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Loss of Endothelial Glycocalyx During Normothermic Machine Perfusion of Porcine Kidneys Irrespective of Pressure and Hematocrit

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Background. Normothermic machine perfusion (NMP) is a promising modality for marginal donor kidneys. However, little is known about the effects of NMP on causing endothelial glycocalyx (eGC) injury. This study aims to evaluate the effects of NMP on eGC injury in marginal donor kidneys and whether this is affected by perfusion pressures and hematocrits. **Methods.** Porcine slaughterhouse kidneys (n = 6/group) underwent 35 min of warm ischemia. Thereafter, the kidneys were preserved with oxygenated hypothermic machine perfusion for 3 h. Subsequently, 4 h of NMP was applied using pressure-controlled perfusion with an autologous blood-based solution containing either 12%, 24%, or 36% hematocrit. Pressures of 55, 75, and 95 mm Hg were applied in the 24% group. Perfusate, urine, and biopsy samples were collected to determine both injury and functional parameters. **Results.** During NMP, hyaluronan levels in the perfusate increased significantly ($P < 0.0001$). In addition, the positivity of glyco-stained glycocalyx decreased significantly over time, both in the glomeruli ($P = 0.024$) and peritubular capillaries ($P = 0.003$). The number of endothelial cells did not change during NMP ($P = 0.157$), whereas glomerular endothelial expression of vascular endothelial growth factor receptor-2 decreased significantly ($P < 0.001$). Microthrombi formation was significantly increased after NMP. The use of different pressures and hematocrits did not affect functional parameters during perfusion. **Conclusions.** NMP is accompanied with eGC and vascular endothelial growth factor receptor-2 loss, without significant loss of endothelial cells. eGC loss was not affected by the different pressures and hematocrits used. It remains unclear whether endothelial injury during NMP has harmful consequences for the transplanted kidney.

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Over the past years, extended criteria donor (ECD) organs are used more often, including organs from donors after circulatory death.[1] Unfortunately, the use of ECD organs is often accompanied by decreased organ quality, and these organs are more susceptible to ischemia-reperfusion injury (IRI), which is inevitable in organ transplantation.[2]

Therefore, the necessity arises to assess organ quality before transplantation. Normothermic machine perfusion (NMP) allows for this assessment and might even allow for resuscitation of poor-quality ECD kidneys.[3] However, much is still unknown about rather basic aspects of NMP, such as its effects on endothelial glycocalyx (eGC) injury.

The eGC is a layer of proteoglycans, glycosaminoglycans, and glycoproteins located at the luminal site of endothelial cells. The eGC is involved in many different processes such as regulation of permeability and glomerular filtration, mechanotransduction, and anticoagulant and anti-inflammatory properties.[4–6] Glycocalyx loss is associated with many pathologies, such as capillary leak syndrome, edema formation, accelerated inflammation, hypercoagulation, and loss of vascular responsiveness, resulting in reduced renal function.[4,7,8] Moreover, the severity of the pathologies is associated with the amount of glycocalyx injury.[9,10] Furthermore, the destruction of the glycocalyx plays a major role in IRI, leading to endothelial dysfunction with vasoconstriction and increased inflammatory responses.[11–14] Several studies have described the loss of eGC during transplantation. During reperfusion, syndecan-1 levels in plasma increase significantly after liver transplantation.[13] In kidney transplantation, organs from donors after circulatory death showed increased shedding of syndecan-1 and heparan sulfate compared with living kidney donors.[15]

Although many studies have shown effects of extracorporeal circulation on eGC injury,[11,16–18] the occurrence and role of eGC injury in organ preservation and resuscitation modalities such as machine perfusion are insufficiently studied. Sladden et al[19] described the shedding of eGC products during ex vivo lung perfusion of both porcine and human lungs. They also found that eGC breakdown occurs in both lung donors and recipients and that it is associated with primary graft dysfunction.[20]

Because NMP is a promising technique, evaluation of eGC injury during perfusion is needed, especially in the context of different NMP protocols that are currently used, which might affect this injury. To date, there is no consensus on rather basic aspects of machine perfusion for kidneys such as perfusate composition or pressure settings. The hematocrit, the volume of red blood cells (RBC) in relation to the total blood volume, varies between 13% and 33% in protocols currently used.[21] Also, different arterial pressures are used, varying between 40 and 95 mm Hg.[21–23] Both these factors may influence the amount of shear stress and therefore eGC injury.

This study aims to evaluate the extent of eGC injury in marginal donor kidneys during NMP and whether different pressures and hematocrits affect this injury.

MATERIALS AND METHODS

Kidney Graft Preparation and Preservation

Our slaughterhouse kidney machine perfusion model was used, as described previously.[24,25] In short, porcine kidneys were obtained from a local abattoir. No animal ethics committee approval was required because slaughterhouse waste material was used. During exsanguination, ~3 L blood was collected in a beaker containing 25 000 IU of heparin (LEO Pharma A/S, Ballerup, Denmark). To ensure an equivalent to a marginal donor status, kidneys were exposed to 35 min of warm ischemic time and flushed with 1 L of cold (0°C–4°C) 0.9% NaCl (Baxter B.V., Utrecht, the Netherlands). The aorta was cannulated caudally, and a gravitational flush was performed with a pressure of 80 mm Hg. Thereafter, cortical biopsies were taken, and the kidneys were placed on oxygenated (FiO$_2$ 100% at 100 mL/min) hypothermic machine perfusion (HMP) at a pressure of 25 mm Hg (Organ Assist, Groningen, the Netherlands) for 3 h.

Experimental Setup

This study involved 5 different groups (n = 6/group), using 3 different mean arterial pressures (MAP; 55, 75, and 95 mm Hg) and 3 different hematocrits during NMP (12%, 24%, and 36%; **Figure S1, SDC**, http://links.lww.com/TXD/A550). The different pressures were applied in the 24% group and with an amplitude of 20%. The ratio of plasma and RBC addition was calculated, based on measured hematocrits.

Normothermic Machine Perfusion

Following HMP, kidneys were perfused using NMP for 4 h to assess renal function and histopathological changes, including loss of eGC and endothelial cells. A custom-built controller was used to drive a centrifugal pump (Deltastream DP3, MEDOS Medizintechnik AG, Heilbronn, Germany). The MAP was set according to the experimental setup and monitored using a Truewave disposable pressure transducer (Edwards Lifesciences, Irvine, CA). The pulsatility was set at 60/min, and the flow was measured using a clamp-on flow sensor (ME7PXL clamp, Transonic Systems, Inc., Ithaca, NY). Via the oxygenator (HILITE 1000, MEDOS Medizintechnik AG, Stolberg, Germany), 0.5 L/min carbogen (95% O$_2$ and 5% CO$_2$) was supplied. The temperature was maintained at 37°C using a water heating system (Jubalo, Seelbach, Germany).

An autologous, leukocyte-depleted, blood-based perfusate was used (**Table S1, SDC,** http://links.lww.com/TXD/A550). Leukocyte depletion was achieved using a BioR 02 plus leukocyte filter (Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany). The perfusate consisted of autologous blood (plasma and RBCs mixed according to the experimental setup), Ringers' lactate solution (Baxter, Utrecht, Netherlands), glucose 5% (Baxter), sodium bicarbonate 8.4% (Baxter), Amoxicillin-clavulanate (Sandoz, Almere, the Netherlands), dexamethasone (Centrafarm, Etten-Leur, the Netherlands), creatinine (Sigma-Aldrich, St. Louis, MO), mannitol (Sigma-Aldrich), and sodium nitroprusside (Sigma-Aldrich). Continuous infusion of Aminoplasmal (B. Braun Melsungen AG, Melsungen, Germany), sodium bicarbonate (Baxter), and insulin (Novo Nordisk, Bagsværd, Denmark) were supplied at a rate of 20 mL/h, via the arterial line.

Sample Collection

Perfusate and urine samples were collected after 15, 30, and 60 min and every following hour during NMP. At the same time points, arterial and venous blood gas samples were taken. Blood gas samples were analyzed immediately using the ABL800 blood gas analyzer (Radiometer, Brønhøj, Denmark). Perfusate and urine samples were centrifuged (1000g for 12 min at 4°C), and the supernatant was stored at –80°C until analyses.

The biopsies were taken after 3 h of HMP and 4 h of NMP and were either stored in a sonification solution (0.372 g EDTA in 130 mL H$_2$O and NaOH [pH 10.9] + 370 mL 96% ethanol) for ATP analysis,[26] fixated in 4% formaldehyde followed by paraffin embedding for histological analyses, or snap-frozen.

Analyses in Perfusate and Urine

Hyaluronan (HA) was determined in duplo in both the perfusate and the urine samples using a commercial kit (Hyaluronan DuoSet ELISA DY3614, R&D Systems Inc., Minneapolis, MN). In short, samples were diluted 20 times in

reagent diluent (5% Tween20 in PBS). Concentrations of HA in the urine were measured as urine was recirculated. Based on the total amount of HA in the perfusate and the urine that was recirculated, an indication was made about which share of total HA was due to eGC shedding.

Sodium, potassium, creatinine, lactate, lactate dehydrogenase (LDH), and aspartate aminotransferase (ASAT) were determined in perfusate, and sodium, creatinine, and total protein levels in the urine by the Laboratory Center of the University Medical Center Groningen using standard biochemical analyses. The hemolysis index was used as a measure of hemolysis because no other red coloring agents were present in the perfusate. ATP levels, as a measure of energy status, were determined in cortical kidney tissue biopsies, as previously described.[26,27]

Histological Assessment

Biopsies were cut into 4-μm sections and stained for general morphology, the presence of endothelial cells, and eGC. The periodic acid-Schiff (PAS) staining, the endothelial nuclei staining for the transcription factor ETS-related gene (ERG), and the fibrin staining were performed on paraffin-embedded material. The Dolichos Biflorus Agglutinin (DBA) and vascular endothelial growth factor receptor-2 (VEGFR-2) stainings were performed on frozen material. Sections were scanned using a Hamamatsu NanoZoomer 2.0-HT digital slide scanner (Hamamatsu Photonics, Shizuoka, Japan). Histological scoring of DBA, ERG, and VEGFR-2 was performed in (up to) 8 glomeruli and 360 000 μm² of peritubular area (excluding glomeruli). The number of ERG-positive nuclei was counted. Microthrombi, defined as clusters of occluding fibrin threads either with or without trapped RBC, were counted in up to 50 glomeruli.

A detailed specification of the immunohistochemical staining is provided in **Table S2, SDC**, http://links.lww.com/TXD/A550. In short, for the glycocalyx staining, a biotinylated lectin DBA (B-1035-5, Vector Laboratories Inc., Newark, CA) was used.[28,29] For the VEGFR-2 and fibrin stainings incubation with respectively a primary rabbit anti-human VEGFR-2 (antibody Cell Signaling Technology, MA D5B1, #9698S) and a rabbit anti-human fibrinogen (A0080, Dako, Glostrup, Denmark) antibodies was done. The ERG staining was performed using an anti-ERG rabbit monoclonal primary antibody (EPR3864; Abcam).

Loss of DBA and VEGFR-2 was determined by quantifying the number of positive pixels in glomerular and peritubular areas using Aperio ImageScope v12.4.3.5008 (Leica Biosystems, Wetzlar, Germany), using a build-in Positive Pixel Count v9 algorithm. Because DBA was described to stain proliferating tubular cells as well,[30] the thresholds of the Positive Pixel Count v9 tool in Aperio ImageScope had to be adjusted. The Hue value was set to 0 and the Hue width to 0.1. After adjustment, reliable measurements of positive and strong-positive pixels could be made. Positivity was defined as the number of positive and strong-positive pixels divided by the total number of pixels in the selected areas. Subsequently, differences in the positivity of DBA and VEGFR-2, and counts of ERG and microthrombi per glomerulus, between pre-and post-NMP were calculated per kidney.

PAS-stained biopsies were blindly scored by a pathologist (M.C.v.d.H.). Sections were scored for glomerular dilation (grade 0–3), tubular dilation (grade 0–3), simplification of the epithelium of the proximal tubules (grade 0–3), proximal tubular cell necrosis (grade 0–4), proximal tubular cell vacuolation (grade 0–3), brush border loss of the proximal tubular epithelium (grade 0–3), and interstitial edema (grade 0–3). Grades 0–3 ranged from no signs (0) to severe (3). Grades 0–4 ranged from no signs (0), sporadic (1), clusters (2), confluent areas (3), to massive (4).

Calculations

Intrarenal resistance (IRR) was calculated by dividing the MAP by flow in mL/min. The oxygen consumption was determined by calculating the difference between arterial and venous oxygen (both dissolved and bound), using a formula that was described previously.[25] Fractional excretion of sodium was calculated using the following formula:

$$FEsodium = \frac{(Sodium\ urine\ \times\ Creatinine\ perfusate)}{(Sodium\ perfusate\ \times\ Creatinine\ urine)} \times 100$$

Absolute values of measured markers were corrected for the weight of the kidney and expressed per 100 g of kidney.

Statistics

Statistical analysis was performed using GraphPad Prism, version 8.0.1 (GraphPad Software Inc., La Jolla, CA). Continuous variables were analyzed by fitting a mixed model. Fixed effects were time, treatment group, and the interaction of the treatment group with time. Individual kidneys were considered as random effects. A Geisser–Greenhouse correction and a restricted maximum likelihood approach were used. Mann–Whitney U and Kruskal–Wallis tests were used because none of the data were normally distributed. P values <0.05 were considered statistically significant.

RESULTS

Hemoglobin Levels and Hemolysis

Mean hemoglobin levels were respectively 2.2, 4.4, and 7.2 mmol/L, and remained stable throughout 4 h of NMP (**Figure S2, SDC**, http://links.lww.com/TXD/A550). Hemolysis indices increased significantly over time ($P = 0.008$), without differences between groups ($P = 0.284$).

Glycocalyx Loss

Different pressures and hematocrits had no significant effect on the HA levels in the perfusate ($P = 0.756$). During NMP, mean HA levels in the perfusate increased significantly from 35.07 ± 17.81 μg/per 100 g kidney tissue (or 42.72 ± 21.73 ng/mL) after 15 min up to 120.32 ± 27.13 μg/100 g (or 466.35 ± 122.05 ng/mL) at the end of NMP ($P < 0.0001$; Figure 1A). HA levels in the perfusate were significantly higher compared with levels in the urine ($P < 0.0001$; Figure 1B). The mean amount of HA in urine was 3.42 ± 2.56 μg/100 g kidney tissue, whereas the total amount of HA in the perfusate was 107.30 ± 25.87 μg/100 g kidney tissue. eGC shedding was comparable between groups, as HA levels in both perfusate and urine (respectively $P = 0.924$ and $P = 0.360$) were not affected by pressure or hematocrit.

DBA positivity was determined in both the glomeruli and peritubular capillaries (PTC) (respectively Figure 1C–F). Pressure and hematocrit did not influence DBA positivity in glomeruli and peritubular compartments (respectively $P = 0.415$ and $P = 0.596$). However, DBA positivity significantly
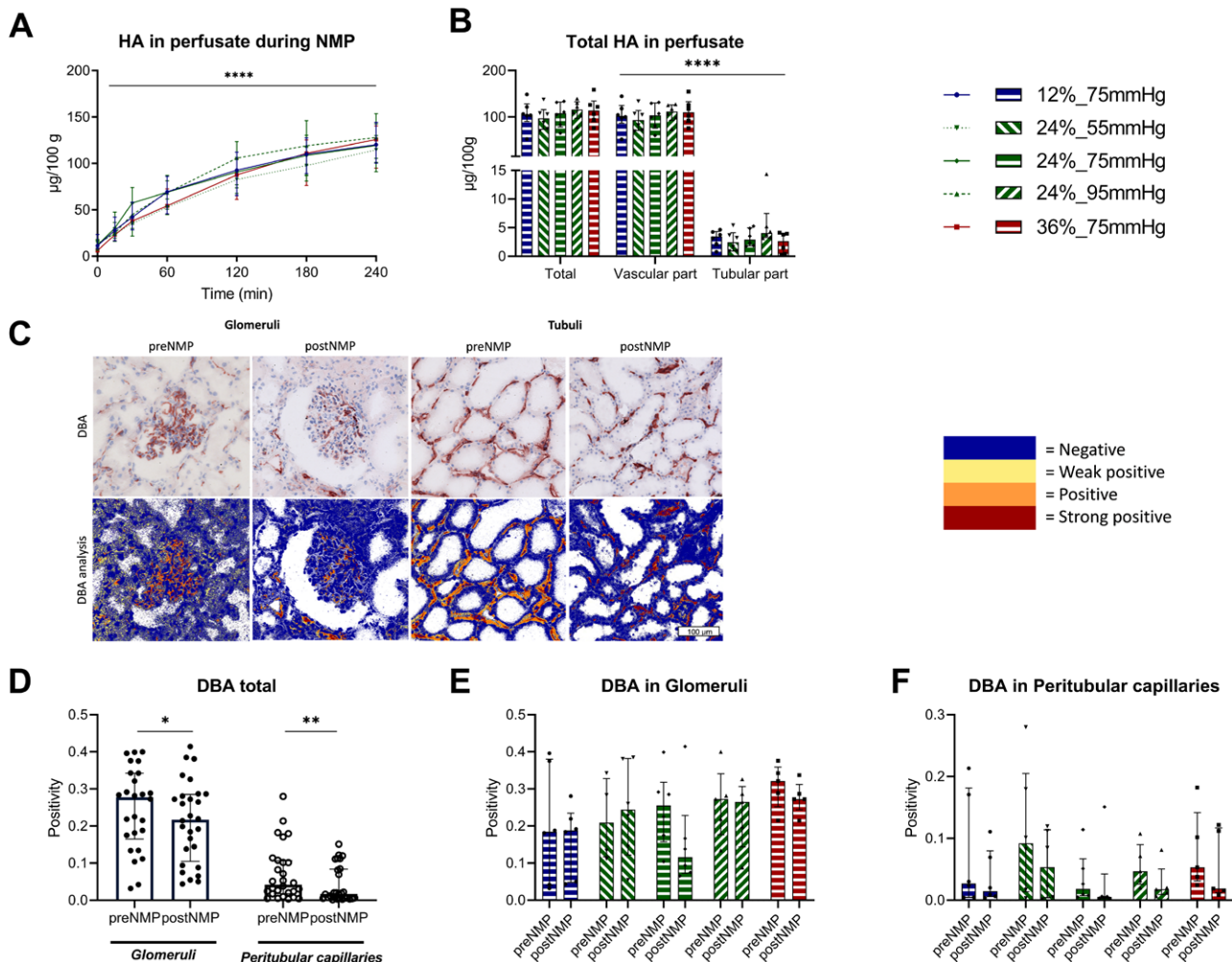
FIGURE 1. Glycocalyx loss during NMP. A, The absolute amount of hyaluronan in the perfusate during NMP, corrected for kidney weight. B, The absolute amount of hyaluronan in the perfusate (vascular part) and urine (tubular part) after NMP, corrected for kidney weight. C, Histological quantification of DBA positivity (number of positive and strong-positive pixels divided by the total number of pixels). D, DBA positivity before and after NMP, pooled. E and F, DBA positivity before and after NMP between the different groups, in respectively glomeruli and the peritubular capillaries. Data are expressed as median ± interquartile range. Time effects: $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$. DBA: dolichos biflorus agglutinin; HA: hyaluronan; NC: negative control, 1% BSA in PBS: phosphate-buffered saline; NMP, normothermic machine perfusion.

decreased during NMP in both compartments (glomeruli: from 0.25 ± 0.11 to 0.21 ± 0.11, $P = 0.024$; PTC: from 0.07 ± 0.07 to 0.04 ± 0.05, $P = 0.003$).

Endothelial and VEGFR-2 Loss

The number of endothelial nuclei was counted in the ERG-stained sections (Figure 2A). No significant decrease in ERG counts was observed after NMP ($P = 0.157$; Figure 2B). No differences between groups were observed in ERG counts in the glomerular and the PTC compartments (respectively $P = 0.349$ and $P = 0.221$; Figure 2C, D). The mean counts of ERG in the glomeruli and the PTC were respectively 52 ± 15 and 145 ± 60 before NMP, and respectively 60 ± 16 and 143 ± 37 after NMP.

Loss of the endothelial marker VEGFR-2 was quantified in the glomeruli only because expression of VEGFR-2 in the PTC was already absent in the biopsies before NMP (data not shown; Figure 2E). VEGFR-2 expression significantly decreased after NMP in all groups that were perfused with 75 mm Hg (HCT 12%, $P < 0.001$; HCT 24%, $P = 0.003$; HCT 36%, $P = 0.003$; Figure 2F).

Kidney Injury During and After NMP

The results of the histological assessment of the PAS staining are displayed in Figure 3A, B. No differences between groups were observed in glomerular dilation ($P = 0.154$), tubular dilation ($P = 0.241$), simplification of the epithelium ($P = 0.540$), tubular cell necrosis ($P = 0.050$), tubular cell vacuolation ($P = 0.251$), brush border loss ($P = 0.516$), and interstitial edema ($P = 0.367$).

The number of microthrombi per glomerulus was significantly increased ($P < 0.001$) after NMP (Figure 3C–E). Moreover, significantly more microthrombi were observed in the 95 mm Hg group compared with 55 mm Hg ($P = 0.041$) and 75 mm Hg ($P = 0.020$) and in the 36% hematocrit group ($P = 0.034$).

Proteinuria increased during NMP ($P = 0.027$; Figure 3F) without differences between groups ($P = 0.217$). The mean cumulative levels of the total protein content increased up to 0.43 ± 0.86 g/100 g kidney after 4 h of NMP.

Weight gain during NMP was significantly higher in both the 12% ($P = 0.044$) and 36% ($P = 0.049$) hematocrit groups when compared with the 24% group (Figure 3G). It was also
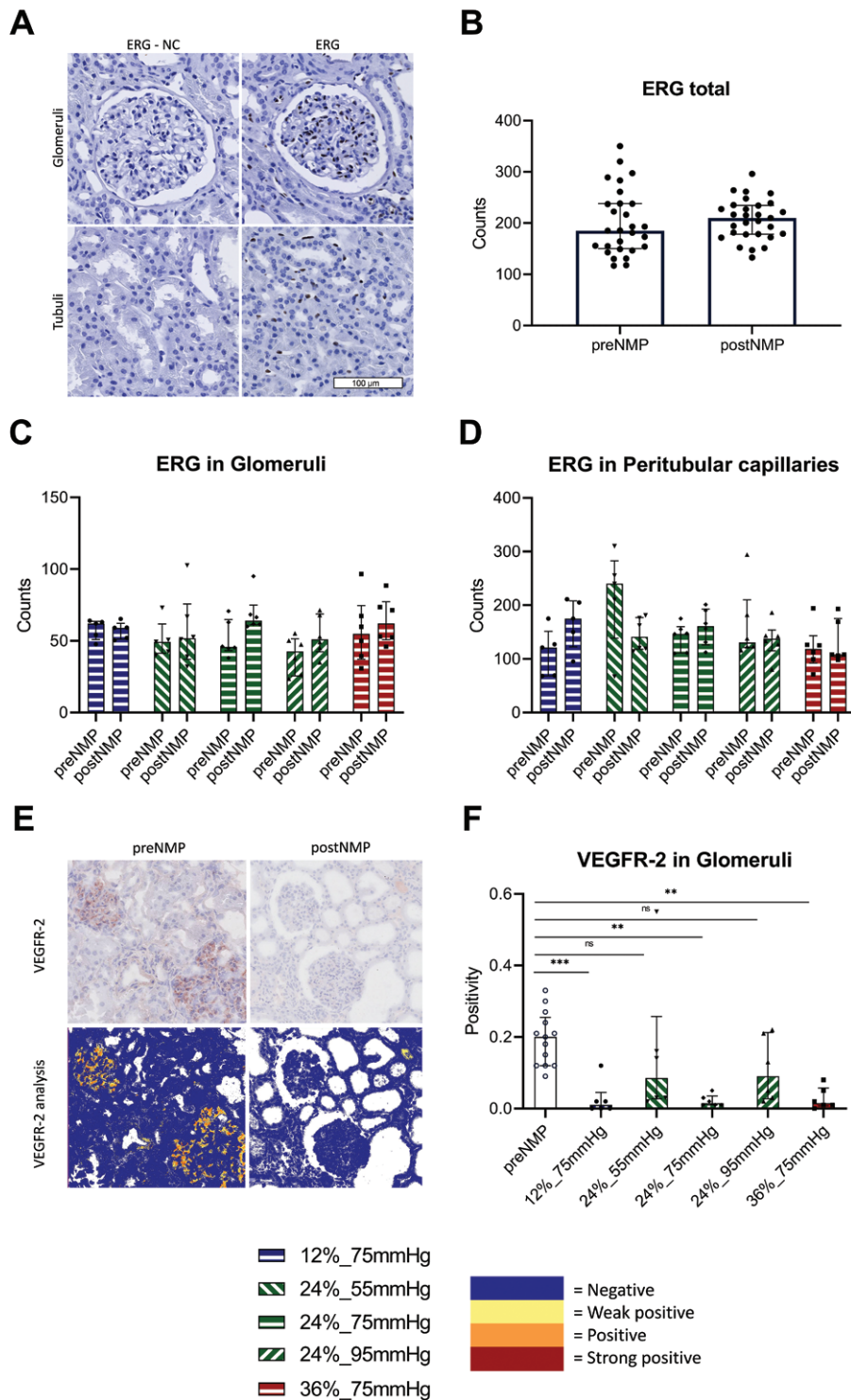
FIGURE 2. Endothelial loss during NMP. A, Histological view of representative ERG-stained biopsies before and after NMP. B, Mean absolute ERG counts before and after NMP, in both glomeruli and peritubular capillaries, pooled. C and D, Mean absolute ERG counts before and after NMP between the different groups, in respectively glomeruli and the peritubular capillaries. E, Histological quantification of VEGFR-2 positivity (number of positive and strong-positive pixels divided by the total number of pixels). F, VEGFR-2 positivity in the glomeruli before (pooled) and after NMP between the different groups. Data are expressed as median ± interquartile range. ERG: ETS-related gene; NC: negative control, 1% BSA in PBS: phosphate-buffered saline; NMP: normothermic machine perfusion; VEGFR-2: vascular endothelial growth factor-2.

significantly higher in the 95 mm Hg group compared with 55 mm Hg ($P = 0.033$).

Mean LDH levels in the perfusate increased significantly over time, from 535.45 ± 359.83 U/L to 832.14 ± 316.01 U/L ($P < 0.001$; Figure 3H). Moreover, LDH levels were significantly higher in the hematocrit 36% group compared with the hematocrit 12% group ($P = 0.032$) and 24% group ($P = 0.008$) after 4 h of perfusion. Mean ASAT levels in the perfusate also increased significantly over time, from 91.31 ± 65.30 U/L to 217.03 ± 79.55 U/L ($P < 0.001$;
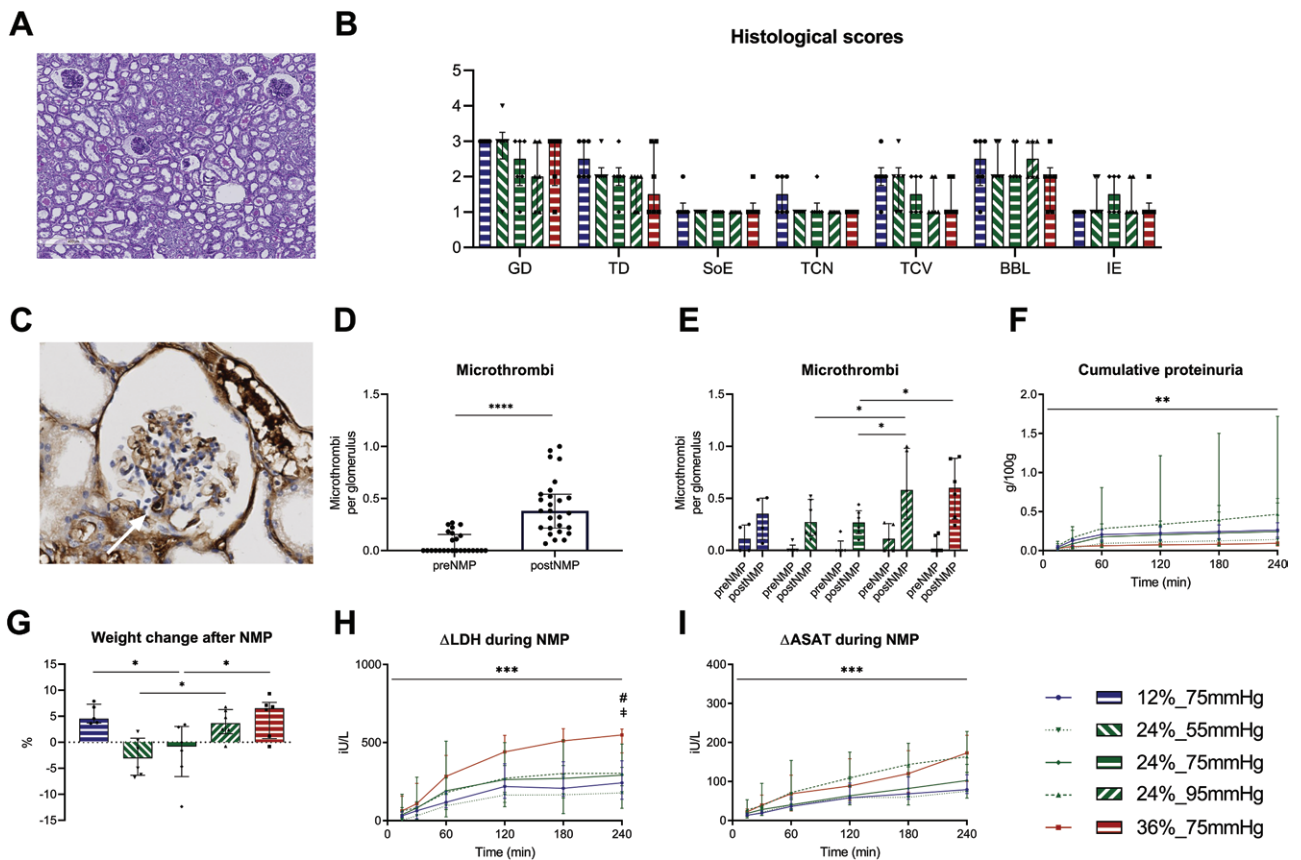
FIGURE 3. Kidney injury during and after NMP. A, Histological view of a representative PAS-stained biopsy B, Assessment scores of PAS-stained sections. C, Representative view of microthrombi in a glomerulus. D, Total microthrombi per glomerulus before and after NMP, and per group (E). F, Cumulative total protein content in urine corrected for kidney weight. G, Weight change after NMP per group. Change of LDH (H) and ASAT (I) concentrations in the perfusate during NMP. Data are expressed as median ± interquartile range. Time effects: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. #$P < 0.05$ Hematocrit 36% vs 12%, ‡$P < 0.01$ Hematocrit 36% vs 24%. ASAT: aspartate aminotransferase; BBL: brush border loss; GD: glomerular dilation; IE: interstitial edema; LDH: lactate dehydrogenase; NMP: normothermic machine perfusion; PAS: periodic acid-Schiff; SoE: simplification of the epithelium of proximal tubules; TCN: tubular cell necrosis; TCV: tubular cell vacuolation; TD: tubular dilation.

Figure 3I). No effect of treatment was observed on ASAT levels in the perfusate ($P = 0.059$).

Kidney Perfusion and Function During NMP

No differences in IRR were observed between the groups ($P = 0.373$; Figure 4A). The mean value of the IRR was 0.50 ± 0.38 mm Hg/mL/min.

Creatinine clearance rates were comparable between groups ($P = 0.745$; Figure 4B). During NMP, the creatinine clearance rates increased significantly over time ($P = 0.002$), with mean rates of 1.22 ± 1.31 mL/min/100 g kidney at the end of NMP.

No differences in urine production were observed between groups ($P = 0.887$; Figure 4C). The mean total production of urine was 164.53 ± 169.33 mL/min/100 g kidney.

The rates of fractional sodium excretion were not affected by pressure or hematocrit ($P = 0.077$; Figure 4D). Fractional sodium excretion dropped in the first hour to $61.27 \pm 29.24\%$ and remained stable during NMP thereafter.

No differences in oxygen consumption were observed between groups ($P = 0.131$; Figure 4E). The mean levels of oxygen consumption by the kidneys increased significantly over time ($P = 0.002$), from 1.08 ± 0.64 mL O_2/min/100 g kidney up to 1.48 ± 1.05 mL O_2/min/100 g kidney.

Finally, levels of ATP were determined in the cortical tissue before and after NMP (Figure 4F). No effect of pressures or hematocrits was seen on ATP levels ($P = 0.879$). ATP levels in all groups were significantly lower after NMP ($P = 0.014$), with mean ATP levels were 9.68 ± 6.11 µmol/g protein before perfusion and 5.99 ± 3.98 µL/g protein after NMP.

DISCUSSION

This study assessed the extent of eGC injury during NMP in marginal donor porcine kidneys and whether perfusion pressure and hematocrit affect the severity of this injury. Both the glomerular and PTC compartments suffered from glycocalyx loss and decreased expression of VEGFR-2. Neither hematocrit nor MAP affected the severity of this injury in this model. Although the loss of glycocalyx and endothelial receptor VEGFR-2 was observed after NMP, no differences were found in the amount of endothelial cell detachment. The number of microthrombi was significantly increased after NMP and significantly higher in the 95 mm Hg pressure and 36% hematocrit groups. Kidney injury increased during NMP treatment independently of the treatment group, whereas renal function remained stable over 4 h of NMP.

To our knowledge, this is the first time that eGC injury was assessed during NMP of kidneys. We found HA levels in the perfusate increased up to 466.35 ± 122.05 ng/mL during NMP. In healthy adults, HA levels in blood serum are on average 30 ng/mL (usually between 10 and 100 ng/mL) and in
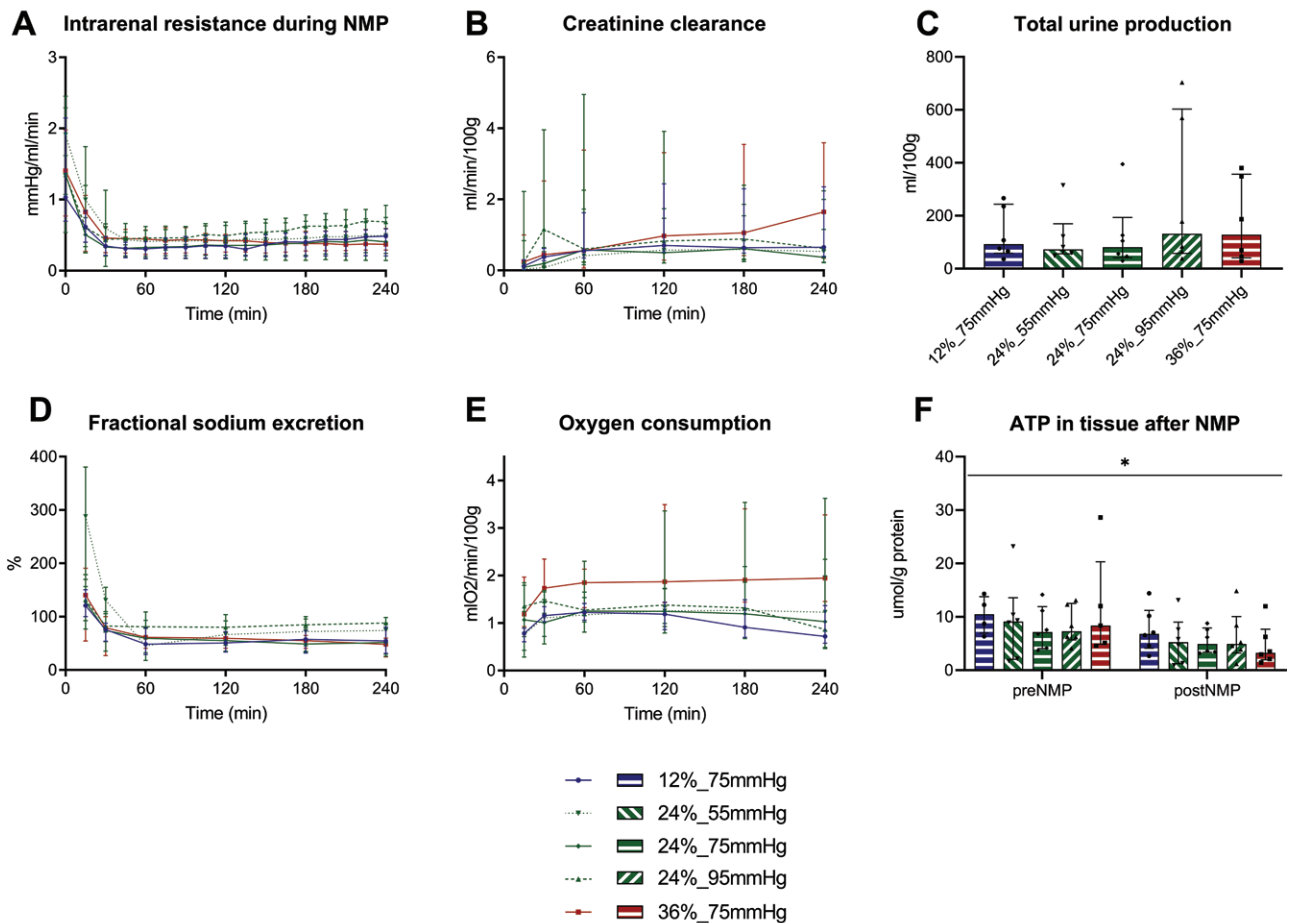
FIGURE 4. Kidney perfusion and function during NMP. Intrarenal resistance (A), creatinine clearance (B), total urine production corrected for kidney weight (C), fractional sodium excretion (D), and oxygen consumption (E) during perfusion. F, ATP levels measured in cortical tissue, before and after NMP. Data are expressed as median ± interquartile range. Time effects: $*P < 0.05$; $**P < 0.01$. NMP: normothermic machine perfusion.

urine 100–300 ng/mL.[31–33] Even when compared with other highly vascularized organs that underwent ex vivo perfusion, these levels are notably high. For instance, Sladden et al[19] showed in ex vivo perfusion of porcine lungs that HA levels of the perfusate did not increase above 300 ng/mL after 4 h of perfusion but often no more than 100 ng/mL. This finding highlights the impact of NMP on eGC loss of the kidney.

It is likely that the increased HA levels in the perfusate are the result of eGC shedding. Although HA may be secreted and expressed apically by proliferating or regenerating renal tubular cells in response to mechanical injury,[34] the results of the quantification of HA in urine and the DBA-staining of cortical tissue, point toward eGC shedding from the vasculature during NMP. The constant number of endothelial cells present in the tissue, based on positive ERG staining, also suggests eGC shedding and not loss due to endothelium detachment.

Because the amount of eGC shedding was not affected by the different pressures and hematocrits used, it is likely the result of other (NMP-related) factors, such as IRI, extracorporeal circuitry, and duration of the perfusion.[11,17,35] Unfortunately, it remains difficult to give a conclusive mechanistic explanation since this study had a more exploratory intention. We did not investigate the effect of the preservation modality on eGC injury in this model. In this study, HMP was used for preservation, because it is considered superior to SCS and is the clinical standard in the Netherlands.[36,37] It, however,

likely that HMP already affected the absolute amount of eGC injury. Although loss of ECs has been described previously,[38] limited literature is available on eGC loss during HMP. It is likely that most eGC shedding will occur during NMP or reperfusion, due to reperfusion injury and temperature increase. However, the total amount of eGC loss during NMP might be different after preservation with SCS and is something that needs further research.

Additionally, besides HA, many other components of the glycocalyx have still not been investigated. In follow-up studies, it might be interesting to evaluate more components of the eGC. For now, it remains unclear if and how the composition of the glycocalyx is affected by machine perfusion.

Besides, long-term effects, such as immunological responses, of eGC shedding during NMP of kidneys remain unclear. A transplantation model could be an interesting follow-up study to investigate the consequences of eGC loss.

Although we aimed for an equivalent to a marginal donor-status kidney, compared with what is found by others, kidney function (represented by creatinine clearance and sodium reabsorption) is relatively poor in our model.[22,24,39] Differences in protocols, in terms of preservation methods and ischemia times, could be an explanation for these differences as we know that these parameters affect renal function.[40,41] However, the eGC and VEGFR-2 loss that we found might also contribute to the poor function. VEGFR-2 is an essential

endothelial marker for proper glomerular structure and function, and loss of endothelial VEGFR-2 is an early event in endothelial dysfunction.[42–44]

Possible consequences of eGC loss were evaluated as well, among microthrombi formation, proteinuria, interstitial edema, and weight gain.[4,5,8] Although we cannot prove a causal relationship in this study, increased microthrombi, proteinuria, and weight gain could be a result of eGC loss.

Next to the extent of eGC injury, the effects of different MAPs and hematocrits on this injury were evaluated. The level of injury was irrespective of the used MAP or hematocrit. The chosen MAP and hematocrits represent the spectrum of settings that are currently used in NMP (40–95 mm Hg).[21–23] No differences between groups in endothelial damage were observed. This finding corresponds with earlier findings that endothelial damage was not affected by different applied MAPs.[23] Therefore, changing the pressures within this range in (clinical) protocols does not seem to result in additional endothelial or glycocalyx injury and might be done equally safely.

Unlike pressure, the effect of hematocrit on kidney function and injury has not been studied extensively. Kidneys are highly metabolic active organs due to active transport processes of electrolytes by the tubules.[43,45] This makes kidneys prone to ischemia and subsequent IRI. Therefore, sufficient oxygen-carrying is essential during NMP. Data on hematocrit are minimal in NMP studies, but a recent study showed no effect of increased RBC concentrations in the perfusate on fractional sodium excretion or creatinine clearance.[46] In our study, no effects on tubular function were seen as well. It could be that the supplied oxygen range is more than sufficient for the marginal donor kidneys in our model. As function is poor in these kidneys, with high fractional sodium excretion rates, and thus reduced energy consumption, a hematocrit of only 12% might still be sufficient for the remaining function. It remains unclear whether the same is true for good-quality kidneys, as these might have higher metabolic rates and might demand more oxygen. However, because NMP is a technique that is particularly used for marginal, ECD kidneys, this is still an important finding.

Observing the course of oxygen consumption during NMP, a hematocrit of 36% may be slightly favored as the rate of oxygen consumption remains more stable throughout the 4 h, whereas oxygen consumption in the other groups had a decreasing trend over time. And as expected, a higher hematocrit also helped with maintaining the pH during NMP (data not shown). This is due to the known buffering capacity of the RBCs. Higher hematocrits, and thus the number of RBCs, might also be the explanation for the slightly higher LDH levels in the perfusate, although hemolysis indices were similar.[47] The amount of renal injury (represented by the PAS staining and total protein in urine) was similar in all pressure and hematocrit groups.

Although the MAP and hematocrit levels did not affect the amount of eGC injury or kidney functionality and viability, the relatively poor function of the kidneys in this study could be due to the eGC loss. However, because both the amount of eGC injury and tubular function were similar in all groups in this study, it remains impossible to be conclusive. Moreover, the use of leukocyte-depleted perfusate could mask inflammatory consequences of eGC injury.

Next, the power of the study is limited due to the small groups, but the study was designed as an explorative study with the aim to detect major differences that would justify clinical follow-up studies. Related to this, a wide intragroup variability is observed. Although this variation complicates the interpretation of the data, it might reflect the actual clinical situation best because human donor kidneys are even more heterogeneous.

Another limitation of this study is that no distinction in the size of HA was made with the assay used. Both low (15–40 kDa), medium (75–350 kDa), and high (>950 kDa) molecular weight forms of HA were detected. The function of HA is size dependent, attributed to the manner of binding to its receptor.[48] In this perspective, it remains unclear what the exact consequences of HA loss during NMP are. A transplantation model following NMP would be a valuable next step, as longer follow-up will be possible.

The optimal setting to evaluate the impact of eGC loss during NMP on the viability or functionality of a kidney is transplantation. Transplantation would allow for the analysis of effects of eGC loss on both kidney function and endothelial inflammation. For instance, the evaluation of eGC loss on signs of vascular rejection would be an interesting next step.

In conclusion, NMP is accompanied with loss of eGC and VEGFR-2 expression, whereas endothelial cell loss seems limited. The different pressures and hematocrits used did not affect the amount of eGC shedding and endothelial receptor loss, whereas microthrombi formation was more substantial in the high pressure and hematocrit groups. It remains unclear whether eGC injury during NMP, irrespective of pressures and hematocrits, has harmful consequences for the transplanted kidney. But based on the extensive literature on the consequences of eGC loss in kidneys and the observed microthrombi formation, minimizing eGC loss during NMP seems worth pursuing.

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