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Continuous *versus* Pulsatile Flow in 24-Hour Vascularized Composite Allograft Machine Perfusion in Swine: A Pilot Study

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ABSTRACT

Introduction: Multiple perfusion systems have been investigated on vascularized composite allografts, with various temperatures and different preservation solutions, most using continuous flow (CF). However, physiological flow is pulsatile and provides better outcomes in kidney and lung *ex vivo* perfusions. The objective of this pilot study is to compare pulsatile flow (PF) with CF in our 24-h subnormothermic machine perfusion protocol for swine hindlimbs.
Methods: Partial hindlimbs were harvested from Yorkshire pigs and perfused with a modified Steen solution at 21°C for 24 h either with CF ($n = 3$) or with pulsatile flow (PF) at 60 beats/min ($n = 3$). Perfusion parameters, endothelial markers, and muscle biopsies were assessed at different timepoints.
Results: Overall, lactate levels were significantly lower in the PF group ($P = 0.001$). Glucose uptake and potassium concentration were similar in both groups throughout perfusion. Total nitric oxide levels were significantly higher in the PF group throughout perfusion

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($P = 0.032$). Nitric oxide/endothelin-1 ratio also tends to be higher in the PF group, reflecting a potentially better vasoconductivity with PF, although not reaching statistical significance ($P = 0.095$). Arterial resistances were higher in the PF group ($P < 0.001$). Histological assessment did not show significant difference in muscular injury between the two groups. Weight increased quicker in the CF group but reached similar values with the PF after 24 h. *Conclusions:* This pilot study suggests that PF may provide superior preservation of vascularized composite allografts when perfused for 24 h at subnormothermic temperatures, with potential improvement in endothelial function and decreased ischemic injury.

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Introduction

Vascularized composite allotransplantation has emerged as a revolutionary treatment option for anatomical restoration after severe disfigurement or amputation. Since the first successful hand allotransplantation performed in 1998 in France,[1] 48 face[2] and more than 130 upper extremities have been transplanted worldwide.[3]

Static cold storage at 4°C is currently the gold standard to preserve solid organs[4] after procurement until the time of transplantation. However, vascularized composite allografts (VCA) are more sensitive to ischemia than some solid organs due to their muscle component[5]: muscle tissue's high metabolic activity makes it especially vulnerable to ischemia, leading to irreversible injuries after 6-8 h of cold storage.[6]

As in solid organ transplantation, machine perfusion has been shown to extend preservation duration of VCA up to 24 h.[7-10] Several parameters are commonly investigated in VCA machine perfusion systems: temperature, cellular *versus* acellular preservation solutions, pressure targets, and oxygenation. Despite pulsatile flow (PF) being more physiologic, most of VCA perfusion systems have used continuous flow (CF).[11] PF has shown better outcomes in reconditioning kidneys prior to transplantation compared to CF,[12] and also has demonstrated beneficial effects in reducing the inflammatory response in lung perfusion during cardiopulmonary bypass.[13] Evidence also suggests that endothelial cells more effectively upregulate endothelial NO synthetase (eNOS) expression and nitric oxide (NO) levels with PF, with better NO/endothelin-1 (ET-1) ratios leading to a more vasoconductive phenotype.[12,14] Conversely, other studies did not conclude any superiority of a type of flow over the other in terms of viability or functional outcomes in kidney[15,16] and lung[17] perfusion. To our knowledge, there are no data in the literature on the impact of PF in VCA machine perfusion preservation. The objective of this pilot study is to investigate the impact of pulsatility in VCA subnormothermic machine perfusion preservation during 24 h in a swine vascularized composite allotransplantation model.

Materials and Methods

Study design

Six partial hindlimbs were randomly assigned into 2 groups: in the control group, limbs were perfused for 24 h with CF, and in the experimental group limbs were perfused with PF. To avoid any bias related to the donor pig, for each swine, one of the partial hindlimb was randomly attributed to either the control or the interventional group. The remaining hindlimb was subsequently attributed to the other group. The study was approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital/Harvard Medical School (protocol #2019N000176).

Surgical model

Three female Yorkshire pigs weighing between 20 and 25 kg were used in this study. We performed a modified heterotopic swine hindlimb transplant model as described previously.[18,19] In each animal, both hindlimbs were harvested as follows: general anesthesia was induced with Telazol 2 mg/kg and maintained with 2% isoflurane inhalation with oxygen. Both limbs and inguinal groins were prepped with alcoholic Betadine and draped in a sterile fashion. A 12-cm wide rounded skin patch was incised on the medial aspect of the knee and immediately sutured to the underlying fascia. Distal to the skin paddle, the saphenous vessels were isolated and ligated. A 10-cm longitudinal incision was then made in the inguinal crease, which was dissected until the femoral vessels were exposed (Fig. 1A). The femoral vessels were skeletonized, and the lateral branches were cut and ligated. The thigh muscles (abductors, adductors, quadriceps) were cut to isolate the femur. The leg muscles were then retracted upward to isolate the tibial bone. An intravenous injection of heparin 100 IU/kg was performed 5 min before the femoral vessels were ligated with silk thread just below the inguinal ligament. The femur and tibia were cut with a handsaw at their distal and proximal third, respectively. Once the flap was free of its bony attachments, the femoral artery was cannulated with an 18G Angiocath and the graft was flushed with 100 mL of 10% heparinized serum. Partial hindlimbs were stored in a sterile organ bag surrounded with saline and placed in a box with ice for transport to the perfusion room. The weight and time of ischemia was recorded for each partial limb. The pig was then euthanized according to the American Veterinary Medical Association guidelines for animal euthanasia.

Ex vivo *subnormothermic machine perfusion systems*

Custom-built machine perfusion (continuous flow)
Our perfusion system included a roller pump delivering CF (07522-20 DRIVE MFLEX L/S 600RPM 115/230; Cole-Parmer, Vernon Hills, IL), a hollow membrane oxygenator (Affinity Pixie Oxygenation System; Medtronic, Dublin, Ireland), and

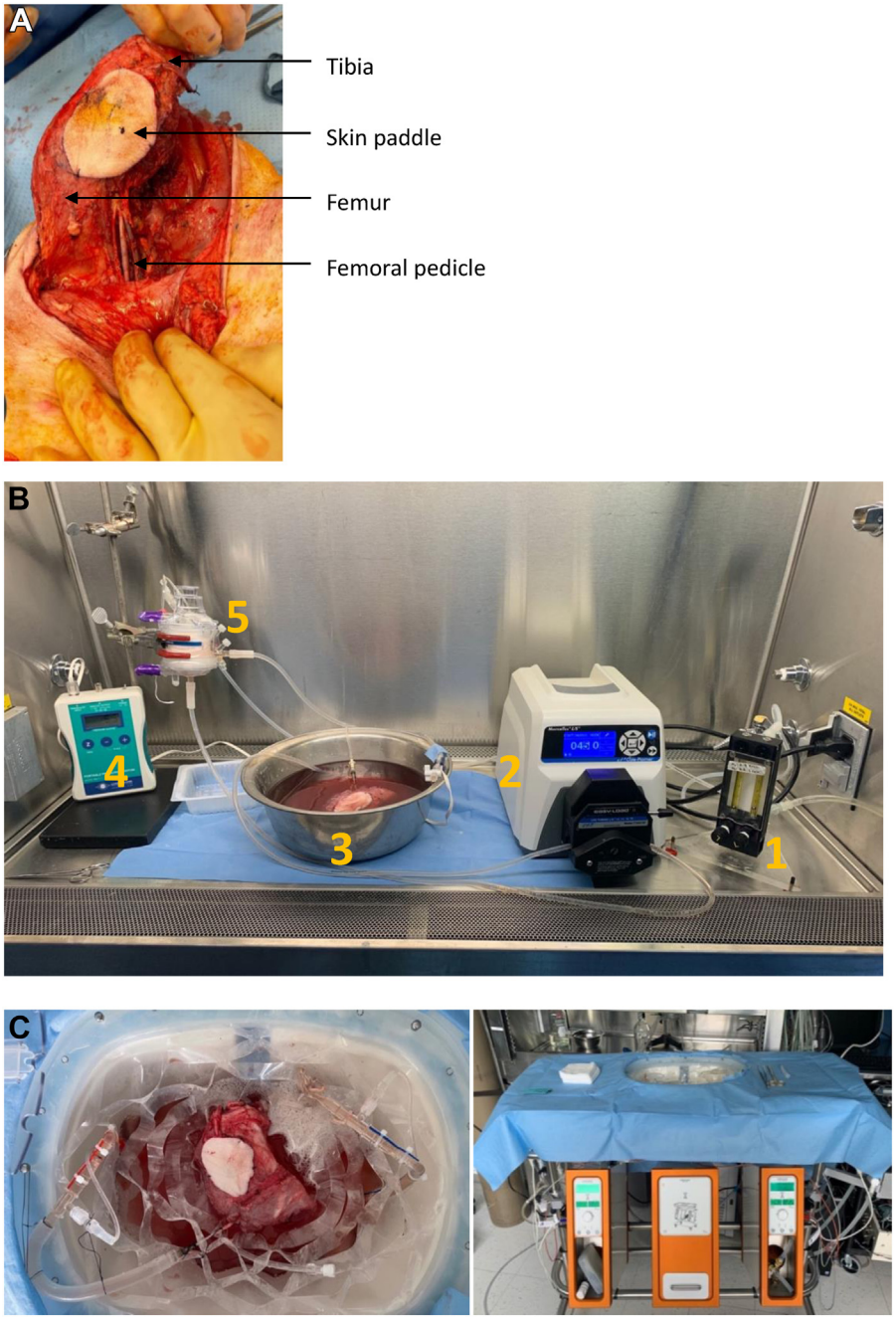

Fig. 1 – Swine hindlimb perfusion setup. (A) The surgical model was an osteomyocutaneous flap harvested on a swine hindlimb. (B) Our custom-built machine perfusion system was composed of an oxygen regulator (1), a roller pump delivering a continuous flow (2), a basin containing the limb and the perfusate (3), a pressure monitor (4), and a hollow membrane oxygenator (5). (C) The Liver Assist machine perfused the limb with a pulsatile flow (60 bpm) through the hepatic artery system (red) while the portal system is shunted (blue).

presterilized size 16 tubing (Masterflex L/S Platinum-Cured Silicone Tubing; Cole-Parmer). A pressure sensor (PM-P-1; Living Systems Instrumentation, St Albans City, VT) was connected to the inlet tubing in shunt with the arterial catheter (18G Angiocath; BD Angiocath, Franklin Lakes, NJ). A steel container served as a perfusate reservoir in which the graft was placed during perfusion, so that the outflow exiting the limb through the femoral vein flowed directly into the reservoir for recirculation through the system (Fig. 1B). The pressure sensor was primed, and the monitor calibrated before starting a new perfusion. Oxygen supply was provided with Carbogen (5% CO_2, 95% O_2) at a flowrate of 0.5 L/min. Once the perfusate was warmed and well oxygenated within the system, the circulating solution was checked for pO_2 and pH with the i-STAT machine (Abbott, Princeton, NJ). If pH was too acidic, sodium bicarbonate 8.4% mg/mL was added as a buffer. The flowrate was manually adjusted during perfusion to maintain a pressure of 40 mm Hg.

Liver Assist machine (pulsatile flow)

The partial limbs were perfused through the hepatic arterial system of the Liver Assist machine while the portal system was shunted (Fig. 1C). The system pump delivered a PF set at 60 beats/min through size 16 silicone tubing. The flow was automatically adjusted by the machine to maintain a stable mean perfusion pressure at 40 mm Hg. Temperature was set to 21°C, and the perfusate was oxygenated with Carbogen (5% CO_2, 95% O_2). The pH was checked and buffered if needed, once the perfusate was warmed and oxygenated.

Perfusate

All partial limbs were perfused with a modified acellular Steen solution (Steen+) developed by our team. Briefly, we reconstituted the original Steen solution usually used for lung perfusions that we modified to adjust to the metabolic specificities of VCA. Human albumin 7% was replaced by bovine albumin 15% to increase the osmotic pressure to combat edema; polyethylene glycol-35 was added at a concentration of 0.5% for its membrane protective effect; heparin (2000 IU/L), insulin (200 IU/L), dexamethasone (16 mg/L), hydrocortisone (200 mg/L), and penicillin-streptomycin (4 mL/L) were also added to the solution. The Steen+ solution was filtered with 0.2 μm filters (FisherBrand FB12566506; Thermo Fisher Scientific, Waltham, MA) and then stored at 4°C. Two hours before perfusion, the perfusate was warmed to room temperature. Two liters of Steen+ solution was introduced in the system at the start of the perfusion, and one perfusate exchange (1 L, 50%) was performed after 12 h of perfusion to limit the accumulation of cellular waste in the solution. In total, 3 L of Steen+ was used for each experiment.

Monitoring

Pressure, flowrate, oxygen, and metabolic parameters were measured at several timepoints throughout the perfusion. pH, pO_2, pCO_2, lactate levels, K+, Na+, HCO_3^-, and glucose were tested from perfusate samples with the i-STAT machine.

Edema was estimated in percentage (%) every 6 h using the following formula:

$$Edema\ (\%) = \frac{Weight\ (t) - Weight\ (t0)}{Weight\ (t0)} \times 100$$

Histology

Muscle tissue samples were collected before and after 12 and 24 h of perfusion. Biopsies were immediately fixed in formalin, processed for routine histopathological examination, and stained with hematoxylin and eosin. Blinded pathological evaluation of muscle injury was performed using the modified version of the Histologic Injury Severity Score proposed by Kruit *et al.*[20] Inflammation, interstitial edema, damaged muscle fibers, and variations in myocyte architectures were scored from 0 to 3. Total score ranged from 0 to 12, with a higher score signifying greater muscle injury.

Endothelin-1 and nitric oxide assays

ET-1 and total NO were assessed at 6, 12, 18, and 24 h of perfusion. Perfusate samples were collected from the venous outflow and processed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

Data are shown in median and range. Values from biochemical analyses, blood gases, machine perfusion parameters, and histological grading were entered into Microsoft Excel for Mac OS X (Microsoft Corp, Redmond, WA). All data were analyzed using GraphPad Prism 9.0 for Mac OS X (GraphPad Software, Inc, San Diego, CA). Statistical differences were assessed with two-tailed nonparametric Mann-Whitney *U*-tests with a significance level $P < 0.05$. Right tailed *f*-test was performed to compare the variances between groups, with a significance level $P < 0.05$.

Results

Vascularized composite allografts characteristics

Six partial hindlimbs were perfused at subnormothermic temperatures, randomly assigned to the CF group ($n = 3$) or the PF group ($n = 3$). The mean graft weight before perfusion and the mean ischemia time, corresponding to the period between vessel clamping and the start of perfusion, were comparable between the two groups ($P > 0.999$ and $P = 0.500$, respectively; Table).

Perfusion parameters

All six grafts were perfused successfully for 24 h. After an initial peak at reperfusion, arterial resistances stabilized after 1 h (Fig. 2A). Arterial resistances were somewhat higher in the PF group compared to CF ($P < 0.001$), in part due to greater

Table – Characteristics of the limbs before perfusion.

		Continuous flow		Median *(minimum to maximum)*
Weight (g)	355	351	398	355 *(47)*
Ischemia (min)	16	15	12	15 *(4)*
	Pulsatile flow			Median *(minimum to maximum)*
Weight (g)	419	373	343	373 *(76)*
Ischemia (min)	14	17	16	16 *(3)*

Both groups had comparable limb weights and ischemia times.

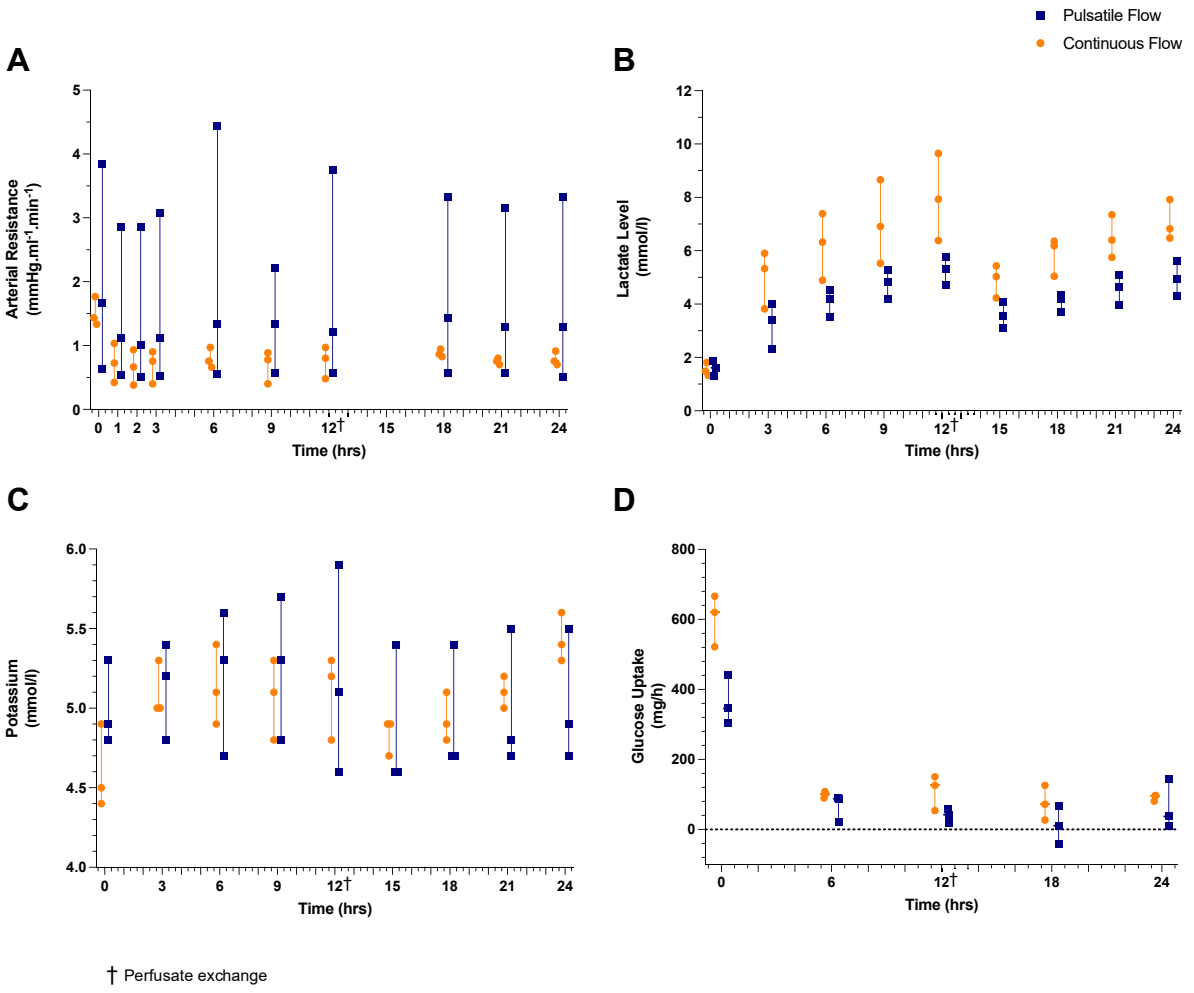

Fig. 2 – Perfusion parameters. Pulsatile flow led to less lactate release and higher arterial resistances compared to continuous flow. (A) Arterial flow rates were slightly higher throughout perfusion in limbs with pulsatile flow ($P < 0.001$), with a higher variability between the replicates (f-test = 16.8825, $P < 0.001$). (B) Lactate levels are lower with pulsatile flow throughout perfusion ($P = 0.001$). (C and D) Glucose uptake and potassium concentration have similar values during the 24 h of perfusion ($P = 0.095$ and $P = 0.719$, respectively).

variability between the replicates (f-test = 16.8825, $P < 0.001$). Overall, lactate levels were significantly lower in the PF group ($P = 0.001$) (Fig. 2B).

Both groups showed a similar trend of potassium concentrations and glucose uptake throughout the 24-h perfusion (Fig. 2C and D). Glucose uptake increased during the first hours and stabilized between 50 and 100 mg/h in both groups throughout the perfusion. The transient decrease in lactate and potassium levels after the 12th hour was a consequence of the perfusate exchange performed at this time.

Endothelial markers

Total NO levels were significantly higher in the PF group throughout perfusion ($P = 0.032$) (Fig. 3). NO/ET-1 ratio also tends to be higher in the PF group, reflecting a potentially better vasoconductivity with PF, although not reaching statistical significance ($P = 0.095$).

Edema

Weight increased quicker in the CF group but reached similar values with the PF after 24 h (14.48% and 12.43% respectively, $P = 0.400$; Fig. 4).

Histology

Mean total Histologic Injury Severity Score in PF and CF was respectively 2.67 and 3.67 at 0 h ($P = 0.600$), 8.00 and 6.00 at 12 h ($P = 0.400$), and 7.00 and 9.00 ($P = 0.400$) at 24 h of perfusion. Figure 5 shows the similar evolution of muscle injury with perfusion time in both groups.

Discussion

Our comparative study demonstrates that it is feasible to perfuse VCA for 24 h in a viable state in a large animal model

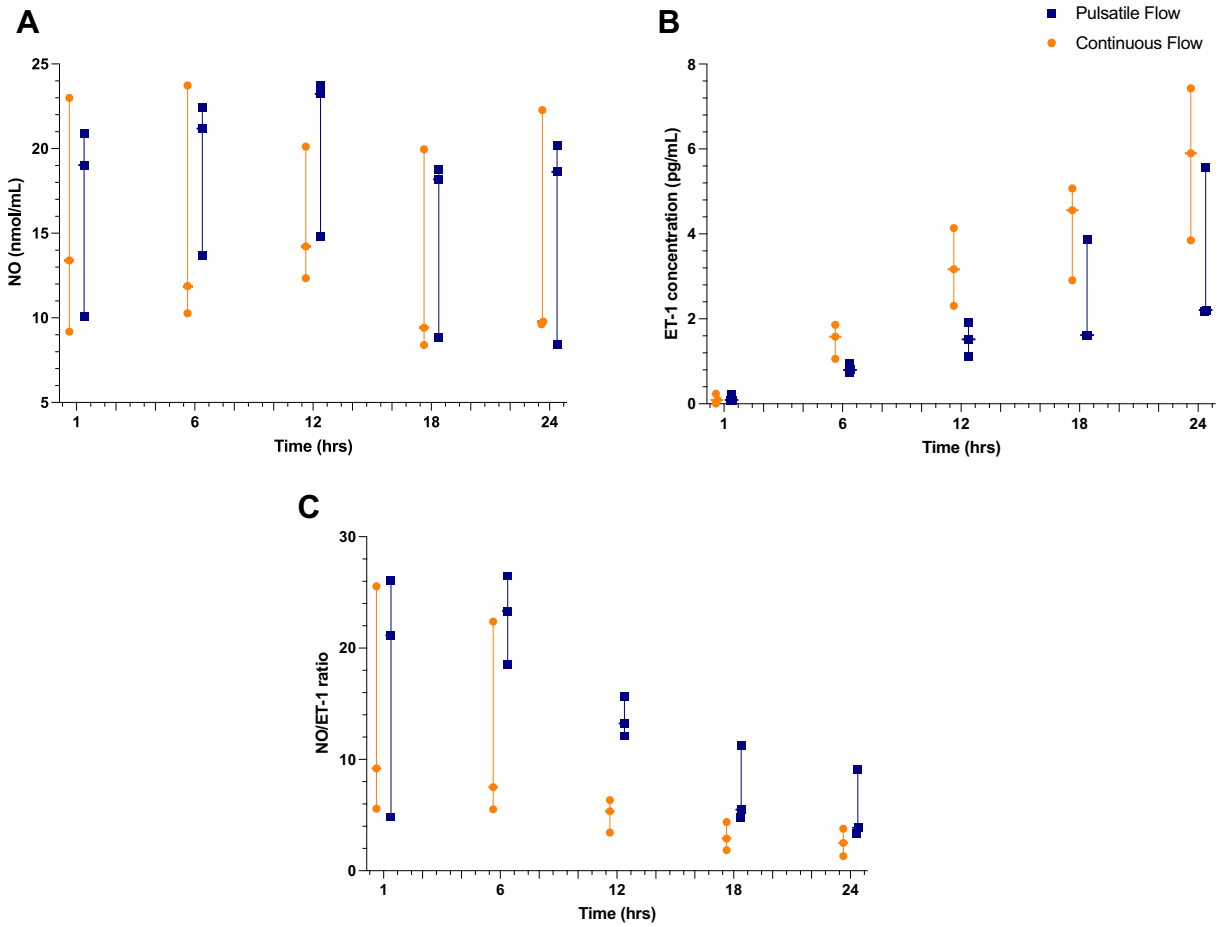

Fig. 3 – Endothelial markers. Perfusate concentrations of (A) NO and (B) ET-1. (C) NO/ET-1 ration. Pulsatile flow group showed higher concentration of NO ($P = 0.032$) and higher trend of NO/ET-1 ratios all over the perfusion, although not significant ($P = 0.114$).

in subnormothermic conditions. By comparing two types of flow in this pilot study, CF seemed to offer better stability of arterial resistances while PF tended to enhance the vasoconductivity of the limbs and decrease the ischemic injury during perfusion, reflected by lesser lactate release overtime.

Most studies aiming to extend the preservation duration of VCA through machine perfusion systems have been performed with CF,[11] providing similar results to ours by reaching up to 24 h of viable preservation of VCAs in large animals[7,8,21] and human limbs.[10] Ozer *et al.*[22] were the only team to use a PF in their 24-h *ex vivo* machine perfusion at subnormothermic temperatures (27°C-33°C) and reported a good survival after transplantation of swine limbs. Several studies compared different perfusion parameters like temperature,[11,23,24] perfusion pressures,[23] preservation solutions[25] (cellular or acellular), or oxygenation,[24,26] but to our knowledge we are the first to investigate which of PF or CF is the most efficient in VCA perfusion preservation.

To date, the influence of pulsatility in organ perfusion preservation is still subject to debate in the literature, with no obvious evidence of its superiority over CF. Sevinc *et al.*[16] did not find improved outcomes after transplantation in human kidneys priorly perfused with PF compared with CF, neither did Luke *et al.*[15] while comparing the two types of flow at subnormothermic temperatures in a swine model. Also, Brandes *et al.*[17] showed the nonsuperiority of PF in improving functional outcomes in swine lungs. Nonetheless, other authors have highlighted the benefits of PF while reconditioning

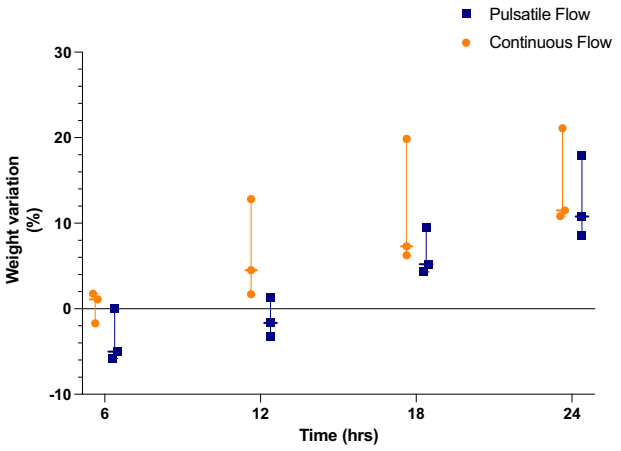

Fig. 4 – Weight variation. Limbs perfused with continuous flow tended to gain weight quicker than pulsatile flow, but edema was similar at 24 h.

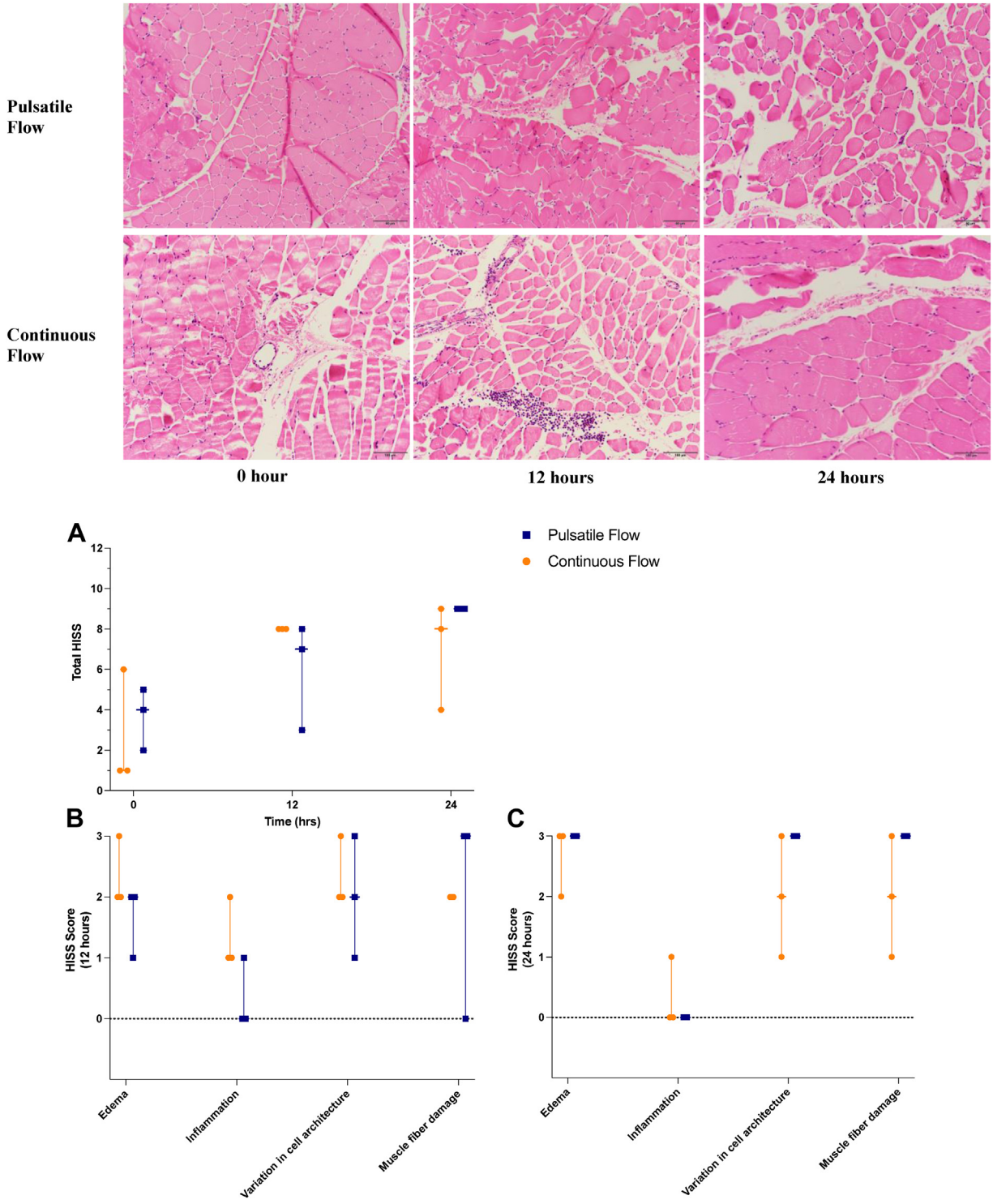

Fig. 5 – Evolution of myocyte damage and HISS scores over perfusion time. There is a similar trend of interstitial edema and myocyte breakdown over time with pulsatile flow and continuous flow (H&E, 20×). The graphs show the evolution of the total HISS (0-12) overtime (A) and the mean histology severity subscores (0-3) at 12 h (B) and 24 h (C) of perfusion. H&E = hematoxylin and eosin; HISS = Histologic Injury Severity Score.

porcine kidneys for 90 min with normothermic machine perfusion after 18 h[12,27] and 20 h[28] of static cold storage. Kidneys reconditioned with PF had a better renal function with higher clearance of creatinine and urea, and less tubular injuries than kidneys perfused in the same conditions with CF. In the Gallinat *et al.* study,[12] the PF group also showed a significantly increased expression of the Krüppel-like Factor 2 (KLF-2), an endothelial transcription factor induced by a laminar shear stress[14,29] that enhances the endothelial function, with higher rates of NO and lesser concentrations of

ET-1. According to the authors, PF induces a better activation of KLF-2 than CF after reperfusion, thus enhancing the endothelial function leading to better outcomes. In their study reporting similar outcomes in kidney reconditioning, von Horn and Minor[28] showed an increased NO/ET-1 ratio in the PF group, reflecting a better vascular conductivity. NO and ET-1 are two main mediators of the vascular tone. When endothelial cells are exposed to a mechanical shear stress in physiological conditions, the eNOS is strongly expressed while ET-1 expression is inhibited, reflecting a higher NO/ET-1 ratio.[14] In our study, PF seemed to correlate with a higher NO/ET-1 ratio, suggesting that pulsatility also improves vasoconductivity in VCA.

PF also resulted in lower lactate levels compared with CF, supporting less ischemic injury. Although edema was similar after 24 h of perfusion in both groups, grafts perfused with a CF tended to gain weight quicker than grafts of the PF group, reflecting a potential beneficial effect of pulsatility in reducing vascular leakage in the first hours of perfusion. In addition, PF may also have a role in reducing the inflammatory response of the organ after reperfusion, as mentioned by Siepe *et al.*[13] in swine lungs. They reported a significant reduction in interleukin 6 expression and caspase-3 activity compared with CF perfusion. In a similar way, we observed less histological signs of inflammation in the PF group. Improved vasoconductivity is related to a better perfusion of organs, diminishes ischemic injury, and enhances tissue viability. In clinical practice, the maintenance of an optimal vasoconductivity of the VCA with machine perfusion between graft procurement and time of transplantation could therefore help to reduce ischemia-related complications such as acute rejection episodes, decreased functional outcomes, and graft loss.

The role of pulsatility in machine perfusion thus remains controversial as there is no clear benefit over CF regardless of the investigated organ. Our findings suggest some benefits with PF in VCA preservation with subnormothermic machine perfusion. Future works should focus on how pulsatility triggers the KLF-2 pathways, and how this would be therapeutically targeted to improve viability of VCA in extended machine perfusion preservation. Finally, temperature represents an important parameter that also needs to be addressed as it may influence the effects of pulsatility on perfusion outcomes.

The major limitation of this pilot study is the limited number of replicates in both groups ($n = 3$). If our results could show a trend with potentially better NO/ET-1 ratio with a PF, more experiments are still required to confirm the actual benefit of pulsatility on enhancing vasoconductivity in VCA machine perfusion preservation. In addition, improved preservation outcomes should ideally be validated in a transplantation model to establish the true advantages of pulsatility in preserving VCA in a preclinical setting. Finally, only female pigs were used in this study due to practical concerns. Although there is no evidence in the literature of gender-based differences in VCA preservation, future studies increasing the number of replicates should also add male animals to address a potential gender bias.

To conclude, PF may provide better outcomes in VCA machine perfusion preservation, with potential improved endothelial function and decreased ischemic injury. Nevertheless, further investigations are still needed to optimize machine perfusion systems for VCA, and special consideration should be given to the type of flow.

Author Contributions

Study design: Pierre Tawa, Marion Goutard, Reinier J. de Vries, Korkut Uygun, Curtis L. Cetrulo, Mark A. Randolph, Alexandre G. Lellouch, and Basak Uygun.

Performed study: Pierre Tawa, Marion Goutard, and Reinier J. de Vries.

Collected data: Pierre Tawa and Marion Goutard

Analyzed data: Pierre Tawa, Marion Goutard, Alec R. Andrews, and Ivy A. Rosales.

Important intelligent input: Pierre Tawa, Marion Goutard, Reinier J. de Vries, Alec R. Andrews, Heidi Yeh, Mark A. Randolph, Alexandre G. Lellouch, Curtis L. Cetrulo, Korkut Uygun, and Basak Uygun.

Manuscript preparation and corrections: all authors.

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Disclosure

Some of the authors declare competing interests. Drs Lellouch, Cetrulo, Uygun, and Ms Pendexter have provisional patent applications relevant to this study. Dr Uygun has financial interests in Sylvatica Biotech Inc, a company focused on developing organ preservation technology. All competing interests are managed by Mass General Brigham in accordance with their conflict-of-interest policies.

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