Accepted Manuscript online: 2022-06-28 Article published online: 2022-10-31

Exceeding the Limits of Static Cold Storage in Limb Transplantation Using Subnormothermic Machine Perfusion

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J Reconstr Microsurg 2023;39:350–360.

Abstract

Background For 50 years, static cold storage (SCS) has been the gold standard for solid organ preservation in transplantation. Although logistically convenient, this preservation method presents important constraints in terms of duration and cold ischemia-induced lesions. We aimed to develop a machine perfusion (MP) protocol for recovery of vascularized composite allografts (VCA) after static cold preservation and determine its effects in a rat limb transplantation model.

Methods Partial hindlimbs were procured from Lewis rats and subjected to SCS in Histidine-Tryptophan-Ketoglutarate solution for 0, 12, 18, 24, and 48 hours. They were then either transplanted (Txp), subjected to subnormothermic machine perfusion (SNMP) for 3 hours with a modified Steen solution, or to SNMP + Txp. Perfusion parameters were assessed for blood gas and electrolytes measurement, and flow rate and arterial pressures were monitored continuously. Histology was assessed at the end of perfusion. For select SCS durations, graft survival and clinical outcomes after transplantation were compared between groups at 21 days.

Results Transplantation of limbs preserved for 0, 12, 18, and 24-hour SCS resulted in similar survival rates at postoperative day 21. Grafts cold-stored for 48 hours presented

Keywords
- preservation
- reconstructive surgery
- VCA
- cold storage
- machine perfusion
- transplantation

* *Shared senior authorship.*

received
October 14, 2021
accepted after revision
June 23, 2022
accepted manuscript online
June 28, 2022
article published online
October 31, 2022

DOI https://doi.org/10.1055/a-1886-5697.
ISSN 0743-684X.

delayed graft failure ($p = 0.0032$). SNMP of limbs after 12-hour SCS recovered the vascular resistance, potassium, and lactate levels to values similar to limbs that were not subjected to SCS. However, 18-hour SCS grafts developed significant edema during SNMP recovery. Transplantation of grafts that had undergone a mixed preservation method (12-hour SCS + SNMP + Txp) resulted in better clinical outcomes based on skin clinical scores at day 21 post-transplantation when compared to the SCS + Txp group ($p = 0.01613$).

Conclusion To date, VCA MP is still limited to animal models and no protocols are yet developed for graft recovery. Our study suggests that ex vivo SNMP could help increase the preservation duration and limit cold ischemia-induced injury in VCA transplantation.

Vascularized composite allografts (VCAs) have become an essential therapeutic solution for many patients suffering from facial defects, upper extremities amputations, or major composite tissue defects not adequately addressable by autologous surgery.[1,2] Nonetheless, VCAs are not always easily allocated; skin color matching, age, length of limb, immunological compatibility, and anatomical defect of the recipient make each VCA transplant unique, with constraints that are not present in any other organ transplant procedure. Based on the Organ Procurement and Transplantation Network Data, even though the number of VCA programs has grown in the past years in the United States, some patients still wait more than 3 years for an adequate VCA donor.[3,4] While indications for the use of VCA grow wider with the establishment of new VCA transplant centers,[5] VCAs limited viable ischemic time is a key hindrance to its broader application.[6]

The gold standard for organ preservation has been static cold storage (SCS) for almost 50 years.[7] The organ is flushed with a cold preservation solution (+4°C) via the arterial pedicle, then placed in media and put in an icebox. This easily implementable preservation method is based on static hypothermia reducing cell metabolism and therefore extending the viable storage duration. Although the metabolism is reduced by 10 to 12 fold at 4°C, it is not completely halted.[8] As VCAs contain muscles, a highly metabolic tissue that is sensitive to cold ischemia, they can endure SCS for a maximum of 6 to 8 hours before transplant.[9] An additional specific concern is the major immunogeneicity of the skin, is the most immunogenic tissue[10] and can be further compromised with ischemic injuries in VCA, thus increasing the risk of graft rejection substantially more than in solid organs.

The development of machine perfusion (MP) in solid organ preservation challenges the dogma of static preservation.[11–14] MP has proven to be a reliable preservation method for solid organs (liver, kidney, heart, and lungs) and recently entered the clinic.[11,15–17] Furthermore, MP grants the extension of the solid organ donor pool and is believed to be a potent tool in reducing ischemia-reperfusion injuries and the risk of graft rejection.[18] MP also allows for treatment of the organ with antiviral agents,[19] cells[20] or even gene therapies[21] before transplantation (Txp), while avoiding the toxicity for off-target sites. Finally, ex vivo perfused organs can be tested for viability and function while on the pump,[22] and repaired before Txp.[23,24]

MP of VCA has been tested in rodent,[25] swine,[26–29] and human limb models,[30] and can be considered in early phases given perfusions in other organs like liver have been done since early 20th century, and the very first transplants in the world actually used machine perfused kidneys.[31] Of note, using only dynamic preservation does not appear practical in the clinical setting, due to possible clinical limitations (expertise and equipment) at the point of procurement. An alternative, hybrid approach is to recover the graft and transport on ice in static cold storage, and then use MP to recover grafts before Txp, which has been a strategy successfully used in liver Txp.[32,33] Combining static and dynamic preservation methods could therefore strike a balance between clinical practicality and longer ex vivo preservation of VCAs.

In this study, we first determined the maximum SCS duration in a rodent hindlimb Txp model that allows successful Txp, which to our knowledge has not been done previously, and setting the baseline for the ex vivo storage limitations. We then introduced subnormothermic machine perfusion (SNMP) as an end-ischemic tool to recover grafts, and tested perfusion parameters as a function varying durations of SCS, identifying candidate protocols for in vivo testing. For validation, we performed Txp of rodent hindlimbs that were SCS preserved with SNMP recovery, compared to SCS controls, in an inbred transplant model which allowed us to focus only on ischemia-reperfusion injury in the absence of rejection.

Methods

Animals

Sixty inbred Lewis rats (250 ± 50 gram) were used for all experiments (Charles River Laboratories, Wilmington, MA). The animals received humane care in accordance with the National Research Council guidelines and the experimental protocols were approved by the IACUC of Massachusetts General Hospital (Boston, MA) and the Animal Care and Use Review Office.

Experimental Approach

The experiments were conducted in three phases: (1) determination of the limit of SCS duration allowing long-term VCA

viability in limb Txp model using an inbred rat population (SCS + Txp groups), (2) evaluation of the effect of 3-hour SNMP after select durations of SCS (SCS + SNMP groups), and (3) comparing clinical and histological outcomes in limb Txp after cold storage alone or MP treatment (SCS + Txp *vs.* SCS + SNMP + Txp groups), as displayed in ►**Fig. 1**. Since the focus here is ischemia reperfusion injury, to avoid immunological issues that can arise from allotransplantation, we used an inbred Lewis- > Lewis transplant model, which is a well-accepted rodent model of Txp with no immune rejection. In total, 60 animals were used (four donors and four recipients in each of the five groups of the SCS-Tx study = 40, four donors for each of the three groups of the SCS + SNMP studies = 12, four donors and four recipients in the SCS + SNMP + Tx study = 8).

Rat Hindlimb Procurement

Rats were anesthetized using isoflurane 5% inhalation with 100% O_2 for induction. General anesthesia was sustained with inhaled isoflurane (1–3%) and anesthesia depth was confirmed with a toe pinch test. The average surgical time was 30 minutes. We procured right partial hind limbs that included a skin paddle, the inguinal fat pad, muscles of the thigh and leg, 10 mm of proximal tibia and fibula, the knee joint, and 10 mm of the distal femur.[34] The femoral pedicle was isolated and ligated 5 minutes after IV administration of 100 IU/mL/kg heparin in the penile dorsal vein. A 24G angiocatheter was then introduced in the femoral artery and secured with an 8/0 nylon tie. The femoral vein was cut after ligation. The VCA was manually flushed with 2 mL (200 IU) of heparin saline at room

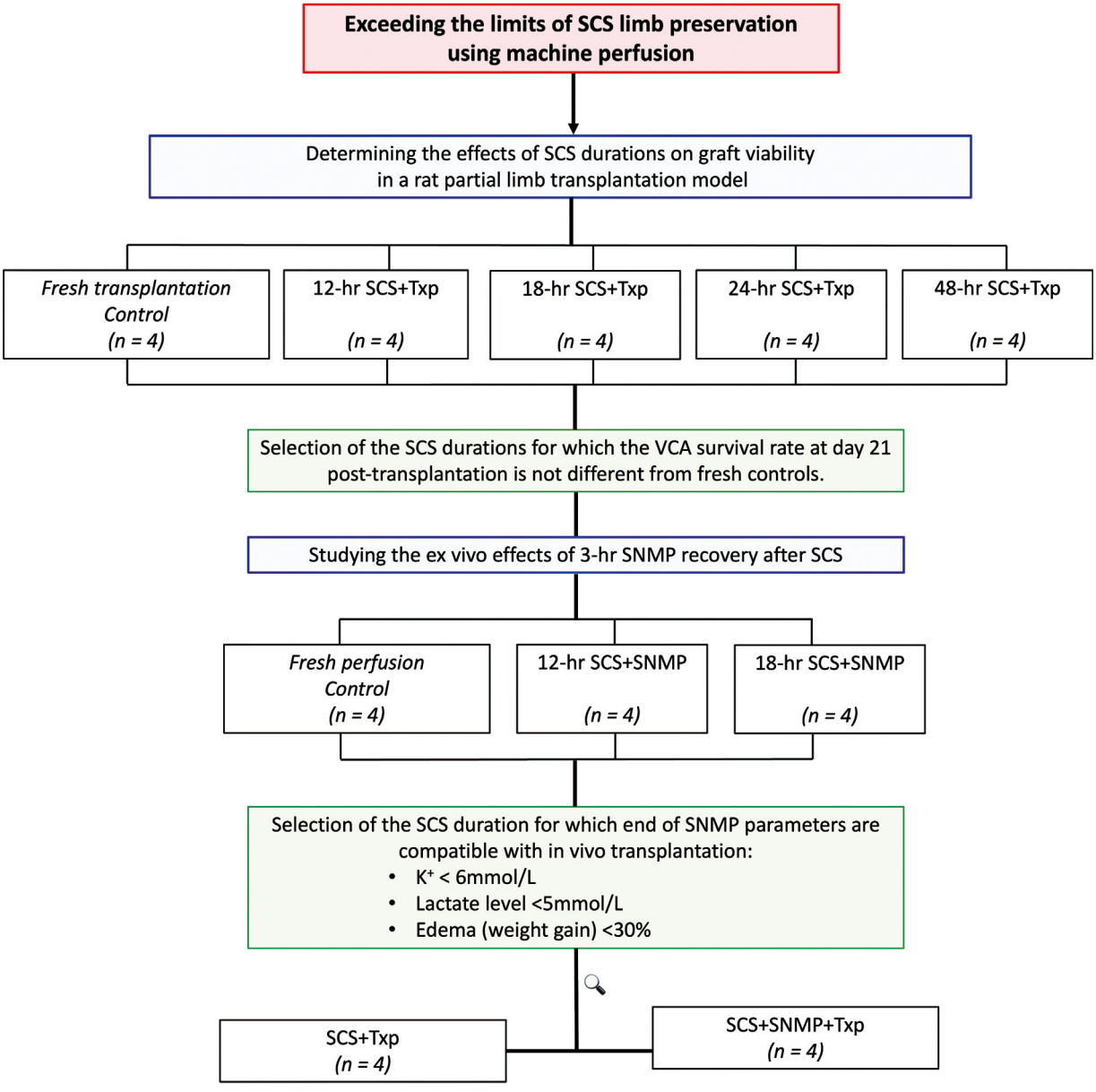

Fig. 1 Experimental design of the study. Sixty animals were used (four donors and four recipients in each of the five groups of the SCS + Txp study = 40, four donors for each of the three groups of the perfusion studies [SCS + SNMP] = 12, four donors and four recipients in the SCS + SNMP + Txp study = 8).

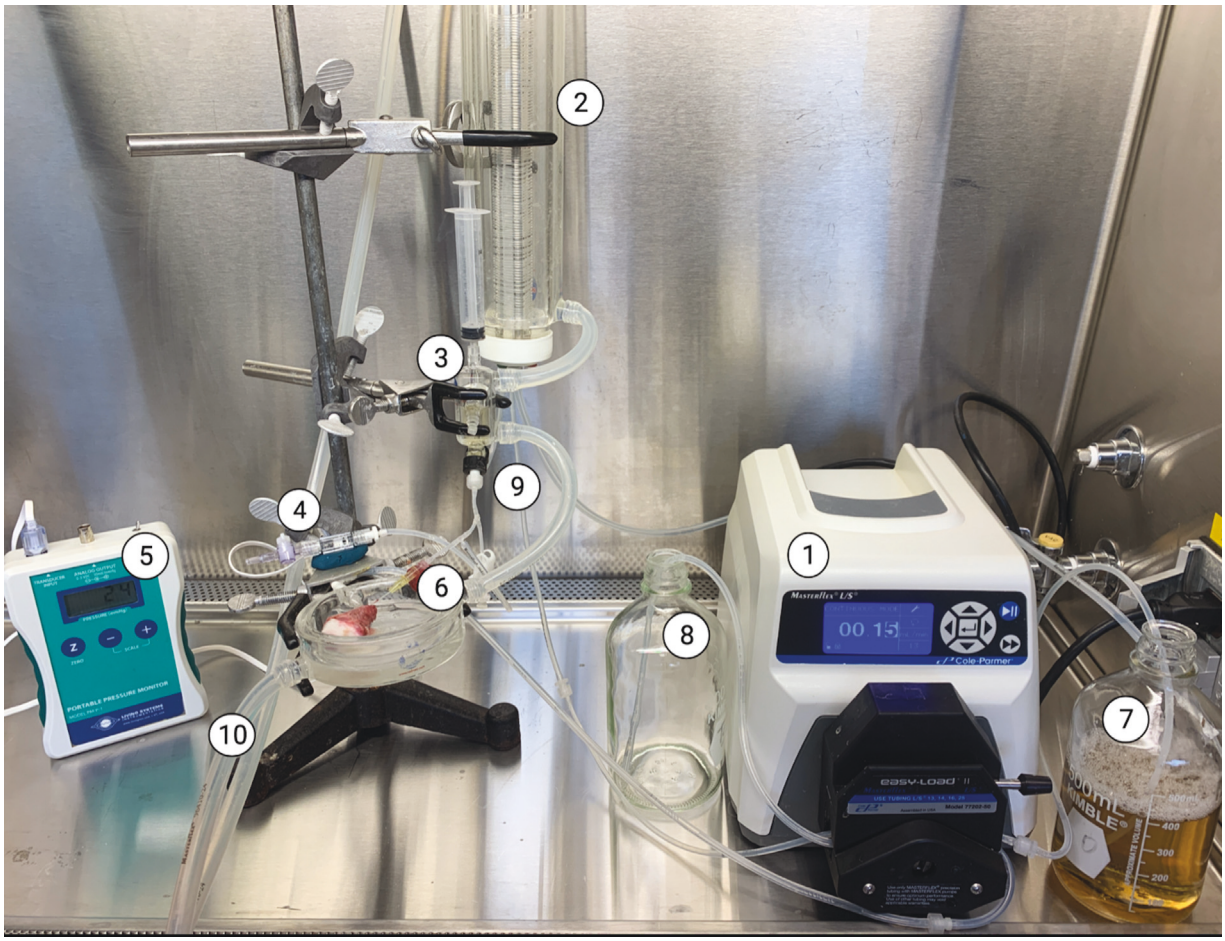

Fig. 2 Machine perfusion set-up for ex vivo rat limb SNMP. (1) Roller pump (Masterflex). (2) Radnoti glass oxygenator. (3) Radnoti bubble trap. (4) Pressure sensor. (5) Pressure monitor. (6) Radnoti organ basin containing a rat hindlimb. (7) Sterile perfusate (Steen +). (8) Empty sterile bottle for outflow drainage. (9) Oxygen inflow (0.5 L/min). (10) Heater tubing containing sterile water at 21°C and circulating in the jacketed components (organ basin, bubble trap, and oxygenator).

temperature and subjected to either SNMP immediately (SNMP only control groups), or cold stored as described below.

Static Cold Storage

Procured rat partial hindlimbs were manually flushed with 5 mL of 4°C Histidine-Tryptophan-Ketoglutarate (HTK) solution at a mean flow rate of 1.5 mL/min. The limbs were weighed and immersed in a sterile bag containing 50 mL of 4°C HTK solution. Limbs were then cold stored in a +4°C refrigerator for different durations (12, 18, 24, or 48 hours) after which they were weighed and subjected to sub-normothermic perfusion or Txp.

Perfusate Composition: Modified Steen Solution (Steen +)

We used a perfusion solution based on the composition of the original Steen solution (►**Supplementary Material 1** for rationale, available in the online version only). We similarly recreated an acellular solution containing dextran and electrolytes. In place of the human serum albumin in the Steen solution, we used bovine serum albumin and added polyethylene glycol-35. Heparin (2,000 IU/L), insulin (20 IU/L), dexamethasone (16 mg/L), hydrocortisone (200 mg/L), and penicillin–streptomycin (4mL/L) were then mixed to the solution. The solution was sterile filtered and stored at +4°C.

200 mL of modified Steen solution (designated as Steen+ in figures for brevity) were used for each perfusion experiment.

Machine Perfusion Set Up and Priming

Our MP system (►**Fig. 2**) was built up in a sterile hood and composed of a roller pump (07522-20 DRIVE MFLEX L/S 600RPM 115/230, Cole-Parmer, Vernon Hills, IL), a heater (Polystat Cooling/Heating Circulating Bath, Cole-Parmer) set at 21°C, inflow and outflow tubing (Masterflex platinum-cured silicone tubing, L/S 13, Cole-Parmer, Vernon Hills, IL), a jacketed membrane oxygenating chamber, bubble trap, and tissue bath (catalog numbers 130144, 130149 and 158400 respectively, Radnoti LTD, Covina, CA). A pressure transducer (PT-F, Living Systems Instrumentation, St Albans City, VT) was connected close to the Angio catheter (BD Angiocath 24G) that is inserted in the femoral artery during perfusion. The inflow tubing was put in a 200 mL sterile bottle of our

Steen+ solution and the perfusion pump was initiated, the outflow was drained in an empty sterile 200 mL bottle.

The pressure transducer was connected to a dummy 24G angiocatheter for flowrates from 0.1 mL/min to 1 mL/min. Oxygen input was started with a flow of carbogen gas to the oxygenator at 0.2 to 0.5 L/min. Once the perfusate was warmed to 21°C and oxygenated, oxygenation and pH of the solution were verified on the i-STAT blood gas analysis machine (Abbott, Princeton, NJ), and $NaHCO_3$ titration was performed to correct for acidosis if needed.

The perfusion was initiated at a flow rate of 0.1 mL/min, and flowrates were adjusted to keep arterial pressure between 30 and 50 mm Hg.

Perfusion Parameters Measured

Once started, perfusion parameters (vascular resistance, flow, and pressure) were recorded continuously; flowrate was manually adjusted on the roller pump to reach a target pressure ranging from 30 to 50 mm Hg. Blood gas and electrolytes in the inflow and outflow perfusate were measured at 1, 2, and 3 hours using the i-STAT machine. Oxygen consumption was calculated using a modified Fick equation using circuit flow, limb weight, and pre- and post-limb oxygen contents. Weight gain was measured at the end of perfusion.

Heterotopic Partial Hind Limb Txp

Twenty-four inbred Lewis rats were used as recipients for Txp, to avoid any immunological interference in graft survival and ischemia reperfusion injuries. The rats were anesthetized using isoflurane 5% inhalation with 100% O_2 for induction. General anesthesia was sustained with inhaled isoflurane (1–3%) and anesthesia depth was confirmed with a toe pinch test. The recipient rat was then prepared for microvascular transfer of the partial hindlimb. The graft was transplanted in a heterotopic fashion on the left flank of the recipient rat as previously described by our group.[34] Postoperative care and analgesia were performed in accordance with the IACUC guidelines in place at Massachusetts General Hospital. During the study period, daily physical examination was performed, and graft pictures taken to ensure clinical evaluation of the animal and graft viability. The presence of epidermolysis on the graft's skin and hair regrowth was evaluated daily. The animal was euthanized in case of self-mutilation (excluded from the study) or vascular failure of the transplanted limb. End of study was performed 21 days post-transplantation and viability of the graft was assessed by a patency test on the graft vascular pedicle. Aspect of the skin and muscle was clinically assessed, and punch biopsies were taken for further analyses.

Histology

For all recipient rats, a 3-mm punch biopsy of the graft including skin and muscle was performed at the end of study. Skin and muscle biopsies were fixed in formalin and processed for routine histopathological examination. Slides were stained with hematoxylin and eosin (H&E) and Masson's Trichrome. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling ; Abcam TUNEL assay kit, 206386) staining was also performed. A blinded evaluation by a pathologist was performed for all biopsy samples and using the Histology Injury Scoring System (HISS)[28] for hypoxia-induced muscle injury, i.e., preservation of muscle architecture, myocyte injury, and apoptosis.

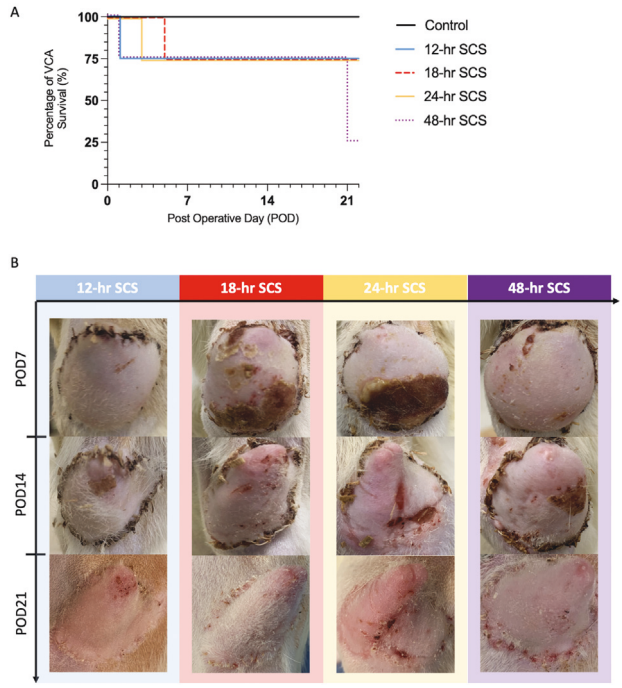

Fig. 3 (**A**) Changes in VCA survival after 0 (control), 12, 18, 24, and 48-hour SCS ($n = 4$ in all groups). After 12, 18, or 24-hours of SCS, similar VCA survival percentages were obtained when compared to control transplantations ($p > 0.05$). VCA failure was found significantly increased at the end of study in the 48-hour SCS group ($p = 0.0032$). (**B**) Post-transplant clinical evolution after 12, 18, 24, and 48-hour SCS. Epidermolysis leaves scarring tissue in the 24 and 48-hour SCS groups, preventing hair regrowth. VCA, vascularized composite allograft.

Clinical Evaluation

A blind grading of postoperative clinical pictures (at postoperative day [POD] 7, 14, 21) was performed by an independent investigator using 0 for no visible skin lesion, 1 for mild epidermolysis (<1/3 of the VCA area), 2 for moderate epidermolysis (>1/3 and <2/3 of the VCA area), and 3 for severe lesions (>2/3 of the VCA area or deep necrosis). Hair regrowth was evaluated at total (0), partial (1) or absent (2), giving an overall clinical score out of 5.

Statistical Analysis

Continuous data are reported as means with standard deviations. A log rank Mantel-Cox test was used to compare VCA survival curves. Differences in perfusion parameters between groups were analyzed using unpaired *t*-tests with Tukey's multiple comparisons correction, or using a mixed-effect analysis when necessary. Comparison of clinical scores was made with multiple unpaired *t*-tests similarly

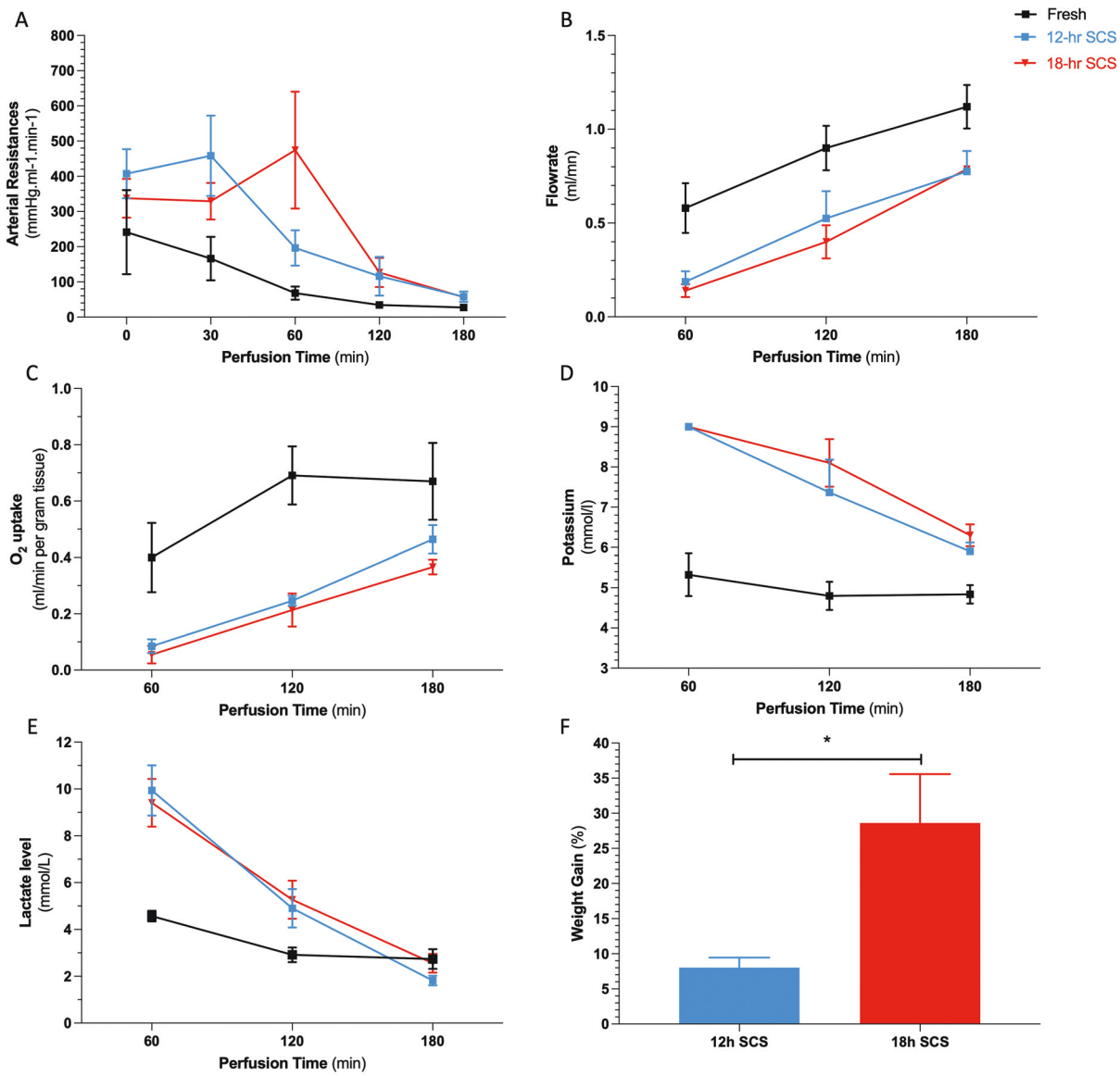

Fig. 4 Post-static cold storage perfusion parameters during a 3-hour SNMP using our modified Steen solution (n = 4 in each group). Panel (**A**) shows high initial resistances after cold storage decreasing after 30 minutes (group 12-hour SCS) and 60 minutes in the 18-hour SCS group. Panel (**B**) shows a steady increase of the flowrate reaching 0.8 mL/min at the end of the recovery perfusion. Oxygen uptake (panel **C**) is lower after a period of SCS compared to fresh control. Potassium and lactate levels (**D, E**) steadily decreased during perfusion. Potassium release was significantly higher in the 12 ($p = 0.003$) and 18 hours SCS group ($p = 0.0002$). Edema (**F**) was significantly higher in the 18-hour SCS group compared to 12-hour SCS ($p = 0.0238$).

with Tukey corrections. All statistical analyses were performed using Prism 9 for Mac OSX (GraphPad Software, La Jolla, CA). *p*-Values less than 0.05 were considered to be significant.

Results

VCA Transplantation after Extended Durations of SCS (12, 18, 24, and 48-Hour SCS + Txp)

As shown in ►**Fig. 3A**, when stored in SCS for 12 hours, transplanted limbs showed 75% survival at 21 days post-transplantation. However, partial epidermolysis occurred in all cases starting between POD 3 and 8. Hair started growing back on a portion of the graft at POD14 (►**Fig. 3B**). Graft survival rate was found similar in the 12, 18, and 24-hour SCS + Txp groups ($p > 0.05$). One visual difference compared to the 12-hour group observed was that in the 18- and 24-hour SCS + Txp groups, as well as in the 48-hour SCS + Txp group, epidermolysis involved most of the skin surface of the graft and no hair regrowth was observed before POD14 (►**Fig. 3B**). In the 48-hour SCS + Txp group, one rat died a few hours after the surgery, the others survived until POD21 when the assessment of the graft viability was performed. Only in this group, two of the grafts did not have a positive patency test on the femoral vein which was found flat, and no outflow or thrombosis was observed even though clinical signs did not predict a vascular failure. Despite good arterial inflow to the graft, the muscles showed necrotic involution.

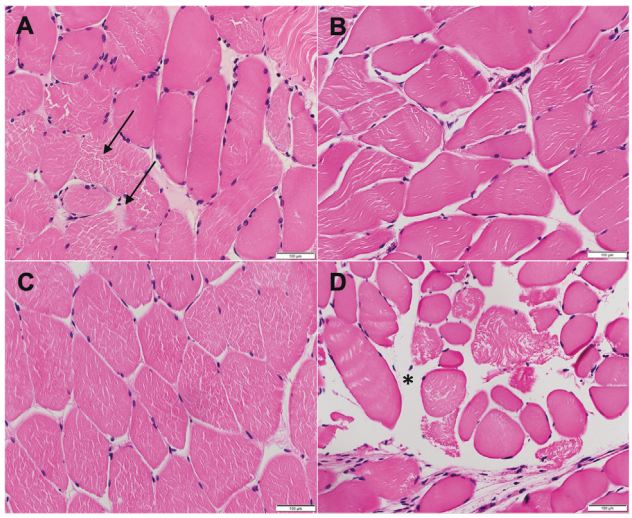

Fig. 5. Pathologic evaluation of muscle biopsies. Muscle biopsies after SCS, before and after SNMP (H&E, 400x). Focal myocyte necrosis (*black arrows*) was present after 12-hour SCS (**A**) and mild edema at 3-hour SNMP (**B**). After 18-hour SCS alone (**C**), there was diffuse myocyte breakdown with extensive myocyte injury and interstitial edema (*asterisk*) after 3-hour SNMP (**D**).

Those findings were unique to the 48-hour SCS + Txp group and consistent with delayed graft failure. The survival rate in the 48-hour SCS + Txp group (►**Fig. 3A**) was significantly lower than in other groups ($p = 0.0032$).

VCA Ex Vivo Perfusion after 12 and 18-Hour SCS: the SCS + SNMP Study

Based on the clinical outcomes, especially the extent of epidermolysis after 24-hour SCS + Txp, the 24- and 48-hour SCS groups were excluded from further studies. Hence, a 3-hour SNMP recovery phase was performed after 12- and 18-hour SCS.

Perfusion Parameters Post-Cold Storage

Arterial resistances slightly increased during the first 30 minutes of perfusion in the 12-hour SCS + SNMP group and peaked at 1 hour in the 18-hour SCS + SNMP group (►**Fig. 4A**). In both groups, resistances then decreased to reach a similar value as the control group at the end of the 3-hour SNMP ($p > 0.05$ between all groups, using a mixed effect analysis). Flowrates rose linearly in all groups, up to 0.8 mL/min in SCS groups over the duration of perfusion (►**Fig. 4B**). Oxygen uptake in the 12- and 18-hour SCS + SNMP groups was significantly lower than in the control limbs ($p = 0.0396$ and $p = 0.0283$, respectively, using a mixed-effect analysis), although they displayed rapid increase over time and reached levels close to the fresh grafts by 3 hours (►**Fig. 4C**). Potassium and lactate levels decreased continuously during the 3-hour SNMP (►**Fig. 4D, E**). All cold stored limbs showed similar lactate levels when compared to the control at the end of perfusion ($p = 0.2518$). After 3-hour SNMP, weight gain was significantly higher in the 18 hour-SCS + SNMP group compared to the 12-hour SCS + SNMP ($p = 0.0238$) group (►**Fig. 4F**).

Histological Analysis after SCS and SNMP

H&E staining of muscle biopsies obtained after 12 and 18 hours of SCS revealed increased ischemic lesions with SCS durations (►**Fig. 5A, C**), represented by myocyte breakdown. After SNMP recovery, interstitial edema remained mild in the 12-hour SCS + SNMP groups (►**Fig. 5B**) but was severe with diffuse myocyte necrosis in the 18-hour SCS + SNMP group (►**Fig. 5D**).

VCA Transplantation after 12-hour SCS and 3-hour SNMP (SCS + Txp vs. SCS + SNMP + Txp)

Based on the perfusion parameters described above as well as the pathology findings, 12-hour SCS was selected for Txp after a recovery phase of 3-hour SNMP. Clinical and histological results were compared with limb Txps after 12-hour SCS (control group).

Clinical Outcomes

Compared to 12-hour SCS + Txp alone, graft recovered with SNMP showed faster recovery from ischemic lesions. In both groups, the success rate was 75%, and partial epidermolysis was observed on all transplanted limbs. In the control group, ischemic skin lesions were noted on all grafts. One displayed partial superficial skin necrosis with persistent scarring lesions at POD21 (►**Fig. 6A**). In two of the transplants, lesions were limited but appeared at POD14 and were not entirely healed by POD21 (►**Fig. 6B,C**). In the experimental group, one transplant presented mild, very limited epidermolysis that healed rapidly with total hair regrowth by POD14 (►**Fig. 6D**). At POD7, two grafts in the 12-hour SCS + SNMP + Txp group showed partial superficial skin necrosis (►**Fig. 6E,F**); both were completely healed by POD14 with partial hair regrowth (one presented with linear lesions consistent with scabbing, ►**Fig. 6E**). Scarce scarring lesions were visible on one of the grafts in the SCS + SNMP + Txp group at day 21, these lesions appeared more severe and on all grafts in the control group, with a worse score in the control group ($p = 0.016$) as displayed in ►**Table 1**.

Histological Analysis

Pathologic evaluation of the skin at POD 21 revealed focal necrosis in one sample in the control group (►**Fig. 7A**). The 12-hour SCS + SNMP + Txp group showed no dermal or epidermal lesions. Evaluation of the muscle demonstrated focal ischemic changes, diffuse atrophy, and focal apoptosis in the control group (►**Fig. 7C**). The hypoxia-induced muscle injury scores of all biopsies from the SNMP recovery group were low, representing no or minimal degeneration (►**Table 2**).

Discussion

This study outlines the impact of extended SCS duration on VCA survival through clinical and histological assessment. In this rat hind limb Txp model, grafts remained viable 3 weeks post-transplantation when preserved for up to 24 hours in SCS. This 24-hour duration represents the longest a rat VCA can be preserved with the gold standard method. However, even though graft survival is not significantly diminished in

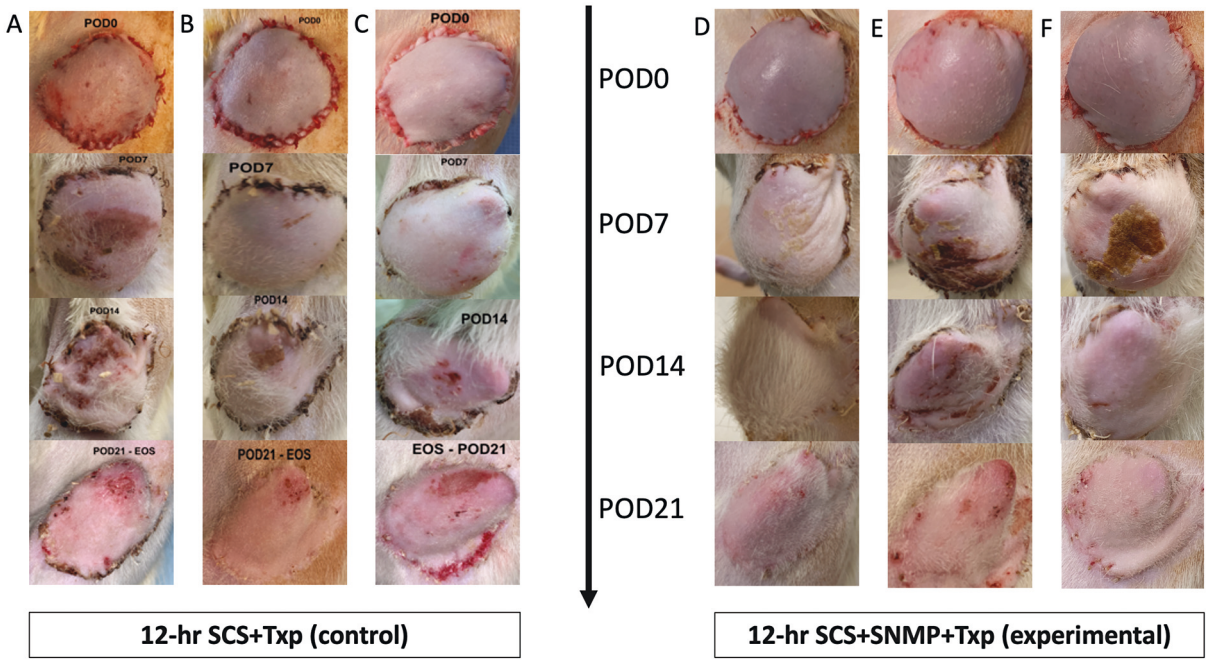

Fig. 6 (A–F) Clinical outcomes. Recovering limbs using SNMP enhances graft quality and accelerate the healing of skin lesions due to extended cold ischemia.

Table 1 Blind clinical evaluation of postoperative pictures

Time	12-h SCS + Txp Mean (±SD)	12-h SCS + SNMP + Txp Mean (±SD)	*p*-Value
POD7	*2 (±1)*	*2 (±1)*	*>0.999999*
POD14	*2.333 (±0.577)*	*1.667 (±1.155)*	*0.421648*
POD21	*2 (±0)*	*0.667 (±0.557)*	***0.016130***

Note: Grading was performed as follows: 0 for no visible skin lesion, 1 for mild epidermolysis (<1/3 of the VCA area), 2 for moderate epidermolysis (>1/3 and <2/3 of the VCA area) and 3 for severe lesions (>2/3 of the VCA area or deep necrosis). Hair regrowth was evaluated at total (0), partial (1) or absent (2). The overall clinical score is out of 5. Better clinical outcomes are found in the 12-hour SCS + SNMP + Txp group at POD 21 ($p = 0.016130$).

this case, our results indicate the quality of the tissues is greatly altered with increasing durations of cold storage. This sets the ground for the need of a novel enhanced 24-hour VCA preservation method that would result in "fresh graft" quality. As recently described in the literature, 6-hour SCS VCA preservation already triggers hypoxia-induced injuries to the muscle in rodent limb Txp.[35] A similar trend in post-SCS limb transplant survival was reported by Arai et al when transplanting cold stored grafts in Euro-Collins solution for 6 to 72 hours.[36] Another concern with SCS is raised by Datta et al who suggest that ischemia-reperfusion induces an inflammatory cascade leading to a systemic vascular endothelial damage. Their study hypothesizes that this reaction observed in the 6-hour cold stored VCA recipients may lead to remote organ dysfunction after Txp.[37] Although the post-transplantation systemic response was not investigated in our study, we did not observe any poor condition of the recipient animal after Txp in all groups.

In development of a new method for organ preservation, the key goals are increasing the duration of preservation and improving the organ's function post-transplantation. Both problems have been addressed in liver preservation using a

Table 2 Pathological evaluation using the HISS[31] for hypoxia-induced muscle injury on biopsies taken POD 21

	Interstitial edema	Inflammation	Myocytes size variation	Myocytes damage	Total
12-h SCS + Txp	1	0	1	1	3
	1	0	3	3	7
	1	0	1	1	3
12-h SCS+ SNMP + Txp	0	0	1	2	3
	1	0	1	1	3
	2	0	0	0	2

Abbreviations: SCS, static cold storage; SNMP, subnormothermic machine perfusion.
Note: All total scores from the SNMP recovery group indicated no or minimal degeneration.

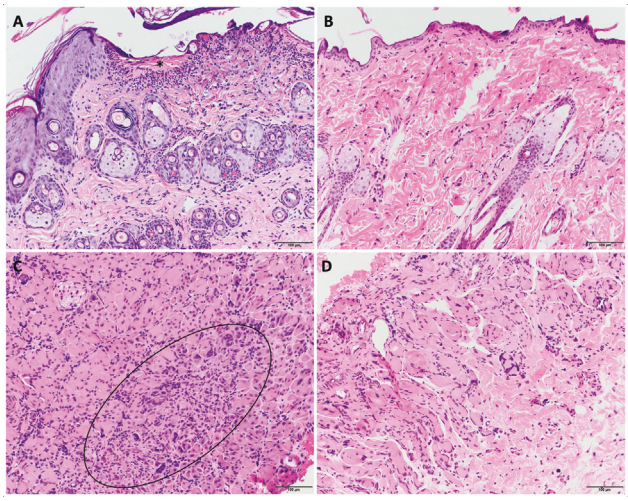

Fig. 7 Pathologic evaluation of skin and muscle biopsies at POD21 (H&E, 400x). In the 12-hour SCS group, the epidermis (**A**) showed focal necrosis and ulceration (*) while the muscle (**C**) showed myocyte injury and atrophy (*encircled*). The 3-hour SNMP group showed normal skin (**B**) while the muscle biopsy (**D**) showed mild interstitial edema.

combination of cold storage followed by a recovery phase with ex vivo MP protocols.[33,38] Bruinsma et al studied the impact of a 3-hour subnormothermic perfusion post-SCS in rat livers[38] and noted rising vascular resistances with longer SCS durations, which was likely due to endothelial dysfunction. Our experiments similarly revealed a peak in resistance up to 1-hour post-reperfusion in the 18-hour SCS + SNMP group; however, our SNMP protocol seemed to allow the graft to recover from cold ischemic injury at least partially, with resistance dropping to the level of no-SCS limb perfusions. Applying 3-hour SNMP after SCS also enabled graft rewarming as well as flushing of accumulated toxic metabolites, reducing the outflow of lactate and potassium levels to the same levels as those in fresh grafts. Nonetheless, the SCS limit for SNMP recovery was set at 12 hours in our study, time after which edema increased drastically throughout the ex vivo perfusion experiment. Notably, the lower oxygen uptake observed during SNMP after 12- and 18-hour SCS represents a possible practical marker of injury to evaluate perfused limb grafts. Reperfusion injuries such as major interstitial edema and myocyte damage were observed on histologic examination of muscle biopsies after 18-hour SCS + SNMP, suggesting that reperfusing the 18-hour SCS graft worsened already existing cold ischemic lesions. Following our perfusion metrics, our SNMP protocol managed to recover our 12-hour SCS grafts, but was not sufficient for 18-hour SCS limbs that developed significant reperfusion injuries.

These ex vivo results were confirmed during the in vivo part of the studies. Applying a 3-hour SNMP recovery phase after 12-hour limb SCS eventually led to improved graft quality at 3 weeks post-transplant. In an orthotopic liver Txp model in rats, Bruinsma et al reported a doubled survival rate (from 50 to 100%) after Txp when applying a SNMP recovery phase on livers that has been subjected to 48 hours of SCS.[38] Although our model did not demonstrate a significant difference in graft survival when using SNMP recovery, this group ultimately resulted in less muscle and skin injuries from a clinical and histological standpoint. These findings are overall consistent with previous rat liver ex vivo SNMP studies.[38–40] Better clinical outcomes were also reported by Van Rijn et al in human liver preservation when performing a 2-hour hypothermic perfusion after 2-hour SCS before Txp compared to 2-hour SCS alone.[33] In renal Txp, hypothermic perfusion succeeded to achieve improved graft function after longer cold ischemic time.[41]

In search for an organ revival strategy, a wide range of different parameters has been used with MP, and shown success. The most important concerns when building an MP are the size of the organ, the type of perfusate, the duration of perfusion and the temperature. All those factors greatly influence the transportability, immediate readiness, and cost-effectiveness of the device. Our priority was to adjust such a device to rat limb perfusion, that can serve for many different experiments, from short-time hypo to normothermic ex vivo perfusion to the loading of cryopreservative agents for supercooling and partial freezing technologies. The presence of an accurate temperature control system on our MP was a key element. Recent studies have shown numerous advantages to using subnormothermic temperature.[42,43] It is a compromise between full metabolism reached at normothermic temperature to low organ metabolism when hypothermic. Normothermic perfusion brings challenges in perfusate composition as it needs to deliver enough nutrients for full metabolism thus requiring added oxygen sources (oxygen carrier and/or blood cells). Hypothermic perfusion has proven efficient with acellular perfusates but is still hindered by the edema observed in prolonged ex vivo organ preservation below 10°C. The choice of a subnormothermic perfusion temperature coupled with the use of an optimized perfusion solution allowed to address the metabolic demand, and avoid massive edema formation and its subsequent tissue lesions in VCA, as previously demonstrated in rat livers experiments.[40]

To date, limited data are available on organ resuscitation after SCS using ex vivo perfusion. However, all seem to lead to favorable outcomes for MP post-SCS, especially in terms of organ function. To our knowledge, this report is the first to suggest VCA perfusion recovery post-SCS, and showing improved graft quality in Txp. One of the limits of our study is the size of the graft. In our study, rat partial hindlimbs weighed between 10 and 20 g, a massive difference of size and weight would obviously bring more challenges to the resuscitation of larger VCAs like human tissues. We can expect that the rewarming of the graft from +4 to +21°C would be longer to achieve in human sized graft. Additionally, the volume of a rat limb makes it more likely to get gas exchanges at its surface and supplementary tissue oxygenation, which would be harder to reach in larger VCA. The key limitation of our study is that our surgical model does not allow for functional assessment as our graft is heterotopic and nonfunctional. Further studies and assessments should be later undertaken to better discern the muscular outcomes that could be found when reinnervation is completed. Testing this new perfusion protocol in orthotopically transplanted limbs[44] is therefore an obvious next step.

Conclusion

With the recent developments in organ preservation technologies for solid organs, it is natural to expect a dramatic change in the field of VCA Txp in coming decade as well. Our study suggests that using MP for VCA recovery would permit using grafts that have been cold stored for more than the current 6-hour limitation. Further investigations are merited with our SNMP protocol as well as others, with the next challenges coming in scale up and shifting the focus to more damaging warm ischemic injury which has been a major bottleneck in expanding utilization in solid organ transplants.

Note

The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense.

Funding

This work was supported by the Office of Assistant Secretary of Defense for Health Affairs, through the Reconstructive Transplant Research Program, Technology Development Award under Awards No. W81XWH-17-1-0680 and W81XWH-19-1-0440. This material is partially based upon work supported by the National Science Foundation under Grant No. EEC 1941543.Partial support from the US National Institutes of Health (R01EB028782) is gratefully acknowledged. Funding from the Shriners Children's is gratefully acknowledged, including the award 986579 to KU, and the support by Shriners Special Shared Facilities that enabled this work.

Conflict of Interest

The authors declare competing interests. Drs. A.G.L., C.L.C., K.U., S.N.T., R.J.d.V., and C.A.P. have provisional patent applications relevant to this study. K.U. and S.N.T. have 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.

Acknowledgments

The authors thank the Shriners Boston Morphology and Imaging Core, Genomics and Proteomics Core, and the Translational Regenerative Medicine Special Shared Facility for access to equipment and expertise.

References

1 Pribaz JJ, Caterson EJ. Evolution and limitations of conventional autologous reconstruction of the head and neck. J Craniofac Surg 2013;24(01):99–107
2 Diaz-Siso JR, Bueno EM, Sisk GC, Marty FM, Pomahac B, Tullius SG. Vascularized composite tissue allotransplantation–state of the art. Clin Transplant 2013;27(03):330–337
3 Cherikh WS, Cendales LC, Wholley CL, et al. Vascularized composite allotransplantation in the United States: a descriptive analysis of the organ procurement and transplantation network data. Am J Transplant 2019;19(03):865–875
4 Wainright JL, Wholley CL, Cherikh WS, Musick JM, Klassen DK. OPTN vascularized composite allograft waiting list: current status and trends in the United States. Transplantation 2018;102(11):1885–1890
5 Mendenhall SD, Brown S, Ben-Amotz O, Neumeister MW, Levin LS. Building a hand and upper extremity transplantation program: lessons learned from the first 20 years of vascularized composite allotransplantation. Hand (N Y) 2020;15(02): 224–233
6 Giwa S, Lewis JK, Alvarez L, et al. The promise of organ and tissue preservation to transform medicine. Nat Biotechnol 2017;35(06): 530–542
7 McAnulty JF. Hypothermic organ preservation by static storage methods: current status and a view to the future. Cryobiology 2010;60(Suppl 3):S13–S19
8 Southard JH, Belzer FO. Organ preservation. Annu Rev Med 1995; 46:235–247
9 Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg 2002;10 (06):620–630
10 Lee WP, Yaremchuk MJ, Pan YC, Randolph MA, Tan CM, Weiland AJ. Relative antigenicity of components of a vascularized limb allograft. Plast Reconstr Surg 1991;87(03):401–411
11 Deng R, Gu G, Wang D, et al. Machine perfusion versus cold storage of kidneys derived from donation after cardiac death: a meta-analysis. PLoS One 2013;8(03):e56368
12 O'Callaghan JM, Morgan RD, Knight SR, Morris PJ. Systematic review and meta-analysis of hypothermic machine perfusion versus static cold storage of kidney allografts on transplant outcomes. Br J Surg 2013;100(08):991–1001
13 Hameed AM, Pleass HC, Wong G, Hawthorne WJ. Maximizing kidneys for transplantation using machine perfusion: from the past to the future: a comprehensive systematic review and meta-analysis. Medicine (Baltimore) 2016;95(40):e5083
14 Tingle SJ, Figueiredo RS, Moir JA, Goodfellow M, Talbot D, Wilson CH. Machine perfusion preservation versus static cold storage for deceased donor kidney transplantation. Cochrane Database Syst Rev 2019;3:CD011671
15 Hosgood SA, Nicholson ML. First in man renal transplantation after ex vivo normothermic perfusion. Transplantation 2011;92 (07):735–738
16 Koerner MM, Ghodsizad A, Schulz U, El Banayosy A, Koerfer R, Tenderich G. Normothermic ex vivo allograft blood perfusion in clinical heart transplantation. Heart Surg Forum 2014;17(03):E141–E145
17 Dutkowski P, Polak WG, Muiesan P, et al. First comparison of hypothermic oxygenated perfusion versus static cold storage of human donation after cardiac death liver transplants: an international-matched case analysis. Ann Surg 2015;262(05):764–770, discussion 770–771
18 Samoylova ML, Nash A, Kuchibhatla M, Barbas AS, Brennan TV. Machine perfusion of donor kidneys may reduce graft rejection. Clin Transplant 2019;33(10):e13716
19 Cypel M, Feld JJ, Galasso M, et al. Prevention of viral transmission during lung transplantation with hepatitis C-viraemic donors: an open-label, single-centre, pilot trial. Lancet Respir Med 2020;8 (02):192–201
20 Chin LY, Carroll C, Raigani S, et al. Ex vivo perfusion-based engraftment of genetically engineered cell sensors into transplantable organs. PLoS One 2019;14(12):e0225222
21 Bishawi M, Roan JN, Milano CA, et al. A normothermic ex vivo organ perfusion delivery method for cardiac transplantation gene therapy. Sci Rep 2019;9(01):8029
22 Renne J, Gutberlet M, Voskrebenzev A, et al. Functional pulmonary magnetic resonance imaging for detection of ischemic injury

in a porcine ex-vivo lung perfusion system prior to transplantation. Acad Radiol 2019;26(02):170–178
23 Lonati C, Battistin M, Dondossola DE, et al. NDP-MSH treatment recovers marginal lungs during ex vivo lung perfusion (EVLP). Peptides 2021;141:170552
24 Wong A, Zamel R, Yeung J, et al. Potential therapeutic targets for lung repair during human *ex vivo* lung perfusion. Eur Respir J 2020;55(04):1902222
25 Gok E, Alghanem F, Moon R, et al. Development of an ex-situ limb perfusion system for a rodent model. ASAIO J 2019;65(02):167–172
26 Duraes EFR, Madajka M, Frautschi R, et al. Developing a protocol for normothermic ex-situ limb perfusion. Microsurgery 2018;38 (02):185–194
27 Fahradyan V, Said SA, Ordenana C, et al. Extended ex vivo normothermic perfusion for preservation of vascularized composite allografts. Artif Organs 2020;44(08):846–855
28 Kruit AS, Brouwers K, van Midden D, et al. Successful 18-h acellular extracorporeal perfusion and replantation of porcine limbs—histology versus nerve stimulation. Transpl Int 2021;34(02):365–375
29 Ozer K, Rojas-Pena A, Mendias CL, Bryner B, Toomasian C, Bartlett RH. Ex situ limb perfusion system to extend vascularized composite tissue allograft survival in swine. Transplantation 2015;99 (10):2095–2101
30 Werner NL, Alghanem F, Rakestraw SL, et al. Ex situ perfusion of human limb allografts for 24 hours. Transplantation 2017;101 (03):e68–e74
31 Belzer FO, Ashby BS, Gulyassy PF, Powell M. Successful seventeen-hour preservation and transplantation of human-cadaver kidney. N Engl J Med 1968;278(11):608–610
32 Dutkowski P, Schlegel A, de Oliveira M, Müllhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol 2014;60(04):765–772
33 van Rijn R, Schurink IJ, de Vries Y, et al; DHOPE-DCD Trial Investigators. Hypothermic machine perfusion in liver transplantation - a randomized trial. N Engl J Med 2021;384(15):1391–1401
34 Goutard M, Randolph MA, Taveau CB, et al. Partial heterotopic hindlimb transplantation model in rats. J Vis Exp 2021;(172):
35 Gok E, Kubiak CA, Guy E, Kemp SWP, Ozer K. Effect of static cold storage on skeletal muscle after vascularized composite tissue allotransplantation. J Reconstr Microsurg 2020;36(01): 9–15
36 Arai K, Hotokebuchi T, Miyahara H, et al. Successful long-term storage of rat limbs. The use of simple immersion in Euro-Collins solution. Int Orthop 1993;17(06):389–396
37 Datta N, Devaney SG, Busuttil RW, Azari K, Kupiec-Weglinski JW. Prolonged cold ischemia time results in local and remote organ dysfunction in a murine model of vascularized composite transplantation. Am J Transplant 2017;17(10): 2572–2579
38 Bruinsma BG, Berendsen TA, Izamis ML, Yarmush ML, Uygun K. Determination and extension of the limits to static cold storage using subnormothermic machine perfusion. Int J Artif Organs 2013;36(11):775–780
39 Berendsen TA, Bruinsma BG, Lee J, et al. A simplified subnormothermic machine perfusion system restores ischemically damaged liver grafts in a rat model of orthotopic liver transplantation. Transplant Res 2012;1(01):6
40 Tolboom H, Izamis ML, Sharma N, et al. Subnormothermic machine perfusion at both 20°C and 30°C recovers ischemic rat livers for successful transplantation. J Surg Res 2012;175(01): 149–156
41 Guy A, McGrogan D, Inston N, Ready A. Hypothermic machine perfusion permits extended cold ischemia times with improved early graft function. Exp Clin Transplant 2015;13(02): 130–137
42 Arni S, Maeyashiki T, Citak N, Opitz I, Inci I. Subnormothermic ex vivo lung perfusion temperature improves graft preservation in lung transplantation. Cells 2021;10(04):748
43 Arni S, Maeyashiki T, Opitz I, Inci I. Subnormothermic ex vivo lung perfusion attenuates ischemia reperfusion injury from donation after circulatory death donors. PLoS One 2021;16(08): e0255155
44 Sucher R, Oberhuber R, Margreiter C, et al. Orthotopic hind-limb transplantation in rats. J Vis Exp 2010;(41):2022