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A Novel Hypobaric Perfusion Method to Remove Microthrombi in Kidney Grafts with Prolonged Circulatory Arrest: A Pilot Study on a Porcine Model

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Background. Intragraft microthrombi prevent complete organ perfusion, thereby compromising the viability maintained by preservation solutions or machine perfusion. Herein, we developed and evaluated a hypobaric perfusion method for flushing microthrombi from kidney grafts with prolonged circulatory arrest in a porcine model. **Methods.** Porcine renal grafts with 1-h warm ischemia were flushed with heparin-containing perfusate in a normobaric environment (control group) or a hypobaric environment of –20 to –30 mm Hg (hypobaric perfusion group) for 10 min using a gravity drip from a 1-m height. Perfusion parameters, histological findings in ex vivo blood perfusion experiments (2 control and 4 hypobaric perfusion kidneys), and safety in allogeneic porcine transplantation experiments (1 donor to 2 recipients) were evaluated. **Results.** The –20 mm Hg hypobaric perfusion group exhibited greater maximal flow than the control group (20.4 versus 6.9 mL/min; $P = 0.028$). Histological evaluation following 3 h of static cold storage and 10 min ex vivo porcine whole-blood perfusion revealed statistically significant reductions in congestion and edema (1.5 versus 3, and 0.5 versus 4 on a 5-point scale, from 0 to 4; $P = 0.014$ and 0.006, respectively) in the medulla along with improved ischemia–reperfusion injury scores (4.0 versus 4.7 on a 6-point scale, from 0 to 5; $P = 0.004$) in the –20 mm Hg hypobaric perfusion group. Kidney grafts perfused under –30 mm Hg hypobaric environment followed by 3 h of static cold storage could be used for porcine allogeneic transplantation without any macroscopic damage to the graft, effect on intraoperative handling, or perioperative adverse events. Thus, the hypobaric perfusion method was considered safe. **Conclusions.** Perfusion in a hypobaric environment may prevent graft congestion, edema, and further reperfusion injury by flushing out erythrocytes occluding the medullary capillaries, improving marginal renal graft quality, and reducing the number of discarded grafts.

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Kidney transplantation is the only curative treatment for end-stage renal failure; however, organ shortages have resulted in the death of many patients on waiting lists.[1-3] To address this severe donor shortage, the use of functionally marginal grafts, including grafts from donations after cardiac death (DCD), has increased, resulting in significant research into how to preserve or restore the function of these grafts.[4,5] Machine perfusion has been shown to enhance graft viability

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by removing intragraft waste products and maintaining organ metabolism by replenishing oxygen and nutrients and is thus now widely used in clinical practice.[6,7] However, microthrombus formation in the organs is a major problem in patients with DCD donors who do not receive anticoagulants.[8,9] The presence of intragraft microthrombi is not only associated with long-term proteinuria and renal fibrosis[10] but can also prevent complete organ perfusion, thereby compromising the viability maintained by various preservation solutions[11-13] or machine perfusion.[6,7] Removing intragraft microthrombi early during perfusion is important to ensure complete organ perfusion; indeed, 1 report showed that flushing the thrombolytic agent before perfusion improved perfusion parameters and reduced the number of discarded grafts.[14]

This study aims to develop a novel nonpharmacological method to improve the removal of microthrombi during the initial perfusion of kidney grafts following prolonged circulatory arrest. Preclinical studies using pig kidneys are important for demonstrating the efficacy of devices in humans, as these effects cannot be investigated in small animals.[15] Our research using pig kidneys investigates whether creating a hypobaric environment by placing the grafts in a sealed container during the initial perfusion can promote microthrombus removal and reduce graft damage during preservation and reperfusion.

MATERIALS AND METHODS

Experimental Animals and Ethics

Animal experiments were conducted with the approval of the Research Council and Animal Care and Use Committee of Keio University (approval no.: 12094-[8]) and Kitasato University (approval no: 21-063). All experiments were conducted at the Fuji Micra, Inc. facility (Shizuoka, Japan) using male microminiature pigs weighing 19–25 kg sourced from Fuji Micra, Inc. The ex vivo whole-blood perfusion experiment involved 4 kidneys subjected to hypobaric perfusion (hypobaric perfusion group) and 2 control kidneys (control group). Two kidneys from 1 donor were transplanted into 2 recipients for the allogeneic transplantation experiment. Animals were handled according to appropriate guidelines[16] and maintained in a temperature- and light- (12-h light/dark cycle) controlled environment, with ad libitum food and water.

Anesthesia and Euthanasia Methods

Animals were fasted for 12 h before surgery, with ad libitum water. After analgesia and sedation with 2.0 mg/kg intramuscular xylazine, general anesthesia was induced with 5% isoflurane and maintained at 1%–3%. Donor pigs were euthanized by phlebotomy after nephrectomy and blood collection, and recipient pigs received postoperative care comprising administration of antibiotics (enrofloxacin 5 mg/kg/day) and analgesics (buprenorphine 0.02 mg/kg twice daily). At the end of the experiment, recipient pigs were euthanized by phlebotomy, after tissue sampling under general anesthesia.

Procurement and Perfusion of Kidney Grafts in the Hypobaric Environment

A midline abdominal incision was made under general anesthesia. Bilateral kidneys with the abdominal aorta and inferior vena cava were procured en bloc after clamping the renal arteries and veins for 1 h to expose them to warm ischemia and divided into right and left kidneys with Carrel patches to the renal arteries at a back table. A 4°C normal saline solution (Otsuka Pharmaceutical Co., Ltd., Japan) containing 2000 U/L heparin (Nipro Co., Ltd., Japan) was perfused from a 16G intravenous catheter (Terumo Corp., Japan) cannulated in the renal artery for 10 min via a gravity drip from a 1-m height. The perfusate flow rate and renal artery pressure were measured using an FD-XS1 (Keyence Corp., Japan).

In the hypobaric perfusion group, the grafts were perfused in a chamber (Figure 1A and B) and maintained at a negative pressure by aspirating air using a vacuum pump unit (Figure 1C) comprising a diaphragm vacuum pump (E.M.P-Japan Ltd., Japan), pressure monitor (ZSE80F, SMC Corp., Japan), and decompression relief valve (SCREEN Co., Ltd., Japan). The ex vivo whole-blood perfusion experiment was performed with a chamber pressure of −20 mm Hg, and the negative pressure was further increased to −30 mm Hg to ensure the safety of the subsequent allotransplantation experiment. In contrast, grafts in the control group were perfused in an environment open to the atmosphere.

After perfusion with normal saline, the grafts were flushed with an organ preservation solution (ETK, Otsuka Pharmaceutical Co., Ltd., Japan), and stored at 4°C in the preservation solution under atmospheric pressure until ex vivo blood perfusion or transplantation experiments. The grafts were weighed on an electronic scale (KF-200, Tanita Co., Ltd., Japan) immediately after procurement, perfusion, and cold storage, and weight change ratios from the value immediately after procurement were calculated.

Ex Vivo Whole-blood Perfusion (6 Kidney Grafts)

Following nephrectomy in donor pigs, a cannula was inserted into the carotid artery, and 400 mL of whole blood was collected in a sodium citrate-added transfusion bag (CPDA bag, Terumo Corp., Japan) and stored at 4°C. After 3 h of static cold storage, the grafts were perfused with autologous blood warmed to 37°C for 20 min via a gravity drip from a 1-m height while measuring the blood flow rate and renal artery pressure, after which the grafts were histologically evaluated.

Orthotopic Transplantation to Allogeneic Pigs (1 Donor to 2 Recipients)

The transplantation of allogeneic porcine recipients was performed as previously reported.[17] In brief, the left kidney was removed via a midline abdominal incision, the graft renal artery with a Carrel patch was anastomosed to the recipient aorta in an end-to-side manner, and the graft renal vein was anastomosed to the recipient renal vein in an end-to-end manner. To maintain the anteroposterior relationship of the renal vessels, the right kidney was inverted upside down and transplanted into the left renal fossa. The recipient and graft ureters were anastomosed end-to-end without stents. The recipient's right kidney was also removed. Recipient pigs were euthanized after 3 d of survival using only graft renal function. No crossmatch testing was performed, and immunosuppressive drugs were not administered.

Tissue Processing and Histological Evaluation

Renal cortical wedge biopsy or whole kidney specimens were fixed in 3.7% neutral formalin solution; embedded in paraffin; stained with hematoxylin and eosin, periodic acid Schiff, and Masson trichrome using standard protocols; and histologically evaluated by external pathologists

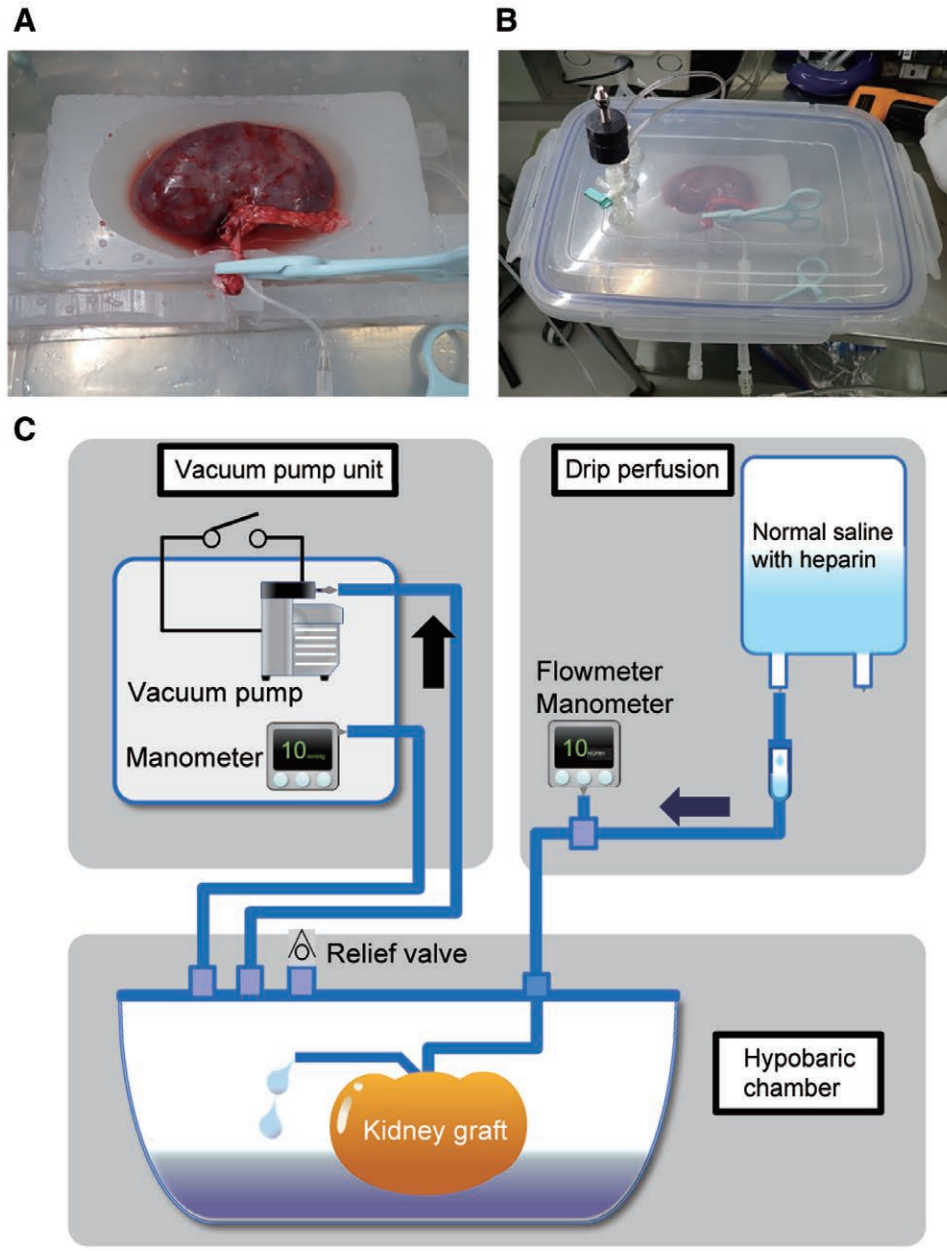

FIGURE 1. Overview of the perfusion in the hypobaric environment. A, Kidney graft fixed in the chamber. B, The sealed chamber with the kidney graft inside. C, Overview of the vacuum pump unit: a pressure monitor and decompression relief valve were connected to the aspiration port of a diaphragm vacuum pump, and the pump was controlled through a control board.

(Septosapie Co., Ltd., Tokyo, Japan). Tubular necrosis, tubular epithelial flattening, congestion of the medullary capillaries, and interstitial edema in the medulla were evaluated semiquantitatively as follows: 0, no change, 1; slight, 2; mild, 3; moderate, and 4; severe. Glomerular mesangial cell lysis and glomerular granulocyte infiltration were quantified as the incidence in all glomeruli. The areas of tubular necrosis, brush border loss, degeneration, cast formation, and tubule dilation were assessed using semiquantitative scores designed to evaluate ischemia–reperfusion (IR)-related changes in the kidneys (0 = 0%–5%, 1 = 5%–10%, 2 = 11%–25%, 3 = 26%–45%, 4 = 46%–75%, and 5 = 76%–100%).[18] Five fields of view at 200× magnification were randomly selected from the cortex and medulla of each kidney for analysis.

Statistical Analyses

Perfusion parameters and the results of the histological evaluation were analyzed and illustrated using the statistical software GraphPad Prism ver. 9.2.0 (GraphPad Software). Comparisons between the 2 groups were performed using unpaired Welch *t*-test, and a 2-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Improving Effects of Hypobaric Perfusion on Perfusion Parameters

During normal saline perfusion (n = 4 in hypobaric perfusion versus n = 2 in control), although no difference in maximal arterial pressure was observed (Figure 2A), the hypobaric

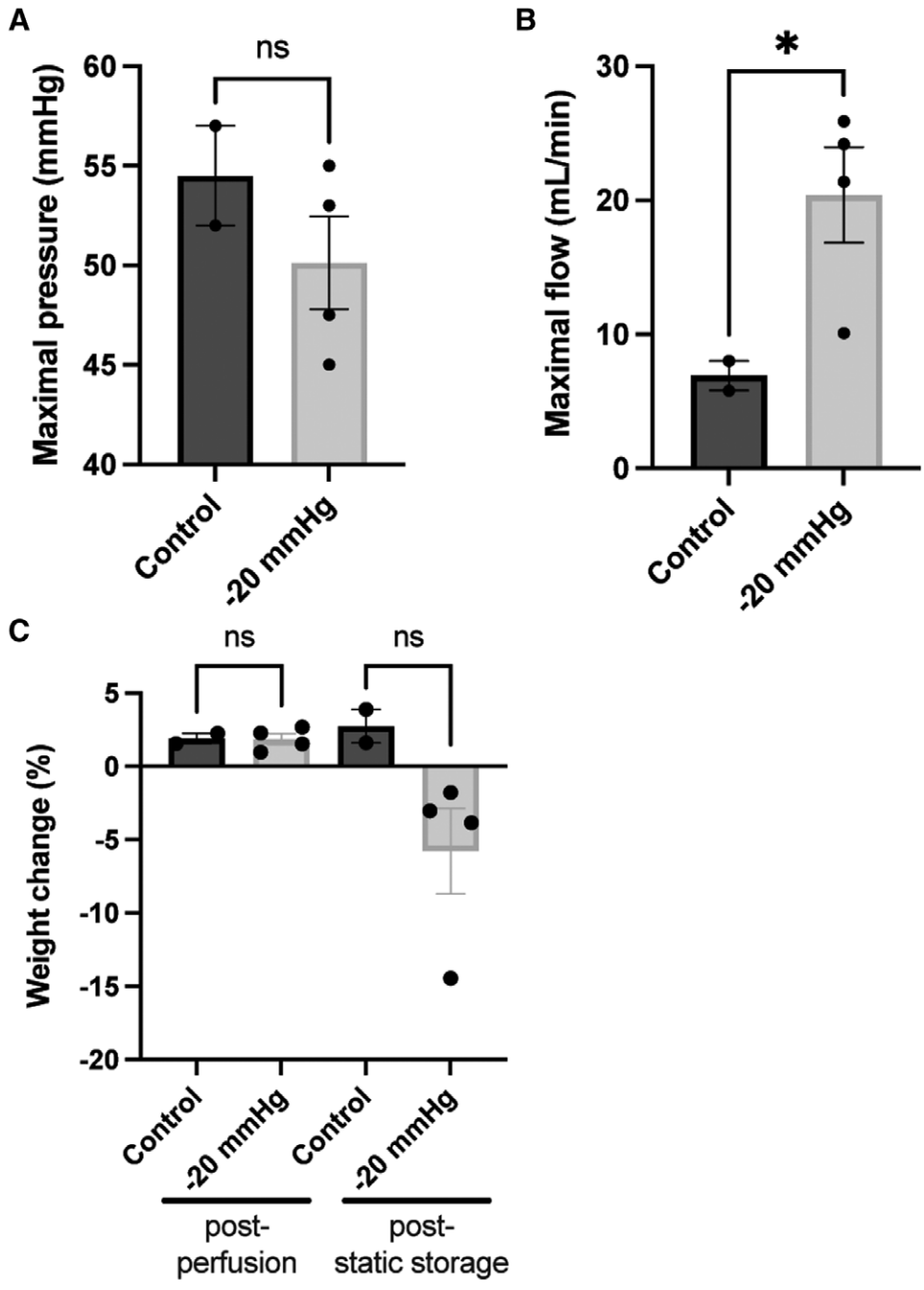

FIGURE 2. The effect of hypobaric perfusion on perfusion parameters. A, Maximal arterial pressure. B, Maximal perfusate flow. C, Graft weight change ratios. Data are mean ± s.e.m. *P = 0.028; ns, not significant; s.e.m., standard error of the mean.

perfusion group exhibited a statistically significant increase in maximal flow (20.4 mL/min in –20 mm Hg versus 6.9 mL/min in control; P = 0.028; Figure 2B). The graft weight increased similarly in both groups after perfusion; however, the graft weight decreased only in the hypobaric perfusion group after 3 h of static cold storage, although the difference was not statistically significant (–5.8% in –20 mm Hg versus 2.7% in control; P = 0.057; Figure 2C).

Effect of Hypobaric Perfusion on Prevention of Congestion, Edema, and IR Injury Following Ex Vivo Whole-blood Perfusion

Ex vivo perfusion with autologous porcine blood after 3 h of static cold storage showed a trend toward a decreased maximal arterial pressure and increased maximal blood flow in the hypobaric perfusion group, although this difference did not reach statistical significance (Figure 3A and B). Histological findings after ex vivo whole-blood perfusion are presented in Table 1. The hypobaric perfusion group showed statistically significant reductions in congestion of the medullary capillaries (1.5 in –20 mm Hg versus 3.0 in control; P = 0.014) and interstitial edema in the medulla (0.5 in –20 mm Hg versus 4.0 in control; P = 0.006), suggesting that the graft weight loss after static cold storage could be attributed to the attenuation of graft edema. The IR injury was significantly reduced in the medullary region (4.0 in –20 mm Hg versus 4.7 in the control group; P = 0.004), although there was no difference in the cortex (Table 2).

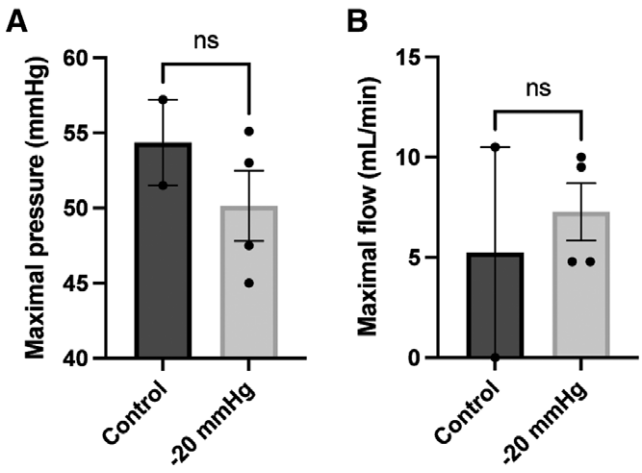

FIGURE 3. The effect of hypobaric perfusion on blood perfusion parameters. A, Maximal arterial pressure. B, Maximal blood flow. Data are mean ± s.e.m. ns, not significant; s.e.m., standard error of the mean.

Verification of the Safety of Hypobaric Perfusion by Porcine Allotransplantation

Porcine allotransplantation experiments were performed using kidney grafts after 1 h of warm ischemia and 3 h of cold ischemia with hypobaric perfusion (n = 2). The maximum flow rate of normal saline was 6.0–7.5 mL/min, and the maximum arterial pressure was 64 mm Hg by −30 mm Hg hypobaric perfusion. No macroscopic damage to the grafts because of hypobaric perfusion was observed, and the intraoperative handling did not differ from that of normal kidneys. Both animals completed the 3-d experimental period, and the serum levels of creatinine and urea nitrogen peaked on the second postoperative day, thereafter showing a decreasing trend (Figure 4A and B). Histological examination showed that the IR injury score in the graft immediately after reperfusion was as high as 5 in both kidneys but decreased to 1.4–1.8 at the end of the experiment (Table 3).

DISCUSSION

In this study, we demonstrated that initial perfusion in a hypobaric environment improved graft perfusion parameters and reduced congestion, edema, and IR injury immediately following reperfusion. In contrast, no perioperative adverse events owing to hypobaric perfusion were observed in the transplantation experiments, confirming the safety of this novel method.

Marginal grafts, including those from the DCD donors, are highly susceptible to intragraft microthrombus formation and severe IR injury after transplantation because of unstable circulatory dynamics during the agonal stage and prolonged ischemia time.[4] Various innovations have targeted the maintenance or improvement of graft function, including a variety of organ preservation solutions (eg, University of Wisconsin solution, Euro-Collins solution, Histidine-Tryptophan-Ketoglutarate solution),[11-13] perfusion techniques (eg, ex situ machine perfusion, regional perfusion),[6,19] hydrodynamic parameters (eg, continuous, pulsatile),[20,21] and perfusion temperatures (eg, hypothermic, normothermic).[22,23] As the superiority of hypothermic machine perfusion over conventional static cold storage has been demonstrated, this technique has been widely used in clinical practice.[6] However, few studies have investigated the effects of using pharmacological agents and enzymes, such as kinases,[14] to flush intraorgan microthrombi, and no studies have focused on investigating the environmental pressure under which perfusion is performed.

In the present study, perfusion in a hypobaric environment improved the medullary congestion of grafts following

TABLE 1.

Histological findings after ex vivo blood perfusion

Pig ID	1	2	3	4	5	6	Mean		*P*
Hypobaric perfusion	–	–	+	+	+	+	–	+	
Tubular necrosis	3	4	4	4	3	3	3.5	3.5	>0.99
Tubular epithelial flattening	3	4	4	4	3	3	3.5	3.5	>0.99
Glomerular mesangial cell lysis (%)	12	16	2	18	0	8	7.0	14.0	0.20
Glomerular granulocyte infiltration (%)	20	5	4	2	12	12	12.5	7.5	0.62
Congestion of medullary capillaries	3	3	1	2	1	2	3.0	1.5	0.014
Interstitial edema in the medulla	4	4	0	0	0	2	4.0	0.5	0.006

TABLE 2.

Ischemia–reperfusion injury scores after ex vivo blood perfusion

Pig ID	1	2	3	4	5	6	Mean		*P*
Hypobaric perfusion	–	–	+	+	+	+	–	+	
Cortex	5	5	4	5	5	5	4.9	4.8	0.47
	5	5	5	5	4	5			
	5	5	5	5	5	5			
	4	5	5	5	4	5			
	5	5	5	5	4	5			
Medulla and corticomedulla	4	5	5	4	5	4	4.7	4.0	0.004
	5	4	4	3	5	4			
	5	5	4	4	4	4			
	5	5	2	4	4	4			
	5	4	5	4	2	4			

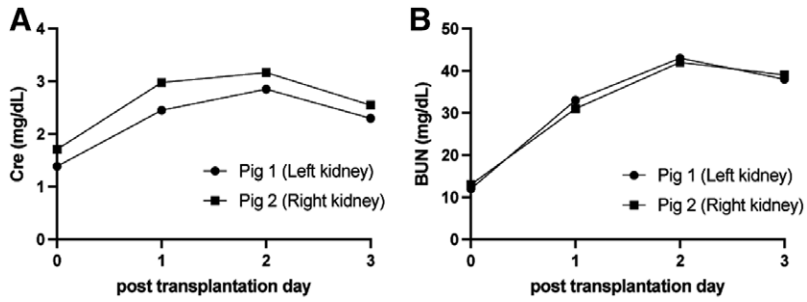

FIGURE 4. Postoperative levels of serum creatinine and urea nitrogen. A, Creatinine and (B) urea nitrogen levels were elevated until day 2 and decreased thereafter. BUN, blood urea nitrogen.

TABLE 3.

Ischemia–reperfusion injury scores after allotransplantation

	Pig 1 (left kidney)		Pig 2 (right kidney)	
	Immediately after reperfusion	3 d after reperfusion	Immediately after reperfusion	3 d after reperfusion
Tubular injury, cortex	5	1	5	2
	5	2	5	1
	5	1	5	2
	5	1	5	2
	5	2	5	2
Mean	5.0	1.4	5.0	1.8

ex vivo blood perfusion. In general, renal arterial occlusion causes blood to fill the capillaries throughout the kidneys, resulting in congestion, which disappears from the cortex and inner medulla during reperfusion and remains only in the outer medulla.[24] This congestion in the outer medulla cannot be prevented by antiplatelet agents or anticoagulants but can be prevented by increasing the blood pressure during reperfusion or by reducing erythrocytes or the hematocrit prior to ischemia, which improves the filtration rate, tubular reabsorption, epithelial damage, renal blood flow, and vascular resistance after IR events.[24-26] It is believed that this congestion does not involve the coagulation system, and that the loss of plasma from the vascular system causes erythrocytes to aggregate, leading to capillary occlusion of the outer medulla.[24-26] Placing the entire kidney graft in a hypobaric environment and dilating the vascular bed inside the graft may have allowed the perfusate to flush out residual erythrocytes. A previous experiment confirmed the dilation of capillaries on the hepatic surface (**Figure S1a, SDC**, http://links.lww.com/TXD/A637) at −20 mm Hg. In contrast, we observed macroscopic organ damage, such as irreversible renal expansion (**Figure S1b, SDC**, http://links.lww.com/TXD/A637) and liver capsular damage (**Figure S1c, SDC**, http://links.lww.com/TXD/A637), at pressures <−40 and −50 mm Hg, respectively.

In our study, edema of the tubulointerstitial space also improved in hypobaric environments, and correspondingly, the graft weight decreased after cold storage in the hypobaric perfusion group. The graft weight, which increased immediately after perfusion with saline, decreased after storage in the colloid-containing preservation solution, and perfusion in a hypobaric environment contributed to this weight loss. This suggests that the hyperosmotic preservation solution may effectively reduce tissue edema by spreading throughout the vascular bed, particularly the medulla, and promoting water movement from the tissue into the blood vessels.[27] Increased graft weight during machine perfusion is an indicator of vascular endothelial damage and tissue edema.[28] The removal of erythrocytes occluding the capillaries of the outer medulla may have restored the blood supply to tubules with a high oxygen demand, further reducing IR injury.[24]

Regarding perfusion parameters, several reports have suggested that graft viability can be predicted by examining various parameters during machine perfusion.[29] Specifically, low flow and high perfusion pressure during machine perfusion can be applied to identify nonviable kidneys.[30] The hypobaric environment may affect perfusion parameters, and whether existing parameters are usable is unclear. However, we observed a decrease in the graft arterial pressure and an increase in blood flow during ex vivo blood perfusion after the hypobaric environment was released, although the differences were not statistically significant. Perfusion in a hypobaric environment may have contributed to the improved graft condition.[30]

This study had several limitations. First, although histological findings were improved by ex vivo perfusion, an improvement in graft function was not observed in the transplantation experiments. The transplantation experiments did not involve any crossmatch testing or the administration of immunosuppressive drugs. The transplantation experiments in this study were designed with a focus on safety, that is, to ensure there were no adverse effects on transplantation because of structural changes or damage to organs caused by hypobaric perfusion. As safety was confirmed in this study, the next step will be to verify the effectiveness of hypobaric perfusion in transplantation experiments using controls for comparisons. Second, because of the relatively small number of animals used in this study, some parameters did not show statistically significant differences. Although we used pigs to closely mimic

clinical conditions, large animal experiments are costly and require considerable time and effort. Further large animal experiments and experiments with human organs are needed to confirm the results of this study and verify the appropriate hypobaric perfusion conditions for clinical application. Finally, the exact mechanism underlying the positive effect of hypobaric perfusion on grafts has not been elucidated. Molecular, biological, and genetic analyses to elucidate this mechanism would contribute significantly to the field of organ perfusion and preservation.[31]

In conclusion, we developed a novel nonpharmacological method of initial perfusion in a hypobaric environment to flush out microthrombi in kidney grafts. We demonstrated its effectiveness in removing intragraft thrombi, preventing edema, and reducing IR injury, as well as the safety during the perioperative period. The concept of "removing microthrombi before perfusing the graft" is effective for both static cold storage, which was examined in this experiment, and machine perfusion, which has been widely used in recent years, and is expected to have a synergistic effect on current preservation methods. Once its usefulness in human organs is established, this novel method could become a reasonable and feasible option in clinical practice. However, further studies are needed to verify the effectiveness of this method in improving graft function after transplantation, elucidate the mechanism, and apply it to other organs.

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