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Review

EX-VIVO LIMB PERFUSION

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Summary

The current gold standard of VCA preservation is static cold storage (SCS), which limits ischemia time to 12 hours for large extremity segments. The current literature provides supportive evidence for the introduction of machine preservation of extremities as an alternative to SCS with potential for translation into clinical practice. The goal of this review is to provide a comprehensive qualitative analysis of published literature on machine perfusion of limbs. We present elements of circuit design, use of animal models, perfusate solutions, temperature and oxygenation during perfusion, strategies to prevent muscle damage, methods to assess viability, and the future of *ex-vivo* limb perfusion. The future of machine preservation research focuses on the application of *ex-vivo* perfusion in scenarios where limb reconditioning or testing for viability prior to transplantation or replantation are warranted.

Key words: machine perfusion, *ex-vivo* normothermic perfusion, hand transplantation, extremity replantation, *ex-situ* perfusion, limb perfusion

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BACKGROUND

Delorme et al. [1] conducted the first known *ex-vivo* extremity perfusion of human lower limbs in 1964. Six lower extremities were perfused using autologous blood. Normal force, duration and of muscle contraction with direct electrical were reported. However, the concept of machine perfusion gained little traction in the 1960s [2] due to the relative simplicity, low cost, and efficacy of limb preservation by cooling. In the last decade, refueling of interest for machine preservation in solid organs, as a mean to increase the number of organs for transplantation, has also inspired research in *ex-vivo* preservation of limbs.

In 1998, the first hand transplant took place in Lyon, France in a patients with a prior traumatic mid-forearm amputation [3]. Later that same year, a 37-year-old male underwent a left hand transplantation in Louisville, Kentucky [4]. To date, more than 150 hand transplants have been performed worldwide [5]. Machine perfusion literature has shown evidence that *ex-vivo* perfusion is a suitable method for reducing ischemia time and preserving limbs for an extended period of time by provision of oxygen and nutrients prior to replantation or transplantation [6-11].

As evidence of machine perfusion's advantages over SCS continues to accumulate, research efforts have focused on translatable animal models, protocol optimization, and system refinement. Nearly 80% of English literature on *ex-vivo* machine perfusion of extremities has been published in the last 10 years, while 41% has been published in the last 3 years; this review encompasses all 34 published original articles (Tab. I).

Table I. Summary of studies involving machine preservation of extremities. There was no species limitation, and all studies published on machine perfusion of extremities are included; the earliest was published in 1964 and most recent in 2022.

Author year institution	Species	Control group(s)	*Ex-vivo* perfusion group(s)	Perfusate temperature	
Burlage et al., 2022 Brigham and Women's Hospital [25]	Rat Hindlimb	1. SCS for 6 hours (n = 10) Transplantation controls: Hindlimbs preserved for 6 h (n = 4), 24 h (n = 5) and fresh controls (n = 5)	Perfusion groups: 1. Perfusion with BSA (n = 4) 2. Perfusion with BSA+PEG (n = 4) 3. Perfusion with HBOC-201 (n = 4) Transplantation group: 1. After 6 h of perfusion with HBOC-201(n = 13)	Midthermic (21°C)	
Figueroa et al., 2022 Cleveland Clinic [35]	Pig Forelimb	1. SCS (n = 12)	Perfusion groups: 1. Perfusion with HBOC-201 (n = 6) 2. Perfusion with RBC (n = 6) Transplantation group: NR	Normothermic (38°C)	
Rezaei et al., 2022 Cleveland Clinic [36]	Human Arm	1. SCS (n = 10)	1. Perfusion (n = 10) Transplantation group: NR	Normothermic (38°C)	
Valdivia et al., 2022 Institute of Transfusion Medicine and Transplant Engineering [33]	Rat Hindlimb	1. SCS for 4h (n = 15)	Perfusion groups: 1. Perfusion with lentiviral vectors (n = NR) 2. Perfusion without lentiviral vectors (n = NR) Transplantation group: NR	Subnormothermic (33°C)	
Amin et al., 2021 University of Manchester [34]	Pig Forelimb	No control groups	1. Perfusion after 2h of SCS (n = 5) Transplantation group: NR	Normothermic (38°C)	
Kruit et al., 2021 Raboud University [7]	Pig Forelimb	1. SCS for 4h with RPL (n = 6)	1. Perfusion with RPL (n = 6) Replantation group: 1. After 18 h of perfusion	Midthermic (16°C)	
Rohde et al., 2021 Cleveland Clinic [37]	Human Arm	1. SCS (n = 7)	1. Perfusion (n = 7) Transplantation group: NR	Normothermic (38°C)	
Amin, et al., 2020 University of Manchester [18]	Pig Forelimb	Experiment 1: No control Experiment 2: 1. SCS for 8 h + perfusion for 4 h	Experiment 1: 1. NMP at 70mmHg (n = 5, other n = 5 for validation) 2.SNMPat 70mmHg (n = 5) 3. SNMP at 50mmHg (n = 5) 4. HMP at 30mmHg (n = 5) Transplantation group: NR Experiment 2: 1. NMP at 70mmHg (n = 5) Transplantation group: NR	Hypothermic (10°C) Subnormothermic (28°C) Normothermic (38°C)	

	Perfusion duration (hours)	Perfusate composition	Perfusate oxygenation	Perfusion pressure (mmHg), flow rate (mL/min)	Perfusate additives	Outcome measures
	6h	BSA, PEG, HBOC-201	95% O_2 5% CO_2	30-40mmHg, NR	Insulin, heparin, dexamethasone, hydrocortisone, antibiotics	MAP, flow, pH, O_2 saturation electrolytes, PaO_2, PvO_2, Glucose, lactate, O_2 consumption, energy change
	22.5±1.71h	HBOC-201 RBC	NR	HBOC: 78.50 ± 10.75 mmHg, 325.00 ± 25.00 mL/min RBC: 85.70 ± 19.90 mmHg, 444.73 ± 50.60 ml/min	Insulin, heparin, vancomycin, albumin, calcium gluconate, methylprednisolone	MAP, flow, pH, O_2 saturation electrolytes, PaO_2, PvO_2, weight, temperature muscle contractility, compartment pressure, Glucose, lactate, O_2 consumption, CK, myoglobin, metHb, albumin, IR thermography, ICG angiography, muscle histology
	41.6 ± 9.4h	pRBC	100% O_2	90mmHg, 0.41± 0.06 L/min	Albumin, heparin, vancomycin, cefazolin, methylprednisolone, dextrose, insulin	MAP, flow, electrolytes, pH, O_2 saturation, weight, temperature, compartment pressure Glucose, lactate, O_2 consumption, CK, metabolomics: taurine, tryptophan; IR thermography, ICG angiography, muscle contractility, H&E histology
	4h	STEEN solution and Sterofundin ISO (1:1 ratio)	95% O_2 5% CO_2	90-120mmHg, 5–9ml/min	$NaHCO_2$, cefazolin. After rewarming: protamine sulfate. Transduced limbs: lentiviral vectors NanoLuc / NeonGreen.	Pressure, flow, temperature, PaO_2 Perfusate cytokine quantification, myoglobin, lactate and LDH activity, artery, muscle, and skin biopsies for bioluminescence detection, thigh skin for culture and isolation of keratinocytes, fibroblasts, and microvascular endothelial cells
	6h	Bovine pRBC	95% O_2 5% CO_2	70mmHg, 356 ± 131.5 ml/min	Albumin, heparin, meropenem, glucose, methylprednisolone, $NaHCO_2$	Perfusate samples were collected at baseline (prior to limb attachment) and from the venous outflow at 3 and 6 h of EVNP Perfusate flow cytometry, cytokine assay, and quantification of circulating cell-free genomic (g) DNA and mitochondrial (mt) DNA via real-time qPCR
	18h	UW solution	95% O_2 5% CO_2	NR, 16±1.7ml/min	Methylprednisolone	Flow, pressure, electrolytes, pH, O_2 saturation, PaO_2, PvO_2, weight, temperature, doppler signal Glucose, lactate, ICG angiography, muscle contractility
	41.6 ± 9.4h	pRBC	100% O_2	90mmHg, 0.41± 0.06 L/min	Albumin, heparin, vancomycin, cefazolin, methylprednisolone, dextrose, insulin	MAP, flow Metabolomics
	6h	pRBC	95% O_2 5% CO_2	NR, 102.3 ± 34.8 ml/kg/min	Bovine serum Albumin, ringers' solution, insulin, Nutriflex, heparin, meropenem, glucose, methylprednisolone, $NaHCO_2$	MAP, flow, electrolytes, weight, temperature, pH, HCO_2, Ca, glucose, hematocrit, Muscle and skin histology, blood gas analysis

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Table I. *continues.*

Author year institution	Species	Control group(s)	*Ex-vivo* perfusion group(s)	Perfusate temperature	
Duraes et al., 2017 Cleveland Clinic [9]	Pig Forelimb	1. SCS for 12h (n = 5)	1. Perfusion (n = 5)	Normothermic (39°C)	
Kueckelhaus et al., 2017 Brigham and Women's Hospital [11]	Pig Forelimb	1. SCS for 4h with RPL (n = 4)	1.Perfusion (n = 3) Replantation group: 1. After 12 h of perfusion (n = 3)	Hypothermic (10°C)	
Puga Yung et al., 2017 University Hospitals and Medical Faculty of Geneva [32]	Pig Forelimb	1. Perfusion of wild-type limbs (n = 6)	1. Perfusion of HLA-E/human CD46 double-transgenic limbs (n = 6) Transplantation group: NR	Subnormothermic (32°C)	
Werner et al., 2017 University of Michigan [8]	Human Arm	No control groups	1. Perfusion (n = 5) Transplantation group: NR	Subnormothermic (30-33°C)	
Kueckelhaus et al., 2016 Brigham and Women's Hospital [24]	Pig Hindlimb	1. SCS for 12h (n = 5)	1. Perfusion (n = 5)	Hypothermic (10°C)	
Ng et al., 2017 Brigham and Women's Hospital [53]	Pig Forelimb	No control groups	1. Perfusion (n = 2)	Subnormothermic (NR)	
Ozer et al., 2016 University of Michigan [31]	Pig Forelimb	1. SCS for 6h with RPL (n = 4)	1. Perfusion (n = 4) Replantation group: 1. After 24 h of perfusion (n = 4)	Subnormothermic (27-32°C)	
Araki et al., 2015 University of Tokyo [29]	Rat Hindlimb	1. SCS for 6h with RPL (n = 4)	Perfusion groups: 1. Perfusion with ETK (n = 4) 2. Perfusion with ETK/HbV (n = 4) Replantation groups: 1. After 6 h of perfusion with ETK (n = 4) 2. After 6 h of perfusion with ETK/HbV (n = 4)	Subnormothermic (23-27°C)	
Ozer et al., 2015 University of Michigan [10]	Pig Forelimb	1. SCS for 6h with RPL (n = 3)	1. Perfusion with RPL (n = 4) Replantation group: 1. After 12 h of perfusion (n = 4)	Subnormothermic (27-32°C)	
Müller et al., 2013 Bern University Hospital [40]	Pig Forelimb	Normothermic (36°C)	Perfusion groups: 1. 6 h ischemia + 12 h perfusion (n = 7) 2.12 h ischemia + 5 h perfusion (n = 6) Replantation groups: 1. 12 h perfusion + replantation (n = 11) 2. 6h ischemia + 12h perfusion+ replantation (n = 8)	Subnormothermic (32°C)	

	Perfusion duration (hours)	Perfusate composition	Perfusate oxygenation	Perfusion pressure (mmHg), flow rate (mL/min)	Perfusate additives	Outcome measures
	12h	Heparinized autologous blood	100% O_2	Gradually increased during the first hour to reach a physiologic arterial pressure	THAM, albumin, glucose, vancomycin, methylprednisolone, insulin, CO_2, nitrogen	O_2 saturation, surface temperature, muscle temperature, compartment pressure, electrolytes, CBC PaO_2 $PaCO_2$ pH, lactate, glucose, CK, myoglobin, weight, CK, myoglobin, ICG angiography, Muscle, skin, and nerve histology, muscle and nerve functionality (electrical stimulation)
	12h	Perfadex	NR	30 mmHg, adjusted to maintain BP	Dextrose, insulin, methylprednisolone	Blood gas analysis Muscle histology and electron microscopy for quantification of IRI and hypoxia markers, lactate, myoglobin
	12h	Heparinized whole human blood	NR	NR, NR	Heparin	WBC count, PBMC count, muscle histology and immunofluorescence analysis of NK cell infiltration and percentage, NK cytotoxicity through immunoassay
	24h	pRBC	40–60% O_2, 5-10% CO_2, 30-55% N_2	93 ± 2 mmHg, 310 ± 20 ml/min	Albumin, $NaHCO_2$, THAM, calcium chloride, heparin, dextrose, insulin, methylprednisolone, antibiotics	MAP, flow, temperature pH, PaO_2, $PaCO_2$, O_2 saturation, $NaHCO_2$, Na, K, Hemoglobin concentration, skin temperature at palm. lactate, myoglobin, muscle histology, fiber contractility, muscle function, and the maximum isometric contractile force of permeabilized single muscle fiber, neuromuscular electrical stimulation
	12h	Perfadex	NR	30 mmHg, adjusted to maintain BP	THAM, dextrose, insulin, glucose, methylprednisolone	pH, $PaCO_2$, base excess, K, Ca, glucose uptake muscle histology and IHC
	3h	William's E medium	NR	NR, 270-320 ml/min	Dexamethasone, insulin, heparin	MAP, flow ATP, lactate clearance analysis, perfusate biochemical analysis
	24h	Autologous blood	95% O_2, 5% CO_2	60-80 mmHg, NR	Glucose, insulin	MAP, flow, weight, temperature, O_2 delivery and consumption, acid-base status, electrolytes, glucose, muscle histology and fiber contractility, lactate, blood gas analysis, functional electro stimulation
	6h	ETK HbV	NR	NR, 1	NR	PaO_2 $PaCO_2$ Muscle histology, lactate, walking track analysis, walking appearance
	12h	Autologous blood	95% O_2, 5% CO_2	60-80 mHg, 10-120ml/min	Glucose, insulin	Pressure, flow, weight, PaO_2 $PaCO_2$ Electrolytes, pH, glucose, blood gas analysis, lactate, single muscle fiber contractility, neuromuscular electrical stimulation
	12h	Autologous blood	21% O_2	NR, 100-150 ml/min	Methylprednisolone, glucose, insulin	Muscle, nerve and blood vessel histology, immunofluorescence, serum cytokine and complement immunoassay, blood gas analysis, neuromuscular electrical stimulation

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Table I. *continues.*

Author year institution	Species	Control group(s)	*Ex-vivo* perfusion group(s)	Perfusate temperature	
Constantinescu et al., 2011 Bern University Hospital [43]	Pig Forelimb	1. SCS for 12h (n = 8)	1. Perfusion (n = 8)	Subnormothermic (32°C)	
Tsuchida et al., 2003 Kumamoto University [47]	Rat Hindlimb	1. WS (n = 6)	Perfusion groups: 1.Perfusion (n = 6) 2. No perfusion (n = 6) Replantation groups: 1. After perfusion for 5 h (n = 6) 2. After WS for 5h (n = 6)	Subnormothermic (25°C)	
Tsuchida et al., 2001 Kumamoto University [48]	Rat Hindlimb	1. WS for 4h (n = 4) 2. WS for 5h (n = 4)	Perfusion groups: 1. Perfusion with EC at 40 mmHg (n = 4) 2. Perfusion with EC at 100 mmHg (n = 4) 3. Perfusion with UW solution at 40 mmHg (n = 4) 4. Perfusion with UW solution at 100 mmHg (n = 4) Transplantation group: NR	Subnormothermic (25°C)	
Gordon et al., 1992 California Pacific Medical Center [26]	Dog Hindlimb	1. Storage at 13°C for 12h (n = 6)	1. Perfusion (n = 6) Transplantation group: NR	Midthermic (13°C)	
Domingo et al., 1991 Centre de Cirurgia Experimental de la Mutua Sabadellenca [19]	Dog Hindlimb	NR	Perfusion groups: 1. Perfusion (n = 9) Replantation group: 1. Immediate replantation (n = 6) 2. Perfusion for 24 h + replantation(n = 6)	Hypothermic (NR)	
Usui et al., 1985 Sapporo Medical College [27]	Dog Hindlimb	1. Storage at room temperature (n = 15) 2. Storage in iced water (n = 10)	Perfusion + Replantation groups: 1. Intermittent perfusion with FC at room temperature + RPL (n = 9) 2. Intermittent perfusion with FC in iced water + RPL (n = 6) 3. Continuous perfusion with FC at room temperature+ RPL (n = 6) 4. Continuous perfusion with FC in iced water + RPL (n = 5) 5. Continuous perfusion with HA in iced water + RPL (n = 5)	Midthermic (room temperature)	
Delorme et al., 1964 Massachusetts General Hospital [1]	Human Leg	No control groups	Perfusion with human blood (n = 6)	NR ("cooled" and "rewarmed")	

Abbreviations in the table in order of appearance: SCS: static cold storage; BSA: bovine serum albumin; PEG: polyethylene glycol; HBOC-201: hemoglobin-based oxygen carrier-201; O_2: oxygen; CO_2: carbon dioxide; NR: not reported; MAP: mean arterial pressure; RBC: red blood cell; PaO_2: arterial partial pressure of oxygen; PvO_2: venous partial pressure of oxygen; MetHb: methemoglobin; IRT: infrared thermography; ICG: indocyanine green; pRBC: packed red blood cells; CK: creatine kinase; H&E: hematoxylin and eosin; $NaHCO_2$: sodium bicarbonate; LDH: lactate dehydrogenase; EVNP: *ex-vivo* normothermic perfusion; qPCR: quantitative polymerase chain reaction; RPL: replantation; UW: University of Wisconsin solution; ICG: indocyanine green; Ca: calcium; NMP: normothermic machine perfusion; SNMP: subnormothermic machine perfusion; N_2: nitrogen; THAM: tris Hydroxymethyl amino methane; EMG: electromyogram; CBC: complete blood count; CMAP: compound muscle action potential; HTK: histidine-tryptophan-ketoglutarate solution; KCl: potassium chloride; NaCl: sodium chloride; $CaCl_2$: calcium chloride; $MgCl_2$: magnesium chloride; NaH2PO4: sodium dihydrogen phosphate; NaOH: sodium hydroxide; K: potassium; ATP: adenosine triphosphate; MRI: magnetic resonance imaging; MRA: magnetic resonance angiography; C1-INH: C1 esterase inhibitor; tPA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor-1; BP: blood pressure; HLA: human leukocyte antigen; CD46: cluster of differentiation 46; WBC: white blood cell; PBMC: peripheral blood mononuclear cells; ETK: Extracellular Trehalose Kyoto; HbV: hemoglobin vesicles; WS: warm storage; CPK: creatine phosphokinase; EC: Eurocollins solution; FC: fluosol-43 diluted with Hartmann's solution; HA: Hartmann's solution; GOT-m: glutamic oxalacetic transaminase.

	Perfusion duration (hours)	Perfusate composition	Perfusate oxygenation	Perfusion pressure (mmHg), flow rate (mL/min)	Perfusate additives	Outcome measures
	12h	Autologous blood	21% O_2	33 mmHg, 100-150 ml/min	Insulin, glucose	Pressure, O_2 saturation, compartment pressure, temperature blood gas analysis, Electrostimulation, nerve stimulation, histology and immunofluorescence, skin and muscle color, capillary refill
	5h	UW	NR	100 mmHg, 1-2 ml/min	NR	Muscle ATP analysis, serum CPK analysis, reperfusion blood flow, and vascular endothelial exocrine function
	4h 5h	EC UW	NR	40/100 mmHg, NR	NR	ATP
	12h	UW solution	NR	70mmHg, NR	NR	Muscle histology and magnetic resonance spectroscopy
	24h	Preserved blood	NR	> 100 mmHg, 500 ml/min	Ringer's lactate solution, Rheomacrodex, mannitol, $NaHCO_2$, heparin, prednisolone, piperacillin, nitroglycerin	Biochemical analysis, muscle histology
	6h	FC HA	NR	50 mmHg, NR	NR	Weight, K, pH Lactate, CK, GOT-m, LDH
	NR ("several hours")	Human blood	Kay-cross disc oxygenator	NR	none	Muscle temperature, pH, $PaCO_2$, PaO_2, contractility, electrical studies on muscles and isolated nerves

RELEVANCE OF ISCHEMIA IN LIMB TRANSPLANTATION

Skeletal muscle is the most metabolically active tissue in the limb and thus most susceptible to ischemic injury. Clinical reports of hand transplantation indicate that shorter ischemia results in superior graft function. Herzberg et al. indicted increased ischemia time (9 h) for modest functional recovery following bilateral hand transplant [12]. Landin et al. reported perioperative ischemic injury (3 h of cold and 3.5 h of warm ischemia) resulting in fibrotic contracture of forearm muscles following transplantation.[13] However, Piza-Katzer et al., in a bilateral hand transplant with shorter ischemia time (2.5-2.8 h), found improved fine motor function and recovery of sensation [14]. Ischemic injury is also documented as a risk factor for acute and chronic rejection after limb transplantation due to activation of immune responses [15]. In experimental models, ischemia time between 1-3 hours have been shown to result in sufficient musculocutaneous reperfusion injury to increase the risk of acute rejection [16].

PERFUSATE TEMPERATURE

Optimal perfusate temperature in *ex-vivo* perfusion has been a topic of controversy in recent years. While multiple studies have shown the superiority of machine perfusion over SCS, there is no consensus on the optimal perfusate temperature. Hence, a wide range of temperatures (4°C to 39 °C) have been tested during machine perfusion of extremities. Karangwa et al [17]. standardized nomenclature for perfusate temperature: hypothermic (0-12 °C) [18-24], midthermic (13-24C°) [21-27], subnormothermic (25-34 °C) [7 15,17,24-32], and normothermic (35-38 °C)[18,30,36-41].

Hypothermic perfusion (0-12 °C)

Kruit et al. [7] successfully replanted six porcine limbs after 18 h of hypothermic perfusion with University of Wisconsin solution, achieving a 3-fold elongation of the current maximum SCS time. Although they found increased muscle damage in perfused limbs compared to the contralateral SCS controls, muscle contractility was preserved. Perfused limbs in their study gained 18.6% weight and SCS 11.6%, but this difference was not statistically significant. Additionally, Amin et al. [18] compared hypothermic (10°C), subthermic (28°C), and normothermic (38°C) perfusion and found that porcine forelimbs could be optimally preserved via hypothermic perfusion, supported by the reduction in markers of cellular injury, inflammation, and better tissue integrity. Gok et al. compared the outcomes of limb transplantation after SCS *versus* hypothermic perfusion with HTK and concluded that hypothermic perfusion preserved limb viability according to amino acid metabolism and energy stores, but failed to restore muscle force [20,42]. In a study by Steward et al., the normothermic perfused limb group maintained a near physiologic pH, while the hypothermic group developed acidosis and increased perfusate potassium, which remained throughout perfusion despite the addition of THAM, which was also associated with hyperkalemia. Muller et al. [40] also found that levels of all studied immune response markers were lower in hypothermic perfusion than normothermic.

Midthermic perfusion (13-24 °C)

As a compromise between hypothermic and normothermic perfusion temperatures, midthermic perfusion has been investigated by a few authors [21-27]. Burlage et al. [25] perfused rat hindlimbs with three different acellular perfusates (bovine serum albumin (BSA), BSA ± polyethylene glycol (PEG), or HBOC-201 (Hemopure, HbO2, Therapeutics LLC)) at 21°C for 6 hours. They reported that energy charge ratios were higher in perfused limbs than SCS and that HBOC-21 (Hemopure, HbO2, Therapeutics LLC) perfusions resulted in significantly less edema. Following perfusion, limbs were also transplanted successfully.

Subnormothermic perfusion (25-34 °C)

Temperatures near normothermia are used in an attempt to decrease the amount of peripheral vasoconstriction and shunting [45]. Gok et al. [46] perfused rat hindlimbs for 6 hours using STEEN (XVIVO Perfusion, Göteborg, Sweden) solution with swine erythrocytes while maintaining the temperature between 30-35°C. They found that gastrocnemius energy stores were maintained at the end of perfusion from metabolomic analysis and that the muscles remained viable, as metabolic activity was maintained. There was no difference in the levels of total high-energy phosphates compared to contralateral cold storage controls. On muscle H&E histology they did not find any qualitative evidence of ischemic necrosis or myocyte degeneration [46]. Werner et al. perfused human limbs with RBCs for 24 hours between 30-33°C with no control group. Muscle fiber cross-sectional area and force of contraction did not change significantly, while histological sections of the flexor carpi radialis also revealed no differences in fascicular architecture or shape throughout perfusion (0, 12, 24 h) [8].

Normothermic perfusion (35-38 °C)

Fahradyan et al. compared subnormothermic (33.0 ± 2.6°C) and normothermic (35.4 ± 1.7°C) conditions. Their study demonstrated that *ex-vivo* normothermic limb perfusion preserves amputated limbs in a near-physiologic state with maintained contractility of the skeletal muscle in response to electrical stimulation, for at least 24 hours [31]. Rezaei et al. perfused 10 human

upper limbs for 41.6 ± 9.4 h demonstrated the presence of active metabolism in and metabolic derangement toward the end of 24-hour perfusion, correlating with mitochondrial structure, swelling, and elongation. They concluded that EVNLP extends preservation time and enables assessment of human limb quality and allows reconditioning [36,38].

PERFUSATE OXYGENATION

Oxygenation is closely related to perfusion temperature as lower temperatures mean lower metabolic rates and therefore lower energy and oxygen demands. Hypothermic perfusion relies on the decreased baseline metabolism and oxygen demand to oxygenate the perfusate through passive diffusion from the air contained within the reservoir [48,49]. Therefore, typical hypothermic perfusion devices do not need to have an oxygenator. However, to support a physiologic metabolic rate, oxygen delivery is necessary, and an oxygenator is typically used in normothermic perfusion. Most normothermic and subnormothermic studies used a membrane oxygenator with 95-100% O_2 [18,24,26,31,32,34,36,37,39,40,50], while only one group used a Paragonix machine with 95% O_2 (Paragonix Technologies, Inc; Cambridge, MA, USA) [18]. Duraes et al. [9] kept the partial pressure of oxygen within physiologic range using a combination of 100% O_2, 7% CO_2, and 93% N_2. Werner et al. [8] had a partial pressure of 300 mmHg by using 40-60% O_2 with 5-10% N_2/CO_2, however.

PERFUSATES

A large variety of solutions have been experimented in *ex-vivo* limb perfusion including commercially available preservatives, and cellular-based solutions with modifications and/or additives [50]. Red blood cell (RBC)-based perfusate are thought to have superior oxygen-carrying capabilities because of the presence of hemoglobin and possibly reduced edema formation due to the closest resemblance to whole blood [52]. However, acellular solutions like University of Wisconsin's [24,27,53], Perfadex [11,25], modified Perfadex [22], histidine-tryptophan-ketoglutarate [20], or STEEN (XVIVO Perfusion, Göteborg, Sweden) [21,23,34,46] have also been used in hypo, mid, sub, and normothermic conditions.

Given the limitations of using human RBC in organ perfusion, including limited availability, need for cross match, mechanical hemolysis, activation of pro-inflammatory proteins, transmission of infectious diseases, and patient refusal to accept human blood products, Said et al. investigated the feasibility of HBOC-201 (Hemopure, HbO2, Therapeutics LLC), ultra-purified bovine hemoglobin polymerized with glutaraldehyde. Under normothermic conditions, HBOC-201 outcomes were similar to RBCs with preservation of muscle contractility and mitochondrial structure [41]. These findings were recently validated by Figueroa et al. [35], who confirmed that the outcomes of normothermic perfusion using HBOC-201 (Hemopure, HbO2, Therapeutics LLC) are similar to RBC-based perfusion.

Kruit et al. performed *ex-vivo* perfusion of six porcine forelimbs, with University of Wisconsin (UW®, Northbrook, USA) solution at 8-10 °C, for 18 hours. All limbs were then replanted. They reported superior muscle contraction, however there was evidence of ischemia-reperfusion injury on histology 12 hours after replantation. They also reported 19% weight increase prior to replantation, which is compatible with the reported histology evidencing muscle injury.

Kuckelhaus et al. [11] used oxygenated STEEN (XVIVO Perfusion, Göteborg, Sweden) to perfuse five porcine limbs for 12 h each, (10-12 °C) after which they found a mean of 44% weight gain. They found less evidence of hypoxic cells on histology of perfused limb muscle samples compared to SCS controls. After 24 hours of perfusion with a modified STEEN (XVIVO Perfusion, Göteborg, Sweden) solution (8 °C), Krezdorn et al. [23] found that porcine limb weight increased 42%, though aside from edema, histology showed preserved muscle architecture. The perfused limbs were then replanted and followed for one week. The SCS control limbs in this study were preserved for 4 hours and showed evidence of freezing damage with minimally disrupted muscle fibers.

Perfusate additives

Common perfusate additives include antibiotics; steroids, to prevent inflammation and maximize cell membrane permeability; anticoagulants, most commonly heparin; pH buffers, such as bicarbonate; glucose; and insulin [7,22,49]. The addition of the and correct dosing of each antibiotic have been derived from clinical indications; their relative efficacies have not yet been investigated. Under normothermic conditions with presence of cefazolin only in the perfusate, we have confirmed growth of pseudomonas during porcine EVLP. Subsequent experiments with the addition of ceftazidime to the perfusate have solved this issue (*unpublished data*). Valdivia et al. [49] studied rat hindlimb preservation, and following perfusion, added streptomycin and amphotericin B to tissue cultures, but did not report on any bacterial or fungal culture results from during the perfusion.

During subnormothermic perfusion, authors investigated additional interventions, such as transgenic expression of HLA-E/hCD46 [32], perfusion in combination with lentiviral vectors [34], and addition of a C1 esterase

inhibitor (C1 INH) [29]. Puga Yung et al. [32] perfused six porcine limbs with transgenic expression of HLA-E/hCD46 using human blood for 12 hours and found that this partially protected porcine limbs from human NK-mediated xenorejection responses compared to the wild-type porcine limbs. Similarly, Valdivia et al. [50] studied rejection through genetic modification with the aim to decrease expression of genes involved in ischemia-reperfusion injury, inflammation, and rejection (e.g., cytokines, but by adding lentiviral vectors to the Steen (XVIVO Perfusion, Göteborg, Sweden) perfusate of rat hindlimbs for 4 hours. Abdelhafez et al. [28] found that limbs pretreated with C1-INH before perfusion had a significant reduction of complement, bradykinin, fibrin, IgM, and IgG depositions, decreased edema formation, and IRI compared to those without after 12 h of *ex-vivo* perfusion. During 24-hour normothermic perfusion of rat hindlimbs, Araki et al. [29] added artificial oxygen-carrying hemoglobin vesicles, which resulted in maintenance of aerobic respiration in the gastrocnemius muscle and successful replantation and functional gait at 3 month follow-up.

Gene expression, genetic markers, and metabolomics

Kruit et al. [7,50] have reported that *ex-vivo* perfused limbs and flaps had similar genetic expression for tumor necrosis factor receptor, regulators of G-protein coupled signaling, hypoxia inducible factor, caspase, and other genes, to their respective cold storage controls, though with small sample sizes. They concluded that machine perfusion preserves limbs in an acceptable state longer than SCS (> 12 hours). However, studies by this group were limited by hypothermic perfusion temperatures and the inevitable downregulation of gene transcription that aligns with intentionally slowed metabolism. There has yet to be a study on genetic expression in normothermic limb perfusion.

Metabolomic analysis entails the identification and quantification of metabolites within a biological sample [52], which allows for pathway analysis downstream of gene expression. Rohde et al. demonstrated preservation of active metabolic activity in normothermic perfusion of 7 human limbs [37]. By 24 hours, there was evidence of deranged metabolism (e.g., tryptophan catabolism, decreased taurine levels) [36]. Dysregulated metabolism at later time points correlated with evidence of altered mitochondrial structure and swelling.

Unknowns

A common finding among *ex-vivo* limb perfusion studies is an improved outcome after at least 6 hours of perfusion when compared to cold storage. Current evidence does not allow conclusions to be drawn about the superiority of one method over another, nor resolve the controversy of presented outcomes across the literature. We can, however, recommend the minimum parameters that should be presented as additional data allow more rigorous analysis of outcomes. In future studies of *ex-vivo* limb perfusion, there should be analysis of basic parameters that are included in most studies to date, such as temperature, hemodynamics, electrolytes, weight, and compared to a contralateral cold storage control. These parameters should be supported by biopsy data (e.g., H&E histology or electron microscopy) and other downstream effects, such as gene expression, metabolomics, protein expression (immunohistochemistry), and muscle contractility. Analyses of these data and correlation with limb outcomes after replantation or transplantation will allow determination of evidence-based viability criteria that can be monitored in real-time, as well as the ability to determine the optimal protocol for limb perfusion. Direct comparisons of slightly different protocols with one factor changed, like temperature or oxygenation, and all others held constant, could provide enough evidence to help us achieve this determination and standardize EVLP protocols in the future.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

AM, MA, DDB: contributed to study design, data collection, interpretation, preparation of the manuscript, and critical revision of the manuscript; VK, AR: contributed to study design and critical revision of the manuscript; BBG conceived the idea, contributed to study design, interpretation, and critical revision of the manuscript.

Ethical consideration

Not applicable.

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