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MAIN TEXT

Renal hemofiltration prevents metabolic acidosis and reduces inflammation during normothermic machine perfusion of the vascularized composite allograft—A preclinical study

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Abstract

Introduction: Recent experimental evidence suggests normothermic machine perfusion of the vascularized composite allograft results in improved preservation compared to static cold storage, with less reperfusion injury in the immediate post-operative period. However, metabolic acidosis is a common feature of vascularized composite allograft perfusion, primarily due to the inability to process metabolic by-products. We evaluated the impact of combined limb-kidney perfusion on markers of metabolic acidosis and inflammation in a porcine model.

Methods: Ten paired pig forelimbs were used for this study, grouped as either limb-only (LO, $n = 5$) perfusion, or limb-kidney (LK, $n = 5$) perfusion. Infrared thermal imaging was used to determine homogeneity of perfusion. Lactate, bicarbonate, base, pH, and electrolytes, along with an inflammatory profile generated via the quantification of cytokines and cell-free DNA in the perfusate were recorded.

Results: The addition of a kidney to a limb perfusion circuit resulted in the rapid stabilization of lactate, bicarbonate, base, and pH. Conversely, the LO

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circuit became progressively acidotic, correlating in a significant increase in pro-inflammatory cytokines. Global perfusion across the limb was more homogenous with LK compared to LO.

Conclusion: The addition of a kidney during limb perfusion results in significant improvements in perfusate biochemistry, with no evidence of metabolic acidosis.

KEYWORDS

ex vivo normothermic perfusion, kidney perfusion, metabolic acidosis, vascularized composite allograft

1 | INTRODUCTION

Acute graft rejection remains a primary limitation to successful vascularized composite allotransplantation (VCA), with over 80% of recipients experiencing rejection within the first year.[1] VCA rejection is complex, involving the donor and recipient immune compartment. The graft inherently consists of both long-lived resident, and highly mobile donor leukocytes that contribute to allorecognition and alloreactivity. Resident donor leukocytes can remain for the lifetime of the graft, but recipient leukocytes also populate the skin and other tissues, creating a highly immunogenic environment unique to VCA.[2-5] Furthermore, tissues are susceptible to ischaemia reperfusion injury. As such, injury is incurred during preservation, transplantation and following reperfusion, and collectively this is considered a potent catalyst for rejection.[6]

The gold standard approach to VCA preservation is static cold storage (SCS), where the vessels of the graft are flushed with cold preservation solution (most commonly University of Wisconsin solution), and the VCA is stored within a sealed bag and submerged in iced water for transit. However, prolonged periods of ischemia directly correlate with delayed graft function and subsequent activation of the innate immune system.[7] Recently, ex-vivo normothermic perfusion (EVNP) has satisfied the need to better preserve solid organs, whilst enabling functional evaluation. This technology is being rapidly adopted into the clinical setting for solid organ transplantation, with several studies demonstrating superior outcomes compared to SCS.[8,9] However, the application of this technology in VCA has been purely experimental to date, with several groups developing perfusion protocols for VCA preservation.[10-17] We have recently reported that normothermic perfusion results in superior graft preservation and less reperfusion injury compared with SCS in a large animal model.[10] However, further work is required to facilitate its safe adoption into clinical practice.

A common, and somewhat speculative limitation to successful VCA perfusion relates to the perpetually increasing metabolic by-products and toxins, when compared with solid organ perfusion.[15-18] Increasing lactate levels beyond physiological range are observed in all published cases (where data is presented). We hypothesize that regulating key metabolites will improve perfusion biochemistry by preventing imbalances associated with hypo-perfusion in the in vivo setting. However, selectively removing metabolites remains a challenge. Attempts to replace perfusate throughout limb perfusion have been evaluated,[19] but this is laborious, time consuming and non-specific. Hemodialysis can be used to regulate hematological, biochemical and acid-base instability but this is reliant upon high performance hollow fiber membranes that target specific solutes based solely on size. As such, life-sustaining substances are frequently removed. The kidney is intrinsically involved in metabolic recycling and clearance in vivo. On these grounds we aimed to (1) replicate physiological hemofiltration using a corresponding kidney in a porcine VCA perfusion model, and (2) evaluate the impact of renal hemofiltration on perfusion biochemistry and hemodynamics. Using this experimental approach, our goal was to determine if renal hemofiltration improves graft perfusion, which would highlight the need to use clinically approved, advanced hemodialysis systems during ex-vivo machine perfusion of the limb.

2 | METHODS

2.1 | Procurement

Landrace pigs ($n = 5$) with a mean weight of 80 kg were sacrificed via Schedule 1 according to the Home Office Scientific Act (1986) as previously described.[10] Briefly, pigs were electrically stunned, followed by exsanguination via severing of the carotid artery and jugular vein. Blood was collected into a sterile receptacle containing 100 ml of isotonic 0.9% saline (Baxter Healthcare, Norfolk, UK) supplemented with 40 000 iU unfractionated heparin (Fannin, Northamptonshire, UK). Following exsanguination, a midline laparotomy

incision was made and both kidneys were excised en bloc. Kidneys were dissected free from the aorta, ensuring maximal length of the renal artery to afford arterial length for cannulation (Arrowflex 7 Fr, Canada) proximal to the renal artery bifurcation. One kidney was selected at random to be used in this study. Bilateral forelimbs were disarticulated at the glenohumeral joint. The dominant brachial artery was cannulated (Arrowflex 7 Fr, Canada) together with the radial collateral artery (vessel cannulae, Andcor, Belgium). Each limb was simultaneously flushed with 1 L 4°C Ringer's Solution supplemented with 10 000 iU heparin to exude residual blood from the vasculature. This marked the end of warm ischemia (time of death after exsanguination until the start of cold preservation flush). Once cannulated, the kidney was flushed with 20 ml of glycerine trinitrate (GTN, 1 mg/ml; Hameln, Gloucester, UK) for vasodilatation followed by 1 L 4°C hypertonic citrate solution, Soltran (Baxter Healthcare, Norfolk, UK) further supplemented with 10 000 iU heparin using a pressure bag set at 70 mm Hg. Finally, the kidney was submerged in 500 ml Soltran in a sealed bag. The randomly selected kidney and the two limbs were then transported back to the laboratory on ice. Cold ischemia was defined as the time from the start of the preservation flush until connection to the EVNP circuit.

2.2 | EVNP

Two identical EVNP circuits were primed with Ringer's solution supplemented with bovine serum albumin (Sigma Aldrich, Dorset, UK), 50 ml of sodium bicarbonate (8.4%), 40 ml 20% mannitol (Baxter Healthcare, Norfolk, UK), 13.2 mg dexamethasone (Hameln, Gloucester, UK), 500 mg Meropenem (Hikma, Portugal), 4000 iU unfractionated Heparin and 30 ml 15% glucose (Sigma Aldrich, Dorset, UK). A target hematocrit of 25%–30% was achieved by adding approximately 800 ml of autologous cell saved leukocyte depleted blood. A syringe driver (Williams Medical Supplies, Swansea, UK) infused GTN (1 mg/ml) at a rate of 10 ml per hour. A second syringe driver infused Nutriflex (B. Braun Medical, Sheffield, UK) supplemented with 6 ml 20% mannitol and 14 ml 15% glucose at a rate of 10 ml per hour. Gas continuously supplied to the circuit was a mixture of 95% oxygen and 5% carbon dioxide. The oxygenator was connected to the water heat exchanger pre-set to a temperature of 38°C ± 1°C. Pressure transducers were filled with Ringer's solution and equilibrated to room air.

2.3 | Preparation

Following a standardized cold ischemia time of 2 h, the kidney was removed from ice and flushed with 250 ml 4°C Ringer's Solution using a sterile catheter bag to exude any remaining Soltran. The two limbs remained on ice for an additional hour before attachment to the EVNP circuit.

2.4 | Perfusion

Following intravascular flush, the kidney was connected to one perfusion circuit at random, via the renal artery cannula (see Figure S1 for a schematic of the perfusion circuits). The target arterial pressure was initially set to 55 mm Hg and then gradually increased by 5 mm Hg every 5 min to reach a target pressure of 75 mm Hg, to prevent endothelial shearing.

A urinary recirculation protocol (as previously described in Ref. [20]) was not used as renal filtration was a key goal of the study. The ureteric cannula was placed in a measuring cylinder and urine output (UO) was measured and replaced like for like with Ringer's Solution throughout the duration of the experiment. One hour into renal perfusion, limbs were removed from ice and randomized to either the circuit containing the kidney (referred to as limb-kidney (LK) perfusion), or perfused on a separate circuit in isolation (limb only, LO—See Figure S2 for a Gantt chart of the protocol). Limbs were perfused via the brachial and radial collateral arteries to achieve maximal perfusion throughout all compartments of the limb. All tissues were periodically bathed on their exposed surfaces with Ringer's solution to ensure tissues were moist and hydrated. Both limbs were perfused for 5 h, with the kidney being perfused for a total of 6 h. This study was designed as a "non-intervention" study meaning that no substrates or solutes were added to the perfusate to maintain physiological parameters. This was to allow a true evaluation of the impact of renal hemofiltration.

2.5 | Infrared imaging

Serial images were obtained using the FLIR ONE Pro (3rd generation) and analyzed using the FLIR Tools and software. Emissivity was set at 0.98, reflective temperature at 22°C, distance 1 m and relative humidity of 50.0% for each image. The proximal section (from the cut edge to the elbow joint) of each limb was marked out before perfusion and used as the region of interest. Using the software, each region of interest was evenly populated with temperature spots. For each image, the mean and standard deviation of the temperature points were calculated, in order to quantify the average and variability of temperature across the limb. These values were then analyzed using a repeated-measures ANOVA approach. The timing of the measurement and the treatment group (LK vs LO), as well as the

interaction between these were included as within-subject effects. *p* Values were calculated using the Greenhouse-Geisser approach. A subgroup analysis including only the first hour of measurements from each experiment was also performed.

2.6 | Sample collection

2.6.1 | Blood gas analysis

A GEM 4000 blood gas analyzer was utilized to assess the biochemistry of the perfusate.

2.6.2 | Perfusate

Serial perfusate samples were collected throughout the experiment. Samples were centrifuged at 1000 *g* at 4°C for 10 min and 1 ml aliquots of plasma were separated and stored at −80°C.

2.6.3 | Biopsy

Biopsies of the muscle, skin and vessels were taken from both limbs at the end of perfusion and stored in 10% neutral buffered formalin (Sigma Aldrich, UK). Sections were cut at five-microns (Leica RM2125, Leica Biosystems, UK) and mounted onto Superfrost™ positively charged microscope slides (Thermo-Scientific, Germany). The slides were de-paraffinized and stained with hematoxylin and Eosin Y (H&E) using the Leica Autostainer XL (Leica Biosystems, Nussloch, Germany). All slides were assessed and graded using the Histologic Injury Severity Score (HISS) grading system by a clinical histopathologist who was blinded to the study groups.

2.7 | Luminex

The concentration of 13 cytokines and chemokines within the perfusate and urine was assessed using a commercially available porcine 13-plex magnetic bead panel (Merck Millipore, Billerica, MA, USA) following the manufacturer's protocol. The plate was read using a Bio-Plex 200 system (Bio Rad, Hertfordshire, UK).

2.8 | qPCR

Extracellular genomic (gDNA) and mitochondrial DNA (mtDNA) was quantified using the appropriate primers (Sigma Aldrich, Dorset, UK). To identify gDNA primers to the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) were used and primers specific for cytochrome B were used to quantify mtDNA. A MicroAmp Optical 348-well reaction plate was used, and qPCR was performed using a QuantStudio 12-flex system with Power SYBR green PCR master mix (Life Technologies, Paisley, UK).

2.9 | Data analysis and statistics

All numerical data were analyzed using GraphPad Prism v.7.0 and SPSS v. 23 (IBM). Data is presented as mean ± standard deviation. Data normality was determined by assessing mean, standard deviation, skewness and kurtosis. Formal evaluation was performed using the Shapiro-Wilk test. For comparison of multiple variables over time in each group two-way ANOVAs were used. Independent samples *t*-test or the Mann Whitney U test were then utilized for a direct comparison between single time-points, dependent on data distribution. Pearson's correlation coefficient was utilized to assess correlations. Data was considered significantly different if a *p*-value of ≤ .05 was observed.

3 | RESULTS

3.1 | Procurement

There was no significant difference observed in warm (20.6 ± 3.0 vs 20.6 ± 2.9 min for LK vs LO, $p = 1.0$) or cold ischemia (195.4 ± 13.7 vs 193.8 ± 12.7 for LK vs LO, $p = .841$) in both LK and LO limbs. Both warm (20.6 ± 3.0 min) and cold ischemia time (133.4 ± 14.7 min) for the randomized perfused kidney were also recorded.

3.2 | EVNP hemodynamics

Both LO and LK circuits perfused without any complications and demonstrated good flows throughout. Flow rates increased more rapidly in the LK group compared to LO and remained significantly higher throughout perfusion (mean flow at 6 h was 496 ml/min ± 78.29 in LK vs 232 ml/min ± 106.6 in LO, $p = .0002$, Figure 1A). In the LK circuit, the flow probe was located on the outflow tubing from the oxygenator before the Y-connector; meaning flow was determined as a total for both the kidney and the limb (see Figure S1). The significant increase in flow was therefore likely to be an artefact of the combined vascular reservoir of the limb and the

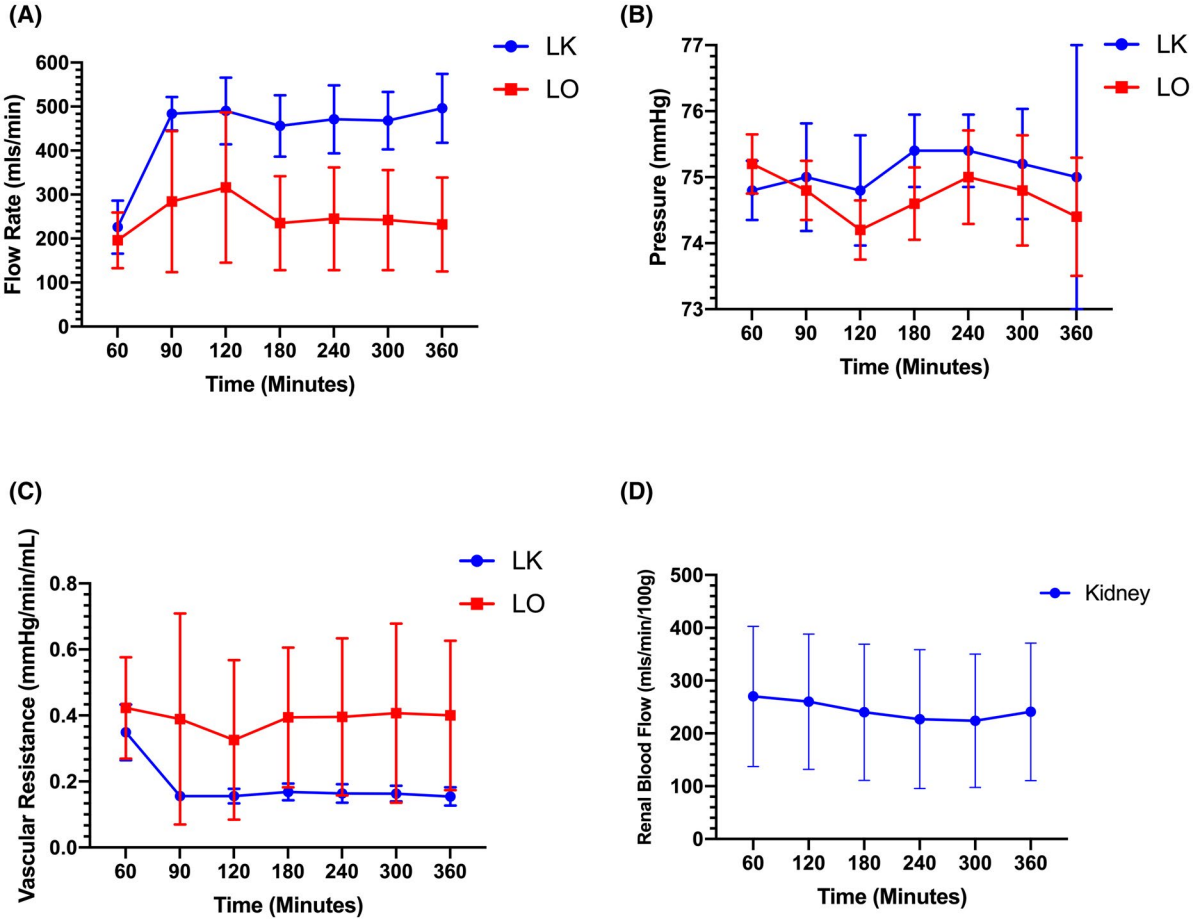

FIGURE 1 Hemodynamic differences between LO and LK perfusion. (A) Flow rate in ml/min, (B) mean arterial pressure (mm Hg), (C) mean vascular resistance (mm Hg/min/ml) in LO vs LK perfusion, and (D) renal blood flow during LK perfusion

kidney, rather than improved hemodynamics. To evaluate this further, we analyzed the mean flow in $n = 5$ kidney perfusions without a limb being attached to the circuit (see Ref. [21]), confirming the mean increase in flow in LK perfusion of 264 ml/min ± 166.2 was comparable to that seen in a kidney alone (241 ml/min/100 g ± 130.2). Mean pressures were maintained at 75 mm Hg via manually controlling pump speed (Figure 1B). Vascular resistance was calculated using the MAP of the circuit divided by flow rate (for LK this was determined as a total for the limb and kidney combined). Interestingly, vascular resistance was lower throughout the experiment in LK perfusion ($p = .0416$, Figure 1C).

3.3 | Limb histology

Muscle, skin, and vessel biopsies were independently reviewed and graded using the HISS grading by a clinical Histopathologist. In both groups tissue architecture was well preserved at 6 h, with no differences observed between either limb.

3.4 | Thermographic infrared imaging

Based on thermal imaging, global perfusion of the kidney was homogenous, with convincing infrared uptake within the first hour of isolated perfusion (Figure 2A). Perfusion remained homogenous throughout the entire experiment following attachment of the limb to the circuit (Figure 2B). For the limbs, trends over time in temperature variability are shown graphically (Figure 2C). Both the average and homogeneity of temperatures were found to change significantly over time as expected ($p < .001$ and $p < .001$ respectively). The average temperature did not differ significantly between LK and LO perfusion ($p = .822$), but temperatures were significantly more variable in the LO group compared to LK group ($p = .002$, Figure 2D), indicating improved, homogenous perfusion in LK perfusion.

3.5 | Perfusate biochemistry

Lactate remained low and stable in the initiation phase (1 h of kidney perfusion) of LK perfusion. Following

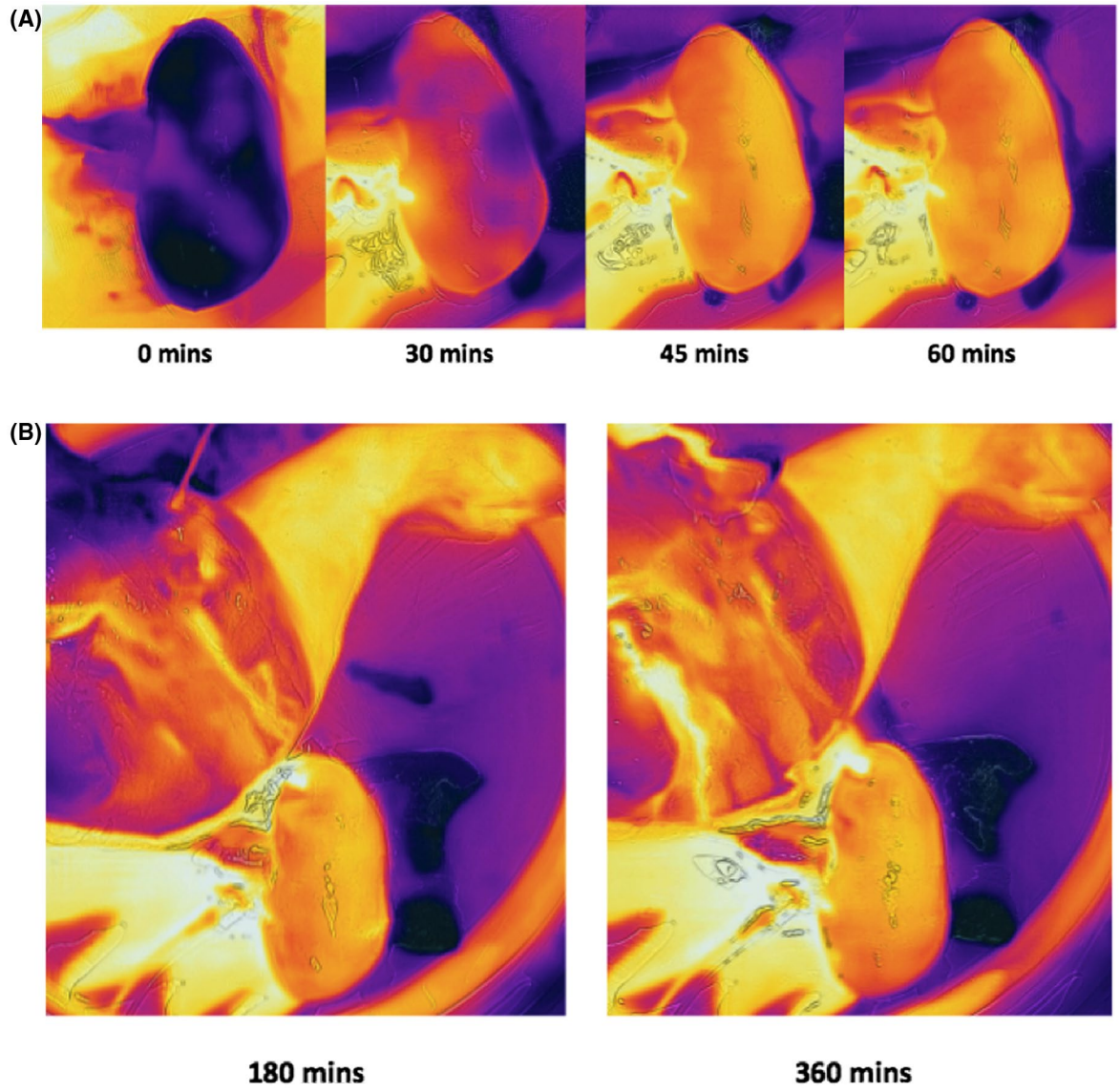

FIGURE 2 Perfusion homogeneity based on topical temperature via infrared (IR) imaging. (A) Representative IR images of the kidney perfused alone in the first hour and (B) hour 3 and 6 following attachment of the limb. (C) Standard deviation of surface temperatures collected from multiple regions of the limb and (D) representative IR images of LO versus LK perfusion

attachment of the limb, lactate increased rapidly from (3.3 mmol/L ± 5.2 to 10.9 mmol/L ± 3.5 within 1 h of LK perfusion, Figure 3A). Lactate levels continued to decrease throughout perfusion, with a final concentration of 7.5 mmol/L ± 1.7 at 6 h. Conversely, lactate levels rapidly increased to 14.6 mmol/L ± 2.2 during LO perfusion, and remained high throughout. The end concentration of lactate during LO was 13.8 mmol/L ± 3.7. Using 2-way ANOVA, lactate was significantly lower in LK compared to LO ($p = .0048$). Taking each timepoint of perfusion as an independent variable via multiple *t*-test, lactate was significantly lower at 3 ($p = .023$), 4 ($p = .022$), 5 ($p = .019$) and 6 h ($p = .009$) of LK compared to LO perfusion.

Lactate is converted to glucose through the Cori cycle by the kidney. We therefore determined glucose levels during perfusion. As expected, glucose concentrations increased during the initiation phase of LK perfusion. When the limb was attached to the LK circuit, glucose levels continued to rise for the remaining 5 h of perfusion, suggesting lactate to glucose conversion was adequate for both tissues (Figure 3B). Conversely in the LO circuit, glucose levels remained statistically lower throughout perfusion ($p = .02$), highlighting a mechanism of action of the kidney. This occurred despite identical supplementation of a 30 ml bolus followed by 10 ml/per hour infusion of 15% glucose to both circuits.

Given that hyperlactatemia disrupts sodium bicarbonate recycling, we then evaluated if stable lactate resulted in physiological levels of bicarbonate during LK perfusion. This was comparable to lactate, with bicarbonate levels

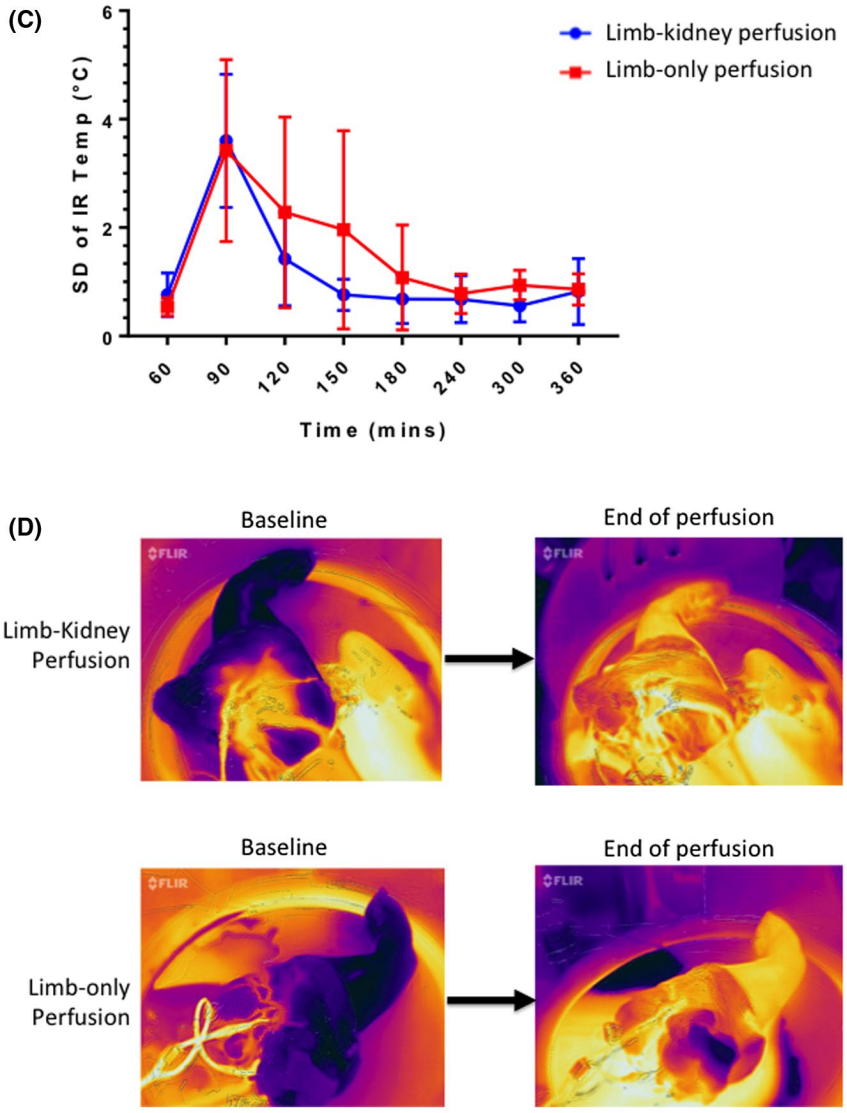

FIGURE 2 (Contiuned)

remaining within physiological range over the 6 h of perfusion (Figure 3C). Conversely, bicarbonate was only within physiological range in the first hour of LO perfusion. In keeping with lactate, this was statistically significant from 3-6 h of perfusion between the groups. As bicarbonate represents a key base molecule during perfusion (as well as systemically), we then evaluated if loss of bicarbonate recycling in the absence of a kidney resulted in a base deficit in the LO perfusate. In keeping with both lactate and bicarbonate, base remained within physiological range in the LK circuit throughout the 6 h of perfusion (Figure 3D). In the LO circuit, a base deficit occurred within 1 h of perfusion, to −9.5 mmol/L ± 6.3 (compared to −2.2 mmol/L ± 7.4 in LK). Base increased in LO, but always remained outside of physiological levels, and this was statistically lower from 3–6 h of perfusion compared to LK perfusion. As acid/base disturbances are a hallmark of metabolic acidosis, we then compared perfusate pH between LO and LK. We found pH was consistently lower in LO across all time points, reaching significance at the end of perfusion (pH 7.44 ± 0.04 in LK and pH 7.35 ± 0.03 in LO, $p = .0057$, Figure 3E).

Given the close relationship between lactate, bicarbonate and base with pH, we then determined relationships between these parameters. In all cases, bicarbonate and base positively correlated with pH in both LO and LK, whereas lactate was inversely correlative (Table 1), suggesting metabolic acidosis was present in LO but not LK.

We then quantified key electrolytes including potassium, calcium and sodium. Only sodium was significantly different between groups, remaining within physiological levels during LK perfusion (Figure 4). Sodium is considered a biomarker of acceptable renal perfusion suggesting adequate function of the kidney via the maintenance of appropriate sodium levels.

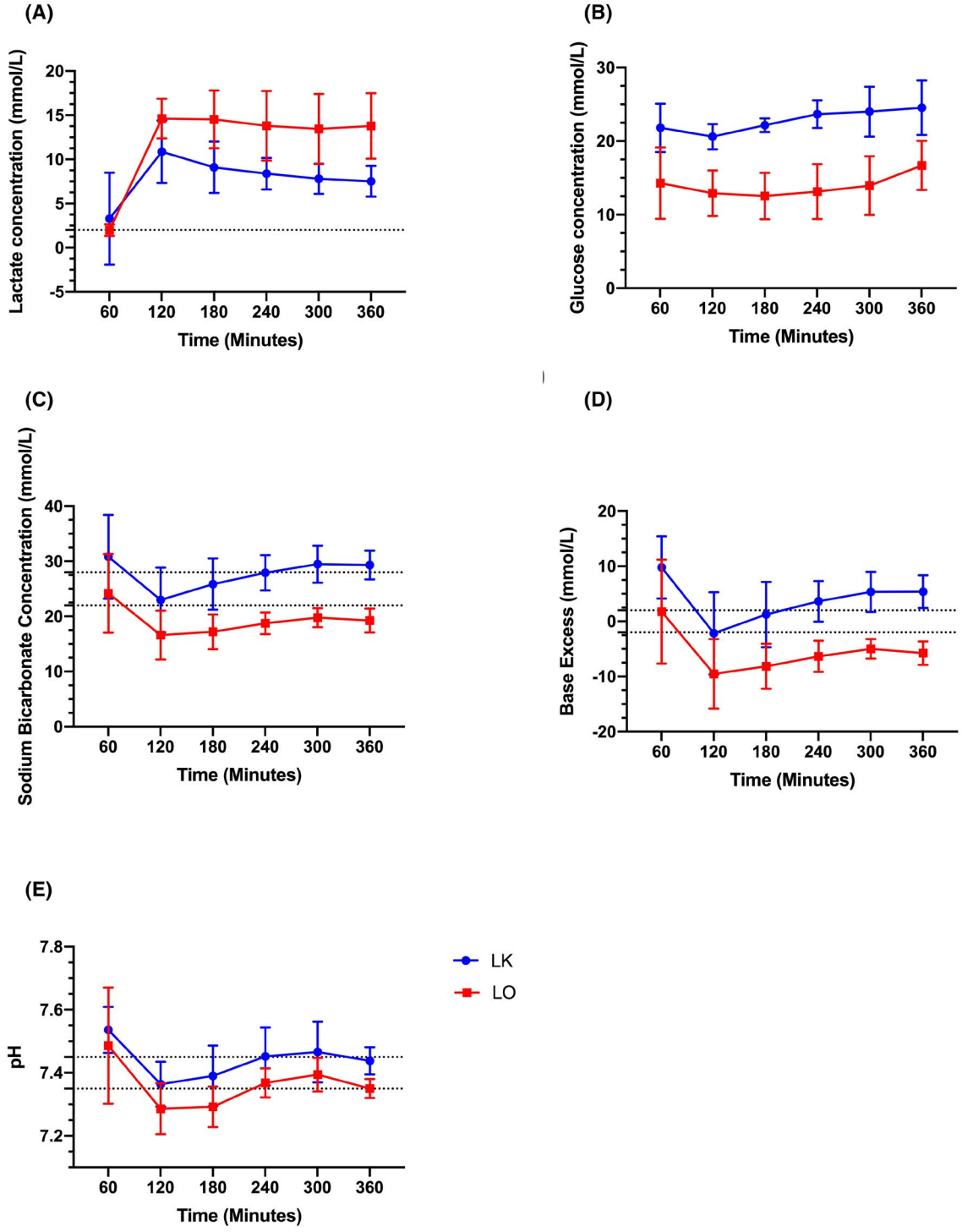

FIGURE 3 Perfusate biochemistry. (A) Lactate, (B) glucose, (C) sodium bicarbonate, and (D) base (all in mmol/L), and (E) pH

3.6 | Inflammatory profile

Nine of the thirteen cytokines and chemokines assessed were detected within the perfusate, increasing over time in both groups. However, only cytokine members of the interleukin-1 family were different between groups. These included IL-1α, IL-1β and IL-18 (Figure 5). We then determined urinary concentrations of IL-1α, IL-1β and IL-18 to assess if urinary secretion was responsible for the lower perfusate levels observed in LK perfusion. However, there was no correlation between perfusate and urine IL-1α, IL-1β and IL-18 (Figure 6). Cell-free gDNA was detected in both groups, but did not differ during perfusion ($p = .707$),

TABLE 1 Correlations between bicarbonate, base deficit/excess and lactate with perfusate pH

	Limb-only	Limb-kidney
	pH	pH
Bicarb	$p = .001$, pearson = 0.979	$p = .01$, pearson = 0.916
Base	$p = .001$, pearson = 0.971	$p = .002$, pearson = 0.967
Lactate	$p = .025$, pearson = −0.868	$p = .004$, pearson = −0.968

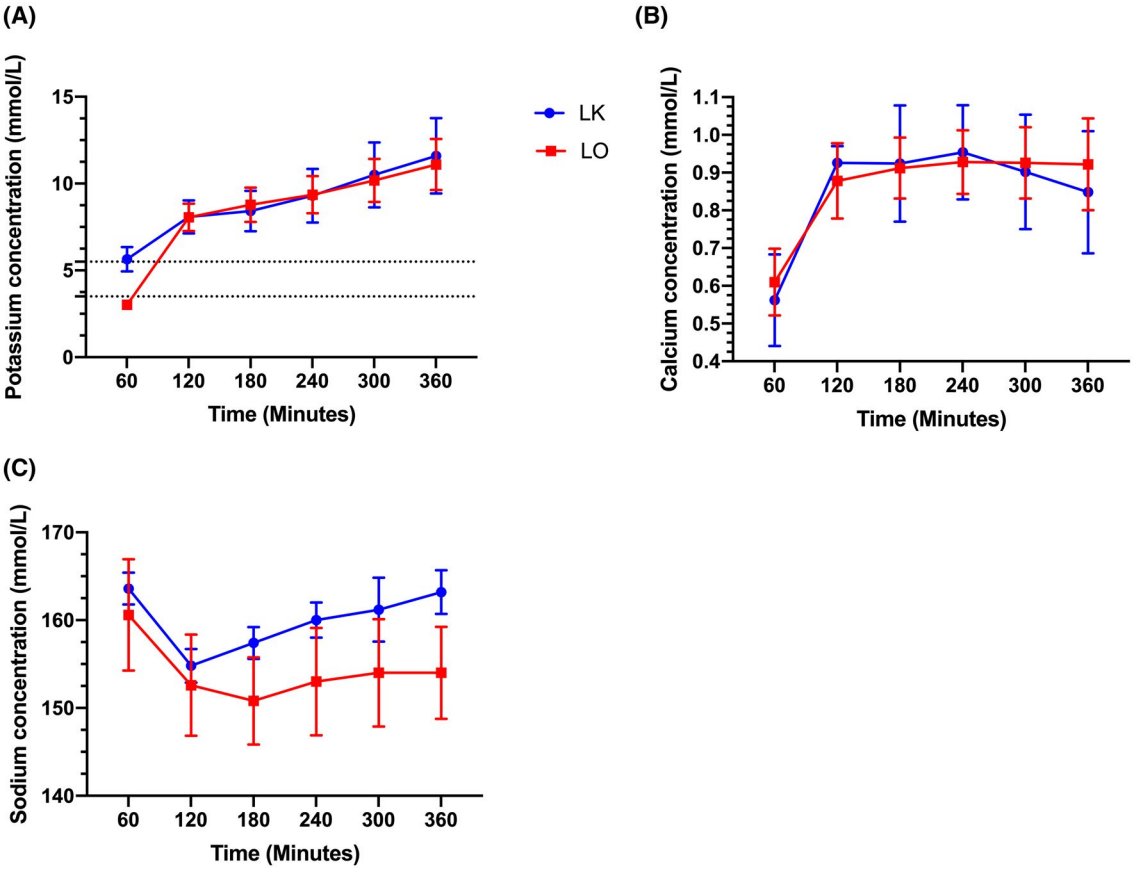

FIGURE 4 Perfusate electrolyte composition. (A) Potassium, (B) calcium and (C) sodium concentrations (in mmol/L) over time in LO versus LK perfusion

whereas mtDNA remained undetectable throughout the experiment in both groups.

4 | DISCUSSION

We have recently demonstrated that normothermic machine perfusion results in improved VCA preservation when compared to SCS.[10] However, a major obstacle to successful NMP of the limb is metabolic acidosis due to a persistent increase in lactate. Metabolic acidosis disrupts several pathways critical for cellular homeostasis, including ADP-ATP conversion, the absorption and processing of fatty acids, and several enzymatic reactions.[22-24] Lactate is a multifunctional molecule involved in several pathways including glucose recycling and lactate conversion to pyruvate, the latter of which is consumed by mitochondria to generate ATP, with CO_2 and water released as a by-product. The lack of these reactions can contribute to acidosis during perfusion, in the absence of a kidney and liver. Furthermore, in patients with metabolic acidosis, hyperlactatemia disrupts bicarbonate recycling contributing to base deficit.[25] Base deficit is a common feature of VCA perfusion and is intrinsically linked to acidosis.[10] To combat this, bicarbonate is often supplemented as a bolus at the start of perfusion, with continuous infusions and additional boluses to control base deficit.[11-18] Bicarbonate supplementation rapidly stabilizes base, but often into base excess, which in turn may be reduced via increasing CO_2 concentrations in the circuit oxygenator. The

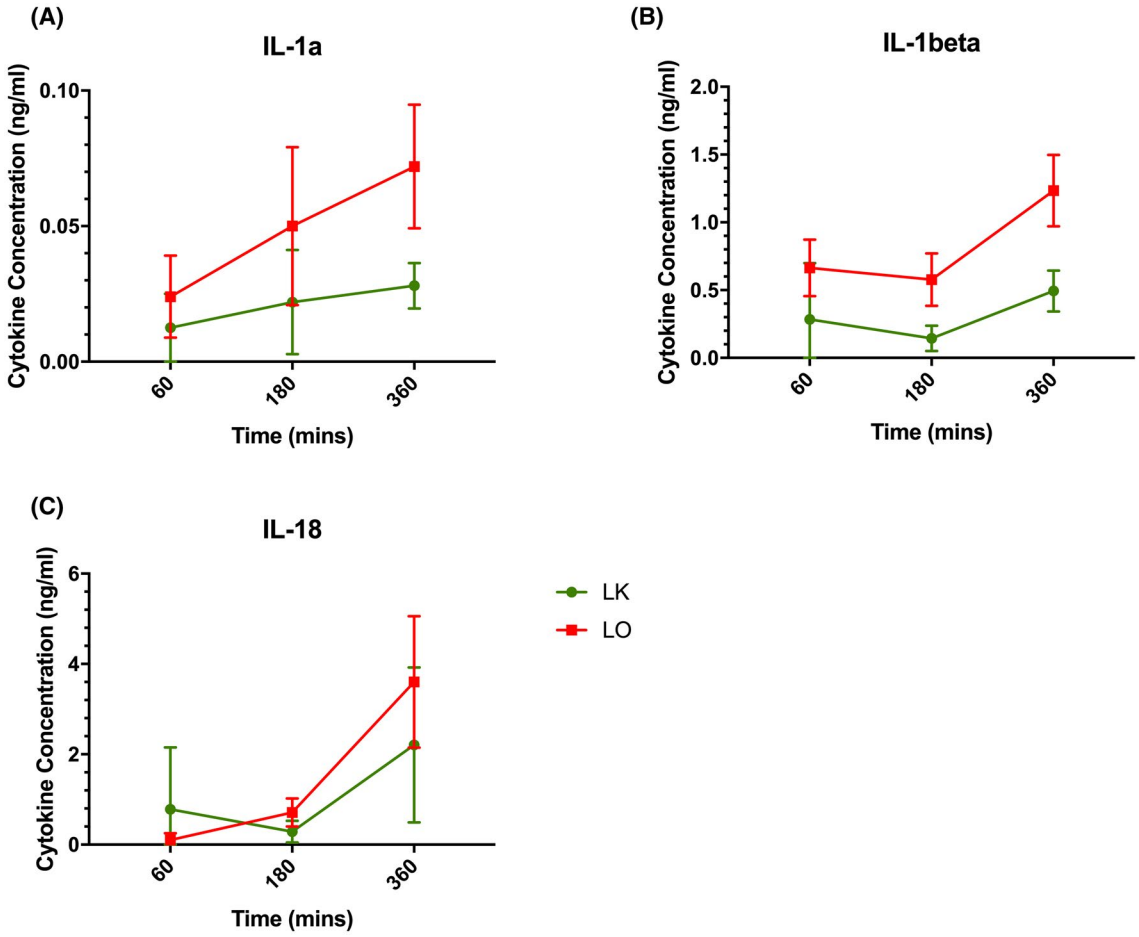

FIGURE 5 Perfusate cytokine profile. (A) IL-1α, (B) IL-1β and (C) IL-18 concentration (ng/ml) in the perfusate of LO and LK circuits

relationship between CO_2, bicarbonate, lactate, glucose and pH is highly complex, as acidosis drives electrolytes from the muscle leading to hyperkalemia. Several groups control this with glucose and insulin supplementation, creating a pendulous scenario incomparable to the stable physiological control of these systems in vivo. The net result of this approach is a disrupted environment, which again is a common feature of VCA perfusion. Despite significant understanding of these processes in vivo, replicating and maintaining a biochemically stable perfusion environment has not been a goal in the development of VCA (and all other organ) perfusion protocols thus far.

In an attempt to address this, we hypothesized that the addition of superior hemofiltration to a VCA perfusion would be of benefit via renal gluconeogenesis and lactate conversion. However, current hemofiltration devices are inadequate in maintaining perfusion biochemistry, as simply removing substances below a specific size from the circuit would not support metabolic regulation and control pH. Indeed, hemofiltration inherently removes key substances that are essential for perfusion homeostasis, including bicarbonate[26] and albumin.[27] It was on these grounds that we attempted to evaluate the impact of renal hemofiltration during VCA perfusion, as this would provide novel insight into the impact of controlling acidosis, and highlight the importance of developing novel technology that could be used to more accurately replicate renal hemofiltration. We hypothesized that given the kidney accelerates lactate conversion during acidosis,[28] pH would be stabilized.

We report that the addition of an autologous kidney to a VCA (forelimb) perfusion circuit resulted in significant improvements to perfusion biochemistry. Renal gluconeogenesis was evident, as a significant loss of lactate occurred with a concurrent increase in glucose (despite identical glucose bolus and infusions) in the LK circuits when compared to LO. This suggests that the kidney was converting lactate to glucose via the Cori-cycle (the kidney can excrete lactate in the urine but this is estimated to contribute less than 2% to lactate disposal).[29] The Cori-cycle describes the process where lactate diffused from skeletal muscle during anaerobic metabolism is absorbed by the renal cortex and converted to glucose via pyruvate.[30] The glucose is then diffused from the renal cortex and

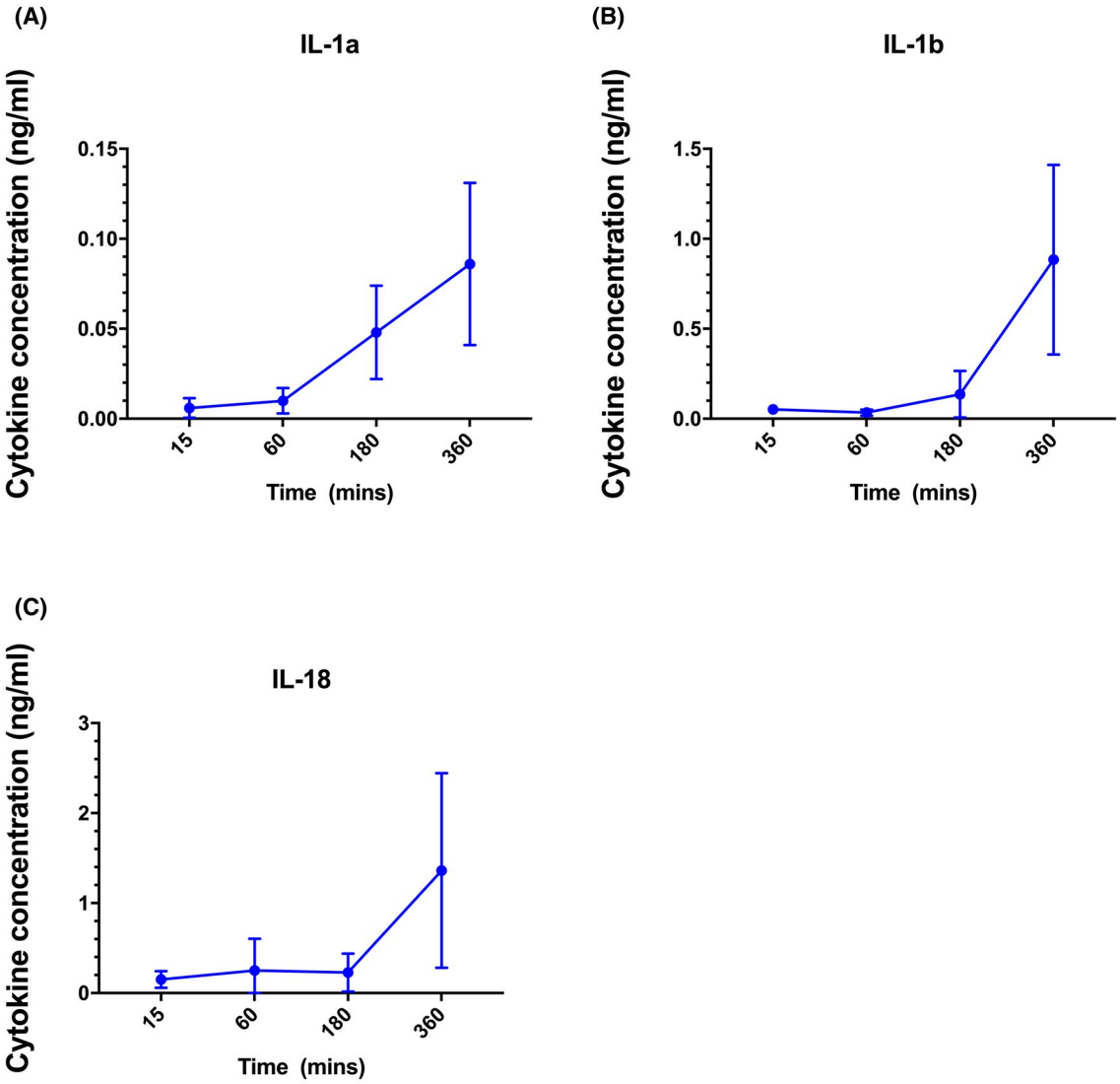

FIGURE 6 Urinary cytokine profile. (A) IL-1α, (B) IL-1β and (C) IL-18 concentration (ng/ml) in urine from the LK circuit

absorbed by the muscle, which consumes the glucose to produce ATP, with lactate and pyruvate as a by-product. This compartmentalized, gluconeogenic cycle enables the continuous recycling of lactate to glucose, and is critical in the control of metabolic acidosis. Furthermore, and perhaps more critical to VCA perfusion, is the requirement for sufficient levels of the Cori cycle by-product, pyruvate within the perfusate. Pyruvate is converted to acetyl-CoA and CO_2 via alanine conversion, resulting in the permanent generation of bicarbonate. Bicarbonate remained within physiological levels throughout LK perfusion, suggesting adequate conversion from pyruvate was occurring. Conversely, bicarbonate levels were outside of physiological range in the LO circuit. This in turn manifested as a base deficit in LO. However, this was not observed during LK perfusion, where base remained within physiological parameters. Logically, a base deficit highlights an acid/base derangement that would likely result in an acidotic environment. This was observed in LO perfusion, where pH of the perfusate was acidotic. Importantly, neutral pH was preserved throughout LK perfusion. It therefore appears likely that renal hemofiltration controls pH via maintaining acid/base balance, through gluconeogenesis and pyruvate to bicarbonate conversion during perfusion.

Following on from this, we sought to determine if the addition of a kidney had any impact on the inflammatory profile of the circuit. We quantified several cytokines over the duration of perfusion, and interestingly, found that only cytokine members of the interleukin-1 family that can be regulated by inflammasome activity were altered. Specifically, IL-1α, IL-1β and IL-18 were significantly reduced in the LK group compared to LO. We hypothesized that this could be via urinary excretion, however, cytokine levels within the urine were minimal, suggesting other

mechanisms were involved. It is therefore more likely that the acidotic environment observed in the LO circuit is responsible for the increase in IL-1 family cytokines. There is evidence that NLRP3 inflammasome activation occurs in the presence of acidosis, increasing the activation of caspase-1 and secretion of IL-1β.[31] Conversely, NLRP3 inflammasome activity is inhibited in an alkalotic environment. However, there are a number of additional inflammasome complexes that can process pro-IL-1β and pro-IL-18, and as such the inflammasome involved in this context requires further investigation. Furthermore, pro-IL-1α is not a direct substrate for the inflammasome effector protease caspase-1, but is cleaved by other caspases and calpains.[32,33] There are thus a number of potential pathways that could contribute to the increase in IL-1 family cytokines observed here. Nonetheless, the IL-1 family cytokines remained low during LK perfusion, but continually increased during LO. Given that IL-1β has been identified as a potential biomarker of donor organ reconditioning in other settings, and IL-1β antagonists have been suggested as potential therapies for tissue reconditioning during machine perfusion, this finding may have wider reaching implications in terms of the cause or effect of increasing IL-1 family cytokines during perfusion.[34]

4.1 | Limitations

There are two primary limitations to the current study. The first is that blood flow was determined globally across the limb and kidney, rather than individually for each tissue (in the LK perfusion). This prevented us from accurately recording blood flow into the kidney and the limb. We determined blood flow based on a significant case series of isolated kidney perfusions and used this as the mean. In future studies, independent flow and pressure monitors should be used on the arterial cannulas to the kidney and to the limb. The second limitation is that we did not record urine production, or other critical markers of renal function, such as creatinine clearance, or markers of renal injury, such as NGAL. This would have provided more insight into the function of the autologous kidney during LK perfusion. However, the inclusion of a kidney was only used as an experimental approach to understand the impact of optimal renal hemodialysis during limb perfusion.

4.2 | Summary

Renal hemofiltration results in the maintenance of physiological lactate, bicarbonate, base, pH, and inflammation. However, positive changes of the VCA histology and perfusion hemodynamics were probably too subtle to show within the study time frame. Based on thermal imaging of the VCA, the homogeneity of perfusion was significantly better in LK compared to LO perfusion. This may be of relevance given that lactic acidosis (type A) occurs either via inadequate oxygen concentrations within the perfusate (which certainly was not the case in this study), or via inadequate perfusion of tissues. Importantly, this finding suggests renal hemofiltration improves the distribution of flow, a process that is critical to the physiological perfusion of donor tissues.

This study highlights the importance of controlling key biochemical metabolites and by-products during VCA perfusion. This is an overlooked yet important consideration for the optimization of limb preservation. We fully recognize that including a donor kidney with a VCA perfusion will never be a clinical reality (nor should it be). However, the findings of this work highlight the need to evaluate clinically relevant approaches to advanced hemofiltration during machine perfusion of the VCA, and potentially, all other organs. However, this creates a complex and currently unrealizable dilemma—existing dialysis technologies are only capable of isolating molecules based on physical characteristics alone, whereas the beneficial actions of the kidney during VCA perfusion are exerted via biochemical processes that maintain "perfusate homeostasis", for which there are currently no existing technologies or approaches. However, the benefit of transmembrane dialysis during perfusion of other organs for transplant has been reported, so this approach should be evaluated during VCA perfusion.[35] Alternatively, replicating biochemical processes to support lactate conversion, gluconeogenesis, pyruvate production, and bicarbonate recycling may represent a superior approach to preventing metabolic acidosis and stepping closer to physiological VCA perfusion.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

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