticed even on day 3, while at 1 ml/min almost on day 7. CCasp-3 expression was observed in all samples and there were no cells in the glomerulus at 2 ml/min FR sample. Accordingly, it was hypothesized that an FR of 2 ml/min induces cell damage of rGEC in the glomeruli and rapidly increases cell death.

3.3. Optimization of perfusion conditions by immune histochemical staining and their analysis

Renal failure has a strong connection with hypertension [20]. Patients with chronic hypertension may be able to damage the glomerular endothelial cells leading to negative effects on the body [21]. When a

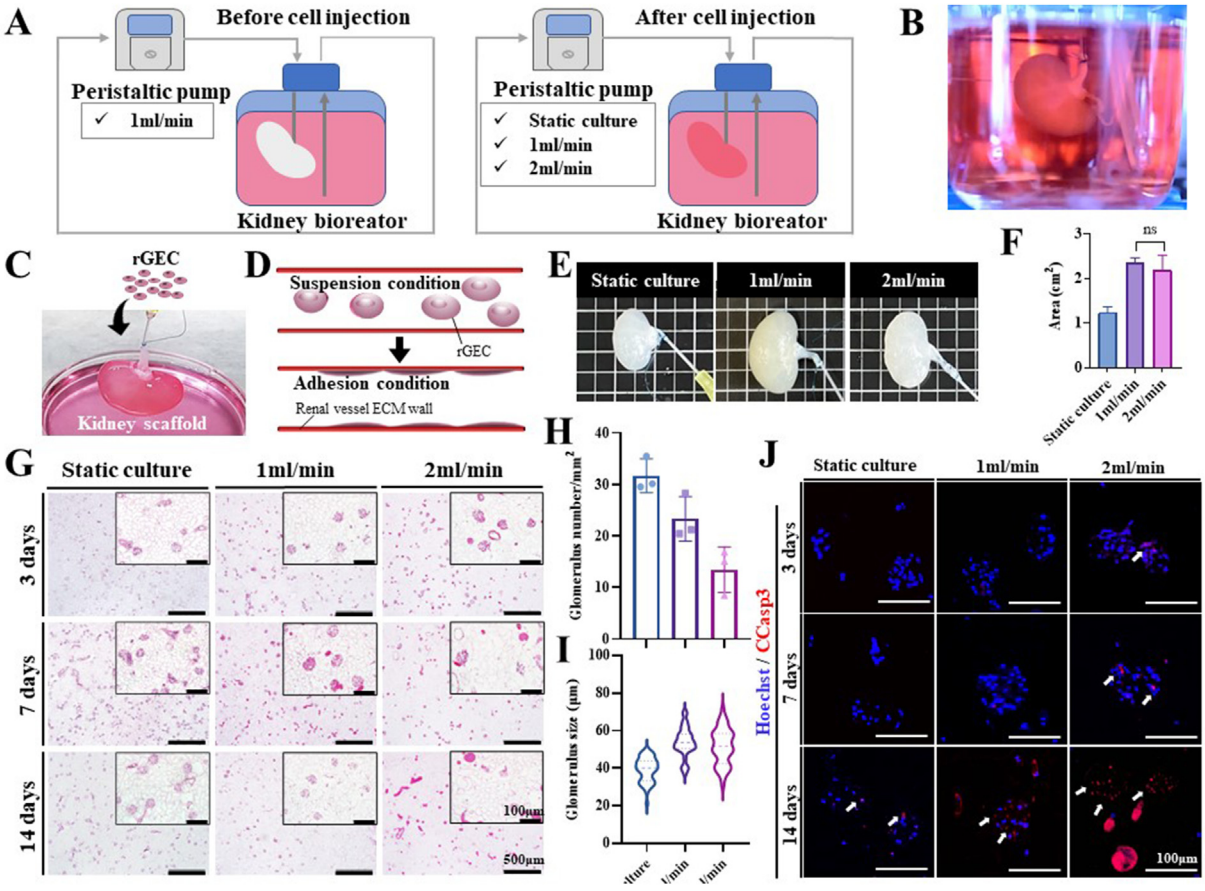

Fig. 2. Recellularization of rGEC in the decellularized kidney scaffold under varying flow rate (static, 1 ml/min, 2 ml/min) and their characteristic evaluation. Histological analysis of renal cortex after H&E staining. Quantified number and size of glomerulus in the cortex which recellularized with rGEC/rBMSC and rGEC in the kidney. (A) Pre and post cell perfusion into decellularized kidney. (B) Photograph of recellularized kidney scaffold during culture. (C) rGEC injection through kidney scaffold artery and (D) schematic image of rGEC recellularization hypothesis which rGEC attachment on renal vessel ECM. (E) Photographs of recellularized kidney scaffold at different flow rate. (F) Quantification of recellularized kidney size. (G) Hematoxylin and eosin (H&E) staining for observing cell distribution at day 3,7and 14 and different flow condition. (H and I) Quantified values for number and size of glomerulus and (J) Immunofluorescence (IF) staining of recellularized kidney scaffold at different flow rate using Hoechst and CCasp3 (White arrow indicate expression of CCasp3).

cultured artificial renal ECM scaffold is grown in high FR, the shear stress increases and results in a large number of relative oxygen species (ROS) that enhance the inflammatory adhesion protein factors such as VCAM-1 and ICAM-1. Therefore, the FR results of the renal scaffolds recellularized with rGEC were analyzed histologically by DAB staining (Fig. 3A and B). As a quantified result of the VCAM-1 marker staining on day 3, expression was significantly higher in FR 2 ml/min, while on days 7 and 14, 1 ml/min FR was found the highest. Compared to the static conditions, the average value was higher under the conditions of 1 ml/min and 2 ml/min and the VCAM-1 protein, which had different expression levels depending on the flow rate, showed high values of 51.36 ± 9.10 on day 3 and 43.44 ± 20.29 on day 7 in a 2 ml/min FR sample, but decreased sharply on day 14 (22.99 ± 14.73) (Fig. 3E). Cell death in the glomerulus might be caused by the high flow rate resulting in low VCAM1 expression in % of the positive area of the quantification. ICAM-1 showed no significant difference in all tested groups on day 3 of culture (36.30 ± 11.73), but on day 7 (58.01 ± 15.04) of perfusion, the 2 ml/min FR sample showed a higher value than in static culture and 1 ml/min FR sample. On day 14, the expression level was lowest under static conditions and highest in 1 ml/min FR (Fig. 3B and F). However, in this result, as with VCAM-1, reducing the number of cells in the glomerulus is likely to have a significant effect. eNOS is a protein that has the function of decomposing active factors such as nitric oxide (NO) in vascular endothelial cells. As a result of staining with a protein marker determining the function of vascular endothelial cells, the highest concentration 72.27 ± 11.80 for 2 ml/min FR was found on the day 3 of perfusion culture. On day 7 of perfusion culture, the 1 ml/min FR sample expressed the highest level (55.67 ± 12.70) among the 3 groups and although it showed a lessening trend in long-term culture on day 14, it was still higher than the 2 ml/min FR (Fig. 3C, and G). Tie1 as a protein involved in angiogenesis has the property of being cleaved from endothelial cells when inflammation occurs. As a result of staining with the Tie1 marker, 1 ml/min was maintained well on days 3, 7, and 14, while the FR 2 ml/min sample showed the highest level (65.21 ± 18.86) in the initial culture and then showed a decline in the trend on day 14 (21.92 ± 13.73) (Fig. 3D and H). Thus, it was demonstrated that a perfusion rate of 1 ml/min caused less injury to vascular endothelial cells in the recellularized renal ECM scaffold compared to the FR 2 ml/min FR, which could survive longer. As a result of culturing at a 1 ml/min FR, inflammation increased in comparison to static culture but compared to a 2 ml/min FR, rGEC inflammation and cell death decreased and endothelial cell function was increased. The entire mechanism is illustrated in Fig. 3I.

3.4. 2D Co-culture of rGEC and rBMSC and their RT-PCR evaluation

To optimize the co-culture cell mixing ratio of rGEC and rBMSC, the

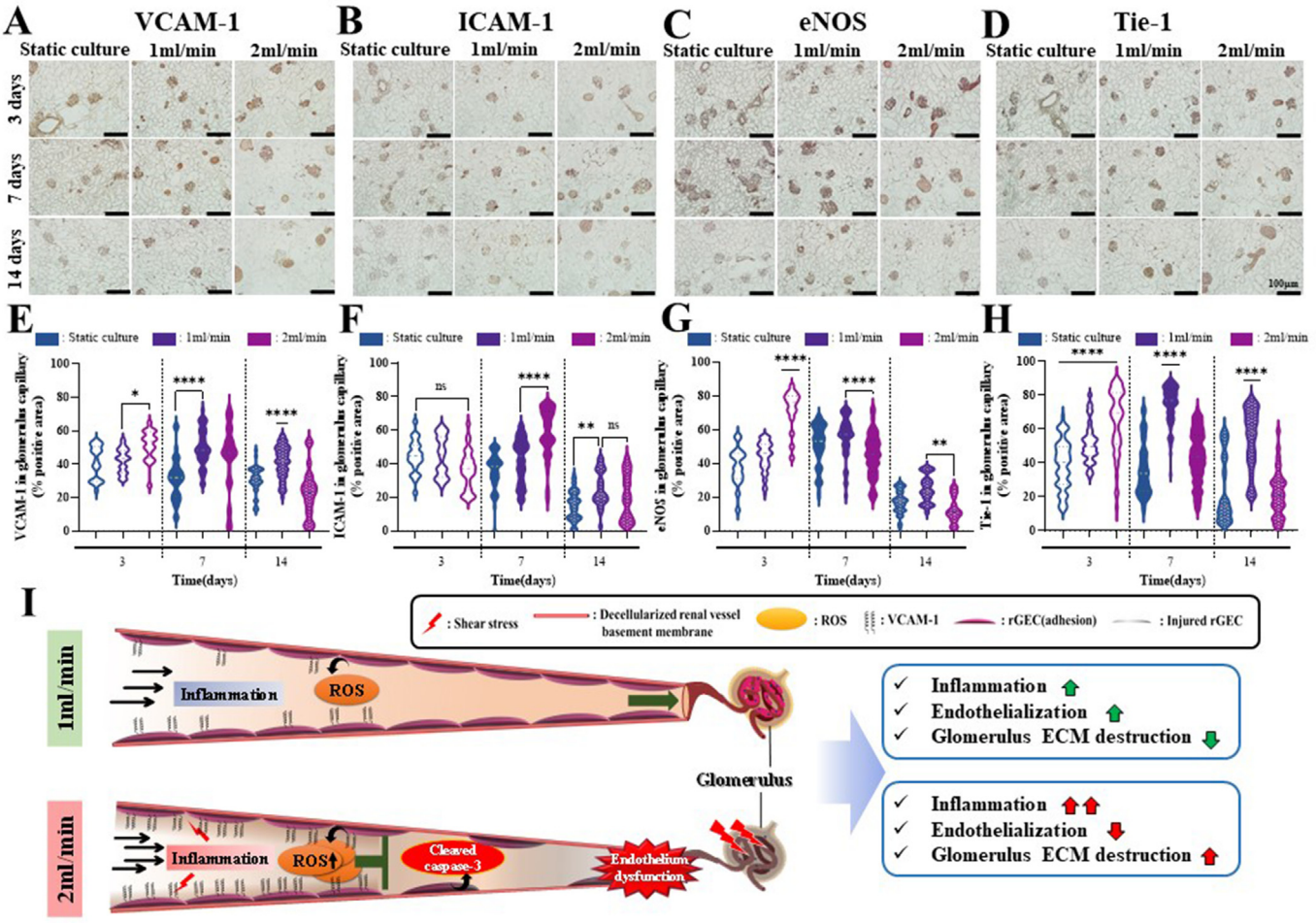

Fig. 3. Immunohistochemistry (IHC) staining of inflammation. Quantification of related protein (VACM-1, ICAM-1,Tie-1), vascular endothelium marker (eNOS) of recellularized rGEC kidney scaffold under varying flow rate (static, 1 ml/min and 2 ml/min) and period (3 days, 7 days and 14 days). (A) VCAM-1 (B) ICAM-1 (C) eNOS (D) Tie-1 DAB staining (E–H) Quantified data for A-D and (I) Schematic illustration of mechanism of cell death in glomerulus microvessels of recellularized kidney scaffold at different flow rate. *($p < 0.0001$ ****, $p < 0.005$**, $p < 0.05$*).*

2D culture was first investigated prior to co-culture in the renal ECM scaffolds. The mixing ratios of rGEC:rBMSC were kept as 100:0, 75:25, 50:50 and 75:25. Co-culturing of the rGEC and rBMSC samples on the 2D plate were cultured for 3, 7 and 14 days to optimize the ratio which can be further applied for renal vessel recellularization. 3 groups of 75:25, 50:50, 25:75 along with pure rGEC (considered a control) were observed for cell network behavior using confocal microscopy and are presented in Fig. 4A. Network formation was observed on day 7 of the culture and became dense on day 14. The expression level of VEGF-A, vWF, CD31, CD105, Tie1, Tie2, Ang1 and Ang2 are related to cell stability, endothelial cell markers, microvascular markers and angiogenesis growth factor. VEGF-A is secreted by mesenchymal stem cells and the obtained results showed that co-culturing of rGEC:rGEC + rBMSC (25:75) had the highest expression level of 1.3 ± 0.11 compared to the control group rGEC:rGEC + rBMSC (100:0) (Fig. 4B). vWF and CD31 are phenotype markers for endothelial cells that is the reason for higher expression with high concentration ratio. vWF was calculated highest in rGEC:rGEC + rBMSC (100:0) (1.00 ± 0.10) and the trend of vWF was quite similar to each other with the highest expression at rGEC:rGEC + rBMSC (75:25) ratio among the co-culture conditions. CD31 noted a maximum in rGEC:rGEC + rBMSC (75:25) (1.08 ± 0.18). So, large number of endothelial cells were included in the ratio of 75:25 had higher and more stable angiogenesis/neo-vessel formation tendencies were observed in the fluorescence-stained result than the ratio 25:75. This result was confirmed by RT-PCR analysis. CD105 is expressed in the micro-vessel of endothelial cells and is also an essential marker of MSC. It is well known that lower expression level of CD105 results low micro-vascularization. As the result of Figs. 4E and 3 groups except for the rGEC:rGEC + rBMSC group (100:0), all combinations of co-culture expressed increased marker levels ranging between 2.05 and 2.91. This finding supports the cells' ability to microvascular-like structure, as shown in Fig. 4A. Tie1 and Tie2 expression levels showed the angiogenesis behavior in the samples. Especially, under the rGEC:rGEC + rBMSC 75:25 co-culture conditions Tie1 has a higher expression level (1.44 ± 0.27) than Tie2 (0.62 ± 0.09). Vascular growth factors, such as angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) were also evaluated because Ang-2 is a context-dependent antagonist of the Tie-2 receptor, which is pro-inflammatory and promotes endothelial cell apoptosis and causes cytoskeletal changes that increase the inter endothelial gap. In rGEC: rGEC + rBMSC 75:25 the highest Ang1 level was recorded at 0.37 ± 0.13 and Ang2 at 0.49 ± 0.39 among the co-culture conditions. For Ang2, the highest value was noted for the set of rGEC:rGEC + rBMSC 25:75 co-cultures. Ang-1 stabilizes the endothelium by inhibiting endothelial cell apoptosis and activating and reducing inflammation [22,23].

3.5. *In-vitro stem cell therapy of injured recellularized renal ECM scaffold*

The rGEC recellularized renal scaffolds were perfused for 3 days, then the rBMSC suspension was injected into the renal artery and samples were harvested on days 3, 7 and 14. The experimental steps are

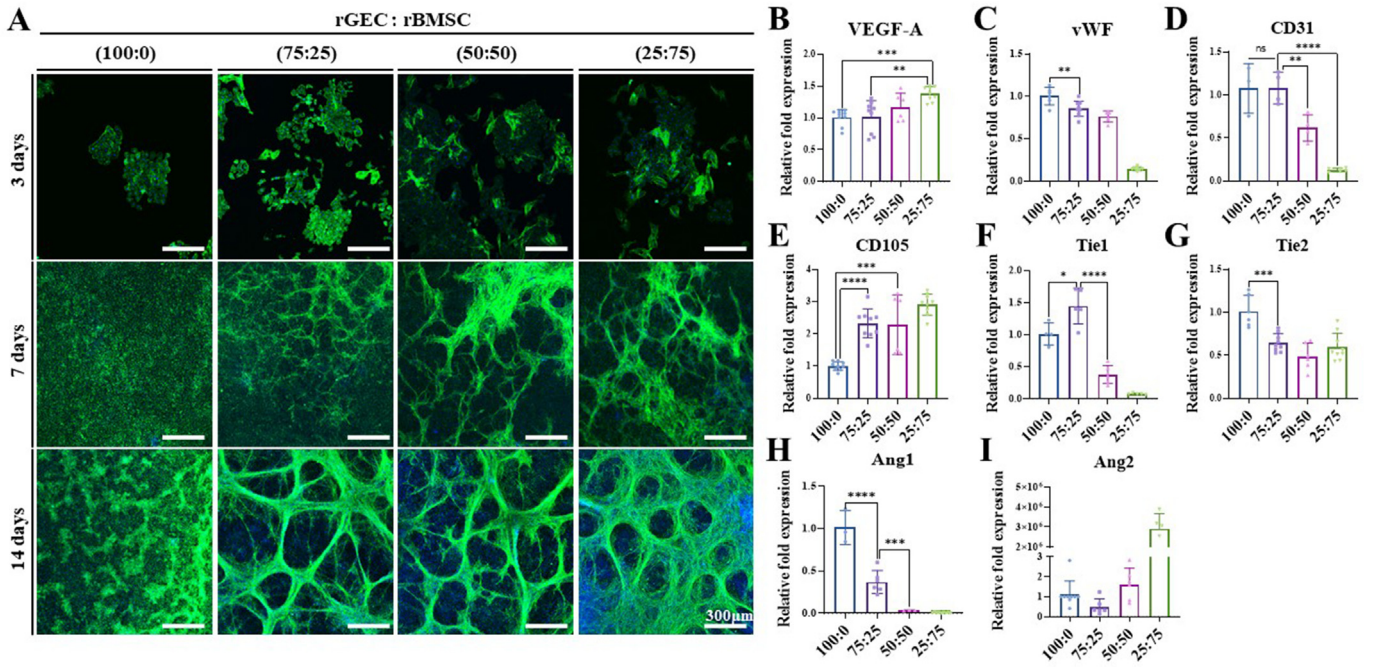

Fig. 4. Selection of co-culture of rGEC and rBMSC on 2D culture. (A) 2D co-culture florescence images of rGEC: rBMSC under different ratio for 3,7and 14 days and their RT-PCR analysis using various blood vessel markers for 14 days of culture. (B to I) Inflammation (vWF), micro vascularization (CD105), endothelial cell marker (CD31), cell angiogenesis and quiescent (VEGF-A, Tie1, Tie2, Ang1, Ang2), of recellularized rGEC/rGEC:rBMSC. ($p < 0.0001$ ****, $p < 0.001$***, $p < 0.005$**, $p < 0.05$*).

summarized in Fig. 5A. For comparison purposes, two different cell culture medium conditions were used for co-culturing rGEC and rBMSC with recellularized renal ECM scaffolds. One medium consisted of DMEM+10% FBS+1% PS and the other was endothelial cell growth medium (ECGM). As a result of tissue staining with H&E, relatively dark cytoplasm was observed in the group cultured in DMEM+10% FBS+1%

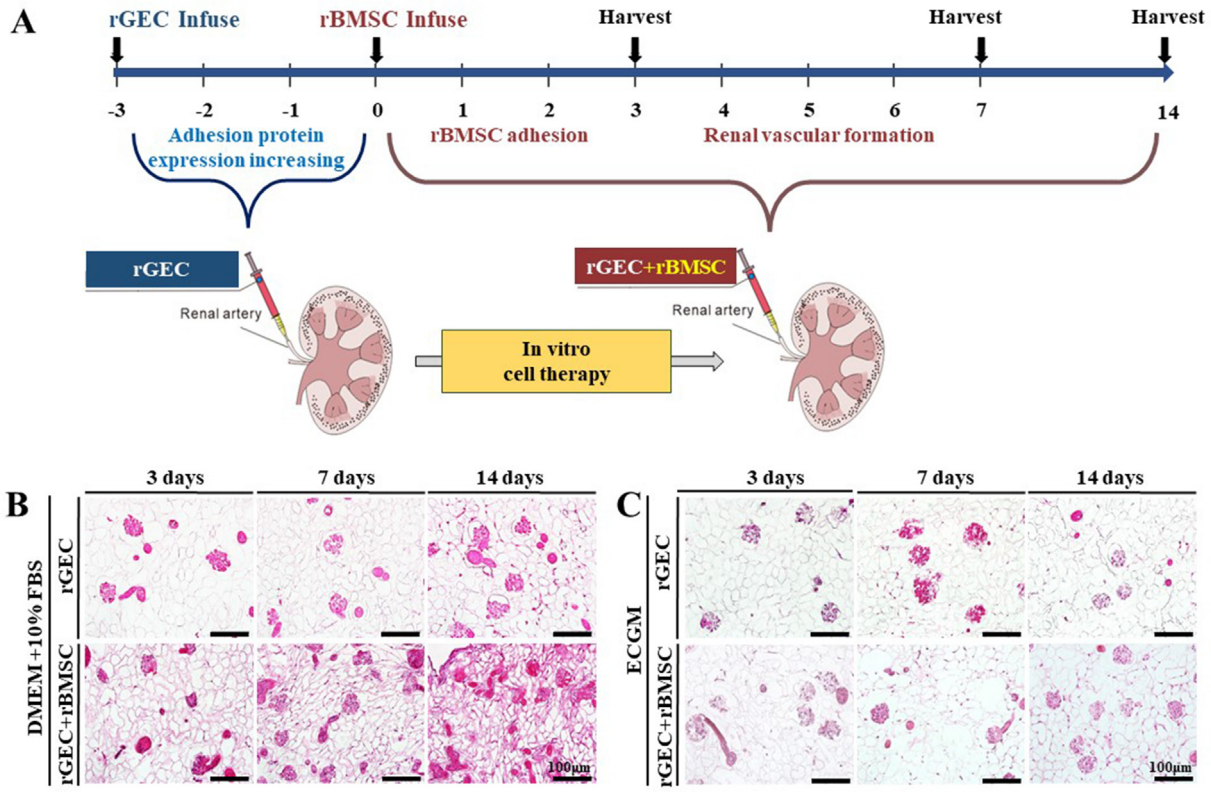

Fig. 5. (A) Recellularization of decellularized whole kidney scaffold using rGEC + rBMSC until 14 days. (B) Hematoxylin and eosin (H&E) stained images for basal media+10% FBS condition and (C) Hematoxylin and eosin (H&E) stained images for endothelial cell growth media (ECGM) condition.

PS medium compared to the group cultured in the ECGM medium. It was experimentally difficult to clearly observe the glomerulus shape in the rGEC + rBMSC co-culture renal ECM scaffold at on day 14 which could be due to the remodeling of the ECM by rBMSC (Fig. 5B). When discussing the rGEC monoculture and rGEC + rBMSC co-culture, samples in endothelial cell growth medium (ECGM), in long-term perfusion culture on day 14, the distribution and migration of cells in renal tissue were higher in rGEC + rBMSC co-culture conditions (Fig. 5C), and cells in the glomerulus microvascular structure were clearly observed and found in abundance.

3.6. Cell death and proliferation analysis in recelluarized renal ECM scaffold vascular injury

Cell death and proliferative capacity by stem cell therapy in recellularized renal ECM scaffolds using rGEC and rGEC + rBMSCs were analyzed by histological fluorescence immunostaining. It was found that the apoptosis marker, cleaved caspase-3 (CCasp3), was less expressed in the glomerular capillaries in the rGEC + rBMSC group (1.60 ± 0.94) compared to the rGEC group (7.11 ± 2.99) on day 14 of the perfusion culture as shown in Fig. 6A and B. The result of Ki67, a cell proliferation marker, was higher on day 7 under co-culture conditions in the rGEC + rBMSC group (13.00 ± 4.93) than in the rGEC group (1.31 ± 1.75) with no significant difference on day 14. Quantitative evaluations are summarized in Fig. 6C and D. The number of cells in the glomerular capillaries increased from day 7–14 in the co-culture conditions (57.77 ± 16.45) while it decreased from day 7–14 in the rGEC monoculture (29.16 ± 9.98). In addition, in the whole renal tissue, the number of cells per unit area was significantly increased in rGEC + rBMSC co-culture conditions (367.01 ± 42.96) and was found to be lower in the rGEC monoculture group (73.1556 ± 7.89) (Fig. 6F).

3.7. Glomerulus capillary inflammation and stability analysis

The inflammatory response occurs in the endothelial cell membrane expressed by perfusion stimulation in the renal scaffold. Glomerulus vessel structure was clearly observed by F-actin and nucleus staining as shown in Fig. 7A. The expression level of VCAM1 in rGECs injured by perfusion was 43.71 ± 19.78. However, in the rGEC + rBMSC co-culture, it was confirmed that the VCAM1 inflammation level was reduced to 27.11 ± 16.88 (Fig. 7B and D). The CD73 response in vascular endothelial cells reduces inflammatory factors such as VCAM1, ICAM1 and TNF-α [22,24]. In the rGEC + rBMSC co-culture recellularized renal ECM scaffold, the expression of CD73 was confirmed to be higher (23.02 ± 13.10) than that in the rGEC monoculture sample (9.17 ± 5.03), which indicates a significant relationship with the VCAM1 expression result (Fig. 7B and D). When analyzing the expression ratios of Tie1 and Tie2 in rGEC and rGEC + rBMSC perfusion co-culture, both groups showed higher expression levels of Tie2, indicating good vascular stabilization. It was confirmed that the rGECs + rBMSCs co-culture (61.04 ± 14.07) under perfusion culture conditions improved endothelial stability compared to rGECs (34.62 ± 19.15). Cell viability, cell proliferation, cell migration and capillary stabilization are shown in Fig. 7C and E. A schematic illustration of the VCAM1 adhesion protein involved in the inflammatory response is presented in Fig. 7F. A key lock mechanism of VCAM1 blockade with the VLA-4 receptor of rBMSC was recognized. The co-cultured rGEC + rBMSC cells were found to be healthier than the monoculture rBMSC. The bioactive factors from rBMSC were found to be helpful in reducing cell death and increasing stabilization with higher cell proliferation and migration.

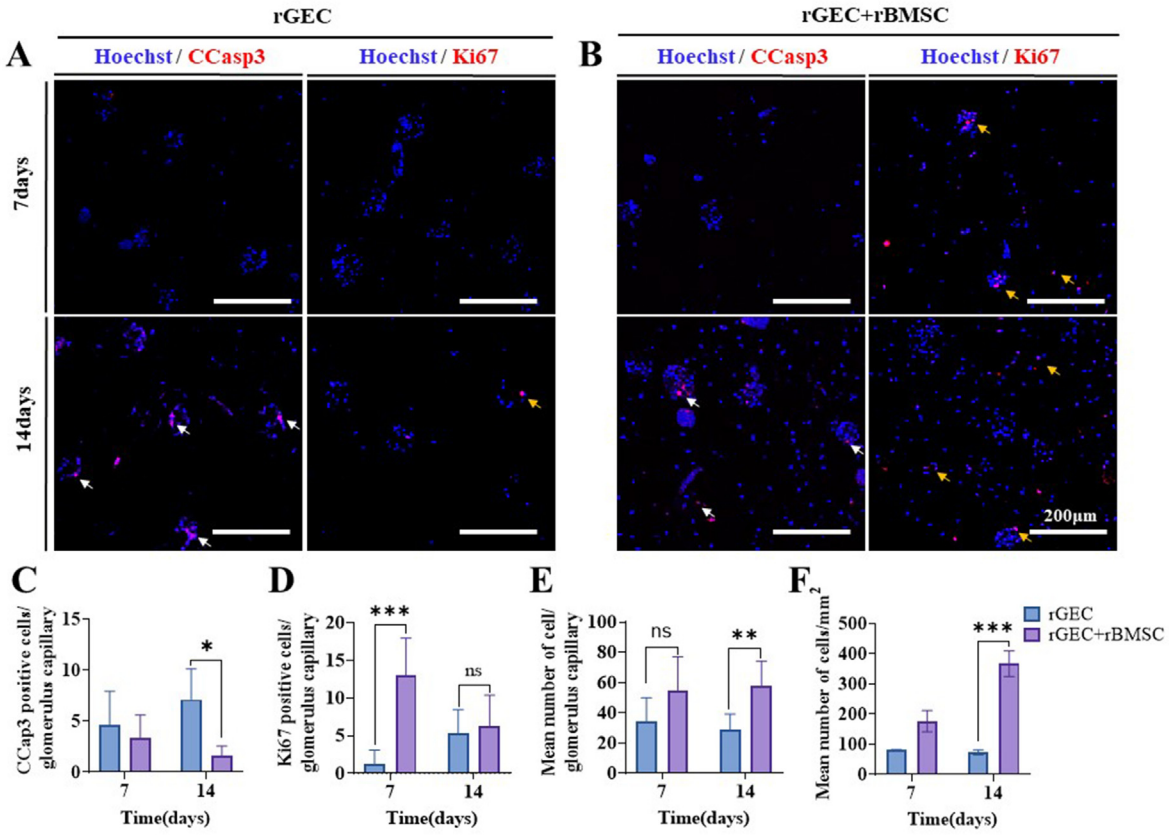

Fig. 6. Immunohistochemistry (IHC) staining (A) rGEC (B) rGEC + rBMSC for cell death (Cleaved Casp3) and proliferation (Ki67) marker for 7 and 14 days. (C to F) Quantification of cell number under different markers. *($p < 0.001$***, $p < 0.005$**, $p < 0.05$**, White arrow indicates expression of CCasp3, yellow arrow indicates Ki67).

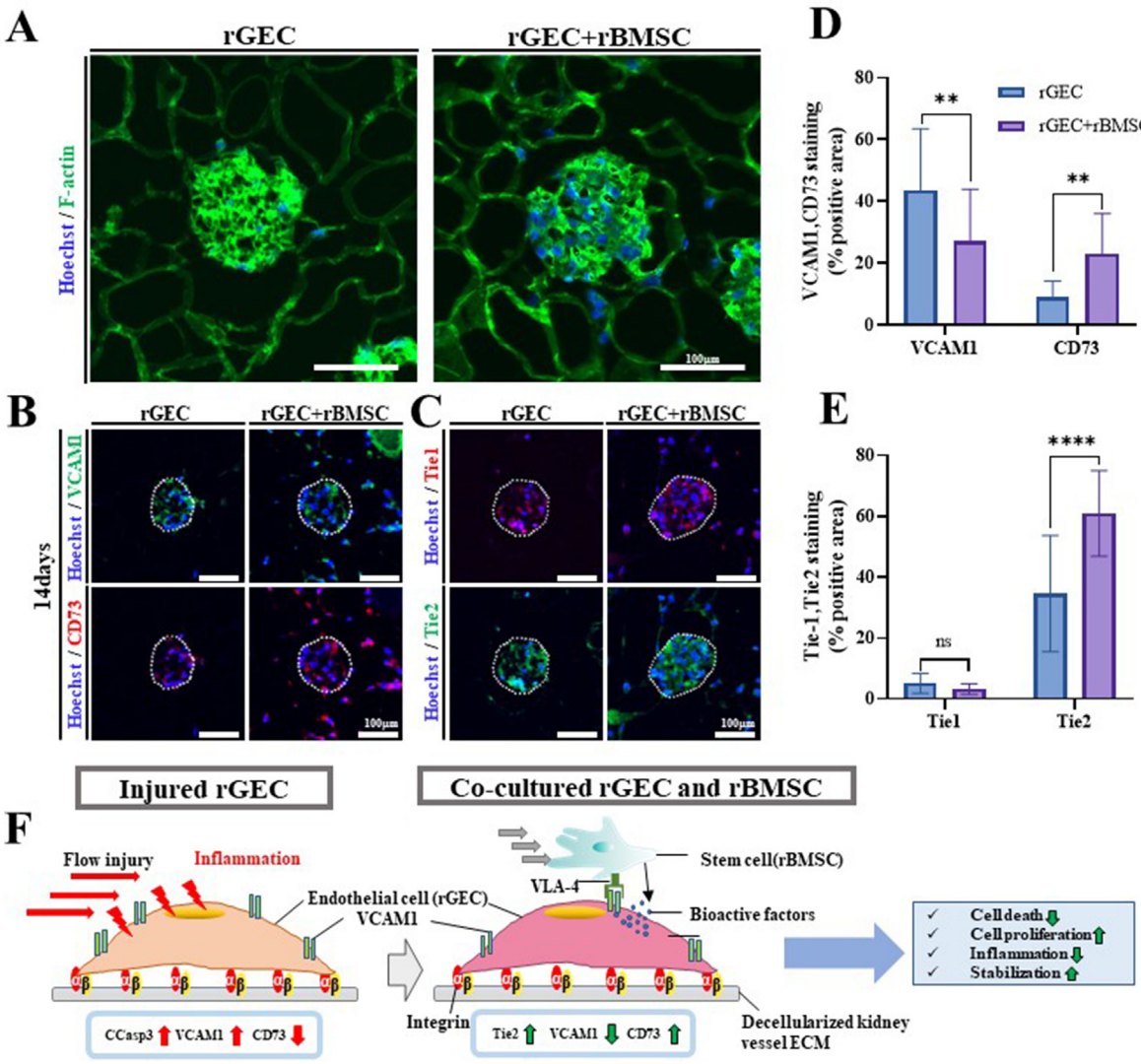

Fig. 7. (A, B and C) Immunohistochemistry (IHC) staining and (D and E) their quantification of rGEC/rGEC + rBMSC for micro vessel inflammation (VCAM1/CD73) and stability (Tie1/Tie2) in the glomerulus and (F) schematic illustration of healing of injured rGEC by the co-culturing of rGEC/rBMSC. *($p < 0.0001$ ****, $p < 0.005$**).*

4. Discussion

Since the kidney is composed of a complex and fine nephron structure, it is essential to consider that the ECM vessel should not be damaged during the decellularization process. Damage to the ECM vessel in the decellularized renal scaffold not only causes cell outflow during recellularization, but also increases the shear stress during perfusion culture. After decellularization, not only the ECM scaffold structure but also the presence or absence of residual vascular-related growth factors (VEGF, FGF, etc.) are important for inducing vascular formation. It is also responsible for supporting endothelial cell retention, adhesion, proliferation and differentiation of adipose stem cells into endothelial cells [25, 26]. Collagen and Growth factors level in kidney scaffold were decreased after decellularization, but it was expected that these parts could be supported by adding rBMSC which released ECM and growth factors such as VEGF, FGF, etc. During co-culturing with endothelial cell in kidney scaffold.

It is vital to maintain the function and stability of blood vessels that transport nutrients and gases through the tissues and induce a smooth fluid flow. In this regard, various methods of vascularization studies in living artificial organs have been reported [27–29]. In another approach, this functional stability is directly related to the vascular status according to the FR of the media perfusion in the ex vivo culture (Fig. 2). Tissue staining results, as shown in Fig. 2 G-J, evidenced that high FR stimulates endothelial cells of renal blood vessels and that glomerular capillary damage causes glomerular endothelial dysfunction. When the recellularized renal ECM scaffold with rGEC is statically cultured and perfused at 1 ml/min and 2 ml/min FR, the smallest glomerulus appears in the static culture. There was no significant difference in the size of the renal area or glomeruli in the samples cultured at different perfusion rates, but some damage of glomeruli was observed in the 2 ml/min FR sample, which is unacceptable. In general, the size of the glomerulus is greater than ~100 μm, but the renal tissue and glomeruli shrunk to less than 100 μm due to the repeated tissue contraction and relaxation during decellularization and recellularization. It was confirmed that perfusion at 2 ml/min FR or greater to increase the size of the glomeruli resulted in the collapse of the renal ECM scaffold tissue (Fig. S1).

Renal failure due to high perfusion flow is closely related to cardiovascular diseases such as diabetes and hypertension [30]. High blood pressure causes damage to the body such as nitric oxide (NO) production in vascular endothelial cells, inflammation related proteins such as VCAM1, ICAM1 and Tie1, with negative consequences in the body.

Research on eNOS has been strongly correlated with hypertension and endothelial dysfunction [31,32]. VCAM-1 and ICAM-1 are proteins expressed on the surface of endothelial cells when an inflammatory stimulus occurs [33] and rapid perfusion culture causes a strong inflammatory response in vascular endothelial cells attached to the glomeruli and causes rapid cell death. eNOS is a protein that functions to decompose active factors such as NO in vascular endothelial cells and determines the function of the vascular endothelial cells. Therefore, when the function of vascular endothelial cells is irregular, the level of protein expression is low [34]. In the present study, as mentioned above, optimal perfusion conditions were established by comparing the protein expression level in vascular endothelial cells with normal and abnormal fluid flow rates during *in-vivo* artificial renal culture. While cultured under high FR conditions, the shear stress increases and a large amount of reactive oxygen species (ROS) is generated, leading to an increase in inflammatory adhesion protein factors such as VCAM-1 and ICAM-1 (Fig. 3) [35]. The Tie1 is cleaved during inflammation, leading to decreased Tie2 phosphorylation and decreased Tie2 expression in an Ang2-dependent manner [36,37]. Tie1 shedding thus contributes to the antagonist role of Ang2 on Tie2 signaling. Therefore, it was suggested that when cultured at an appropriate perfusion rate, a decrease in VCAM1/ICAM1 expression and an increase in eNOS/Tie1 resulted in positive vascularization of the renal scaffold.

It was demonstrated that a FR perfusion of 1 ml/min was shown to cause less injury to vascular endothelial cells in the recellularized renal ECM scaffold compared to 2 ml/min and was reported to be a perfusion state that allowed for longer survival in culture than 14 days. In addition, although inflammation was increased compared to static culture, rGEC inflammation and apoptosis were less than 2 ml/min FR, and as a result of increased endothelial cell function and cell migration, the recellularized renal ECM scaffold was perfused in the bioreactor. The perfusion rate for incubation was selected (Fig. 3I). However, cell death of rGEC increased on day14 of culture due to continued perfusion resulting in high inflammation, higher cell death and lower cell adhesion ability in the glomerulus ECM of the recellularized renal ECM scaffold, which is unacceptable for transplantation owing to a negative effect. Ang1 overexpression is known to increase Tie2 activation and maintain a quiescent EC phenotype while maintaining vessel integrity [38].

The concept of mesenchymal stem cell (MSC) cell therapy is of great interest in repairing damaged tissues [39]. MSCs can reduce the inflammatory response of vascular endothelial cells [40]. In fact, studies relating to the application of adult stem cells (iPSc, adipose-derived stem cell, etc) to *ex-vivo* organ tissues such as heart, lung, and liver other than renal tissue have also been reported, proving the potential of stem cell application studies [41–44]. Therefore, the authors also put forward a hypothesis about the rGEC monoculture the and further rGEC + rBMSC co-culture. First, the ratio of rGEC and rBMSC co-culture in the 2D plate was investigated prior to co-culture in renal ECM scaffolds. The mixing ratio of rGEC:rBMSC is listed as 100:0, 75:25, 50:50 and 75:25. The expression level was analyzed according to the mixing ratio of cells after co-culturing on a 2D plate. VEGF-A, CD31, CD105 (endoglin), vWF, Angiopoietin 1 (Ang-1), Angiopoietin2 (Ang-2), Tie1 and Tie2, were quantified by RT-PCR. The primer sequences are summarized in Table 1. While analyzing the trend of endothelial cell gene expression for the vascularization of the recellularized renal ECM scaffold, the level of CD31 expression in co-culture (rGEC: rGEC + rBMSC (75:25) condition did not differ significantly from monoculture (rGEC:rGEC + rBMSC (100:0) but expression was higher than in other co-cultured groups. The scaffold with fine glomerular capillaries was found suitable for the expression of the microvascular-related gene in 2D co-culture studies. For CD105 expression, sample (rGEC:rGEC + rBMSC) 75:25 showed a higher level than the (rGEC:rGEC + rBMSC) 100:0 control group, therefore it was determined that the capillary structure could be better formed in the 75:25 sample.

MSCs, secrete pro-angiogenic factors such as VEGF-A and Ang-2 [45,46]. During co-culture, higher VEGF-A expression was confirmed as the ratio of MSCs increased under co-culture conditions. According to another study [36], when Ang-2, an agonist of Ang-1 binds to Tie2, under high VEGF level the capillary sprout, vessel remodeling and vessel destabilization occur. In addition, it has been reported that when a large amount of Ang-2 binds to Tie2, it not only increases vessel permeability and destabilization but also disrupts Ang-1/Tie2 signaling, resulting in endothelial cell activation and vascular damage. On the other hand, when the amount of Ang-1 is higher, the amount of Ang-2 is lower, resulting in stable vessel formation by binging to Tie2 [47].

Based on our analysis, all tested groups have vascular formation potential, except for the monoculture (rGEC:rGEC + rBMSC(100:0)) condition. Unstable angiogenesis was observed at 25:75 and 50:50 due to the high expression of VEGF-A and Ang-2. Group 75:25 was found to be capable of stable angiogenesis and vessel formation. When the expression level of Tie2 is higher than that of Tie1, the quiescent of blood vessels is maintained, and conversely, when the expression level of Tie1 is high, vessel formation occurs. The fluid FR in perfusion cultures regulates the expression levels of endothelial cell adhesion proteins such as VCAM-1, ICAM-1, and CD31 (PECAM-1), which bind leukocytes, hematopoietic stem cells and MSCs to the vessel wall [48,49]. It has also been reported to play an important role in immunosuppression [50]. In particular, several studies have reported that MSCs have a deep cross-relationship with adhesion proteins located in the endothelial cell membrane, such as VLA-4/VCAM-1, and many studies report their therapeutic efficacy using the above-mentioned principle [51–53]. Also, MSCs have a higher level of adhesion to damaged endothelial cells than intact endothelial cells [54,55] and have a homing effect on the damaged endothelial layer consisting of four steps: rolling, activation, quiescence and movement into tissues [56].

In the present study, it was hypothesized that damaged endothelial cells induced by the fluid FR of the renal scaffold increase MSC adhesion and recruitment and that MSCs attached to damaged endothelial cells may undergo homing effect in the recellularized renal ECM scaffold. It has been suggested that this may benefit in stable functional angiogenesis, immunomodulation to reduce inflammation, induction of end differentiation and maintenance of cell viability. In addition, this series of procedures has been applied as an *in-vitro* cell therapy concept.

Co-culture conditions at the selected ratio of rGEC:rGEC + rBMSC 75:25 was injected into the decellularized renal scaffold artery, followed by perfusion culture. First, the renal ECM scaffold recellularized with rGEC was perfused and cultured for 3 days, and then the rBMSC suspension was injected into the renal artery. Finally, cultured samples were collected on days 3, 7 and 14. When rGEC and rBMSC were co-cultured with recellularized renal ECM scaffolds, two different culture media were used for comparison purposes. It was first cultured in a medium composed of DMEM+10% FBS+1% PS, and in another, an endothelial cell growth medium (ECGM). The cell culture group using DMEM+10% FBS+1%PS medium was used only for the purpose of cell proliferation, as a control in the experiment. For cell culture at 3 and 7 days, sufficient cell settlement in the glomerulus was confirmed. It is known that in the case of intravascular cell co-culture of renal scaffolds up to 3–7 days seems to be an appropriate period. However, when co-culture of mature endothelial cells and MSCs, continued using DMEM+10% FBS+1%PS medium for a long period of time such as 8–14 days, there was found the collapse of decellularized tissues because of excessive proliferation and activation of cells. Long-term proliferation of cells in microvasculature is not appreciated in past. Due to the use of a specific cell culture medium, the possibility of promoting the regeneration of mature cells without disrupting the structure of the kidney scaffold during long-term co-culture was confirmed in-vitro. As a result of tissue staining with H&E, a volume of relatively dark cytoplasm was observed in the group cultured in the DMEM medium compared to the group cultured in the ECGM medium. Under the rGEC + rBMSC co-culture cell conditions in the recellularized renal ECM scaffold, it was difficult to clearly observe the glomerulus morphology on day 14 of culture. The Tie1/Tie2 results were consistent with the hypothesis of angiogenesis and vascular remodeling. As a result of observing the tissues of rGEC and rGEC + rBMSC samples

grown in ECGM medium, on day14, the distribution and migration of cells in the recellularized renal ECM were greater under the rGEC + rBMSC co-culture conditions, and cells in the glomerulus microvascular structure were also observed. Cell death and proliferation ability were investigated through stem cell therapy using rBMSC in damaged endothelial cell vascular in the renal scaffold. Cleaved CCasp3, an apoptosis marker, was less expressed in the glomerular capillaries in the rGEC + rBMSC group compared to the rGEC group even on day 14. Growth factor secretion and cross-talk increased the cell proliferation, survival, invasion and migration of vascular endothelial cells, suggesting its positive response [19]. When analyzing the cell proliferation marker Ki67, it was concluded that the capillaries in the rGEC:rGEC + rBMSC (75:25) group were expressed at a higher level than that only in the rGEC group on day 7 of perfusion culture and further slightly decreased at day 14. This may be due to the regeneration of vascular endothelial cells in the glomerulus by rBMSC. It was then confirmed that the number of cells in the glomerular capillaries was higher under rGEC:rGEC + rBMSC (75:25) co-culture conditions than in the rGEC (100:0) group. In addition, whole kidney recellularization was observed using rGEC:rGEC + rBMSC (75:25) co-culture conditions and an increase in the number of cells in the recellularized renal ECM scaffold with rapid infiltration and migration on the ECM.

The explanatory mechanism of the inflammatory response occurring in the endothelial cell membrane expressed by perfusion stimulation in the recellularized renal ECM scaffold is shown in Fig. 7F. The VCAM1 adhesive protein involved in the inflammatory response is partially expressed only in endothelial cells and attached to the VLA-4 receptor of rBMSC [54,57–60]. CD73 owing the function of reducing inflammatory factors confirmed via VCAM1, ICAM1, and TNF-α. The expression of CD73 was confirmed higher under cell co-culture (rGEC + rBMSC) conditions, while the VCAM1 expression level of the rGEC injured by perfusion was higher than that of the rGEC + rBMSC co-culture, therefore the results of the identified markers had significant relationships with each other. By examining the ratio of Tie1 and Tie2 expression of rGEC and rGEC + rBMSC in the perfusion co-culture, the expression level of Tie2 of rGEC + rBMSC was higher: supporting the vascular stabilization. Accordingly, it was confirmed that the co-culture of rGECs with rBMSCs under perfusion conditions increased cell viability, cell proliferation, cell migration, and vascularization.

5. Conclusions

In the present work, we have investigated the effect of different perfusion flow rate in whole kidney scaffold and found that there was flow injury during long term cell perfusion in vitro. To overcome this situation, isolated bone marrow derived mesenchymal stem cells were co-cultured with rGEC to reduce inflammation occurred. It was found that co culture therapy inhibited the cell death, suppress inflammation and enhance the overall cell count in recellularized kidney scaffold. In summary, present study proposed that the co-culture of MSCs in the vascular endothelial niche of recellularized kidney scaffold might lead to stable endothelialization and renal cell maintenance for in vivo. Also, our obtained optimized technique might be useful for other decellularized soft organs containing capillary network.

Credit author Statement

Minji Choi: Conceptualization, Methodology, Data curation, Investigation, Writing – original draft, Yubin Yang: Resources, Methodology, Seongsu Park: Formal Analysis & Visualization, Sohanur Rahaman: Formal Analysis, Garima Tripathi: Visualization, Writing – review & editing, Byong Taek Lee: Supervision, Conceptualization, Visualization, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100464.

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