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Review

HYPOTHERMIC PERFUSION OF THE KIDNEY: FROM RESEARCH TO CLINICAL PRACTICE

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Summary

Kidney transplantation is the treatment of choice for patients with end stage renal disease (ESRD). Donor availability is lower than demand, therefore suboptimal grafts retrieved from donors after brain death with expanded criteria donors (EC-DBD) and from donors after cardiac death (DCD) are increasingly used. These organs carry a higher risk of worse clinical outcomes, and subsequently need more advanced preservation systems than static cold storage (SCS). Hypothermic perfusion represents one of the aforementioned strategies.

This review summarizes the main features of hypothermic perfusion: its mechanism of action through analysis of preclinical models and its clinical efficacy in kidney transplantation with a focus on marginal donors. Oxygenated hypothermic perfusion was also evaluated focusing on its potential benefits on cell metabolism and graft immunogenicity. Finally, as hypothermic perfusion not only allows to recover marginal grafts, but may also recondition grafts unsuitable for transplantation, the possible methods of graft evaluation and treatment options during perfusion are described in this review.

Key words: kidney transplantation, organ preservation, machine perfusion, hypothermia, oxygen

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INTRODUCTION

Kidney transplantation is the treatment of choice for patients with end-stage renal disease (ESRD), granting better outcomes compared to dialysis both in terms of patient survival and quality of life [1].

Donors' availability is limited if compared to the request for organs to be transplanted. In fact, a significant proportion of patients dies on the waiting list (mortality rate on the waiting list: 2.4%) or waits for a long time with an average waiting time on list: 3.2 years, reaching transplant in poor health conditions [2].

In 2002, the American United Network for Organ Sharing (UNOS) proposed to increase the pool of donor kidneys to include expanded criteria donors (ECD), for which the relative risk of graft failure (return to dialysis or patient death) was estimated to be 1.7-fold higher than kidney transplant recipients from standard criteria donor (SCD) [3]. ECDs are defined as donors after brain

death (DBD) > 60 years of age or between 50-60 years of age with two of the following characteristics: arterial hypertension (AHT), creatinine > 1.5 mg/dL, or death from a cerebrovascular accident [4].
The original intent of the donor criteria expansion was to list for ECD kidneys only patients for whom the compromise of lower graft survival was offset by a shorter time in transplant waiting list. Nevertheless, in some cases the graft survival of ECD kidneys resulted better compared to that of SCD kidneys.
The kidney donor profile index (KDPI) was developed to improve risk stratification in terms of survival after deceased donor kidney transplant, to provide a more granular index of risk for donor kidneys and to locate them on a continuum basis [5]. Calculation of the KDPI includes ten variably weighted donor parameters: for example, age has the highest negative impact in the KDPI algorithm, especially if a donor is above 50 years or below 18 years [6].
These grafts are more susceptible to ischemia-reperfusion injury (IRI) and have an increased risk of post-transplant complications such as primary non-function (PNF) and delayed graft function (DGF) and are also characterized with unfavorable graft survival rates [7-10]. Moreover, given their immunogenic potential, they have an increased risk of developing acute and chronic rejection in the post-transplant period [11]. Therefore, these organs require more elaborate preservation systems than static cold storage (SCS), in which the kidney is only flushed at the time of procurement using a preservation solution. This method has been the treatment of choice for several years due to its simplicity, low costs and the constant development of more refined perfusion fluids.
The first perfusion machine models were developed in the United States by Humpries et al. in 1960s who successfully performed an auto-kidney transplant on a canine model after 24-hour storage using cryopreserved plasma in a pulsatile perfusion pump and later by Belzer et al. [12,13]. At the beginning of the solid organ transplantation era, perfusion machines were extensively used since they were considered to be the only safe method of storing grafts. In 1969 Collins et al. demonstrated a method for storing and transporting renal grafts on ice in a storage solution. These perfusion solutions progressively became more elaborate providing increasingly better results and thus reducing the success of perfusion machines and establishing the beginning of SCS era [14].
However, during the last twenty years, the increase in marginal grafts utilization has prompted research to find alternative methods of preservation. In 2009 a landmark study on hypothermic machine perfusion (HMP) by Moers et al. was published, starting a new chapter in organ preservation [15].
Currently, *ex-vivo* perfusion machines use different temperature settings (hypothermia, sub-normothermia, normothermia), oxygen support (normobaric or hyperbaric) and application of therapeutic agents (e.g. nutrition, anti-inflammatory and anti-apoptotic additives, mesenchymal stromal cells [MSCs], or MSCs releasing extracellular vesicles [EV]) [16-21].
Contrary to SCS, whose purpose was to allow organ preservation and transportation without damage, the main goal of *ex-vivo* mechanized perfusion is to improve and even recover organs. This setting has led to a paradigm shift, in which not only reliable preservation of an organ with standard criteria is expected, but also damage reduction and improvement in the vitality of a "marginal" organ, through the removal of waste products and the administration of metabolic substrates, can be achieved in order to increase the pool of transplantable organs [11,22].
The aim of this review is to analyze the potential benefits of hypothermic perfusion methods from research to clinical practice and to assess its future perspectives.

HYPOTHERMIC MACHINE PERFUSION

Mechanism of action through pre-clinical models

During hypothermic machine perfusion (HMP) a cold acellular preservation solution (4-8°C) circulates through the renal artery by a perfusion pump [23]. Grafts are connected to the perfusion device by a single-use sterile kit and the vessels are cannulated with a specific cannula. To perform oxygenation of the organs during perfusion, it is possible to use a membrane oxygenator integrated in the perfusion circuit. The flow and pressure values during perfusion are self-regulated, measured by specific sensors and displayed on the device screen [24]. To avoid kidney oedema and endothelial damage, pressure is to be kept between 25 and 30 mmHg into the renal artery [11].
The most used preservation solutions are Belzer MPS® UW (Machine Perfusion Solution University of Wisconsin) or Celsior® (Institut George Lopez, IGL), which both guarantee an adequate intake of nutrients and electrolytes for graft preservation before transplantation [25].
In terms of composition, the UW solution contains osmotic components such as raffinose and hydroxyethyl starch, mixed with a phosphate buffer and a high potassium content, adenosine as a nucleotide precursor, allopurinol, which acts as a free radical scavenger, dexamethasone, insulin and penicillin. The Celsior® solution, on the other hand, is high in sodium, low in potassium and low in viscosity; it is also characterized by a high buffering capacity and contains ketoglutarate as an energy substrate [24].
Various hypothermic machine perfusion devices are currently avaible: LifePort® (Organ Recovery Systems; Itaca, Illinois), Kidney Assist® (OrganAssist; Groningen, Netherlands), Waters RM3® kidney perfusion system (Rochester, Minnesota), Waves IGL® (Lyon, France) [11] and VitaSmart

TM Machine Perfusion (Bridge to Life, Europe) [24]. One of the primary differences between devices is that some are pressure-driven (e.g., LifePort and Kidney Assist), whilst others are flow-driven (e.g., RM3 system). Some devices are portable for administering HMP from the end of organ retrieval. The VitaSmart (Bridge to Life, Europe) is a non-portable multi-organ device to add oxygenation to end-ischemic hypothermic perfusion [27].

Several pre-clinical and clinical models review and meta-analysis have compared outcomes of HMP and SCS when used with DBD, EC-DBD and DCD donor grafts (Tab. I).

The actual mechanisms behind transplantation outcomes improvement when HMP is used are not completely clarified; undeniably, HMP provides the kidney graft with all the needed nutrients and removes toxic metabolites and free radicals [28].

During hypothermia, cellular metabolism is reduced to 5-10% and ATP depletion, oxygen consumption and mitochondrial reactive oxygen species (ROS) production are consequently reduced [29]. Pulsating pressure mechanism carries out shear stress forces upon the vessel's walls, maintaining normal endothelial cells morphology and activating Kruppel-like 2 (KLF2) transcription factor through extracellular-signal-regulated kinase 5, extracellular signal-related kinase 5 e myocyte enhancer factor 2 transcription pathways [30]. Indeed, Gallinat et al. demonstrated KLF2 upregulation, endothelin 1 downregulation and – most of all – upregulation of nitric oxide synthase (NOS) during HMP, leading to vasodilation and microcirculation improvement in the kidney cortex [31]. Chatauret et al. showed the same results, demonstrating kidney cortex microcirculation improvement right after reperfusion in porcine DCD models subjected to HMP [32]. HMP subjected grafts also showed increased levels of Akt and Erk phosphorylation, both fundamental in cellular survival via pro-apoptotic markers downregulation and anti-apoptotic markers upregulation [33].

Besides, despite hypotermia and anoxya, central metabolism pathways are kept active through glycolytic and non-glycolytic pathways; Nath et al. demonstrated increased levels of eight main metabolites such as lactate, glutamate, fumarate, aspartate and acetate in HMP subjected grafts [34].

Finally, this preservation method prevents reperfusion damage after ischemia, caused by several different factors. First, hypotension is frequent in donors, and it often requires administration of vasoconstrictors, leading to renal hypoperfusion and low intake of oxygen and nutrients; cold ischemia and graft manipulation during organ retrieval also play a role in this condition [35]. Moreover, brain death and intracranial hypertension may release great amounts of cytokines and growth factors, worsening renal ischemia [36]. Notably, endothelial cells and kidney tubule cells are most affected by oxygen deficiency and both hypoxia and ATP degradation create ROS and provide an acidic microenvironment, which further damage

Table I. Preclinical hypothermic machine perfusion studies.

Study [ref]	Year	Model	Storage study group	Storage control group	Outcomes study group
Hendriks, Koen et al. [29]	2019	Porcine kidney	HMP	SCS	↓ ROS
Galinat et al. [31]	2014	Porcine kidney	Group 2: SCS + HMP (1hour) Group 3: SCS + HMP (4hours)	Group 1: SCS	↓ Endothelin 1 ↑KLF2 (group 3 > 2) ↑ NOS
Chateuret et al. [32]	2014	DCD Porcine kidney	HMP	SCS	↑eNOS renal cortex ↑ artery vasodilatation
He et al. [33]	2017	Porcine kidney	HMP	SCS	↑ Akt, Erk phosphorylation ↓ Na+, Cl-, BUN, LDH ↓ ATP degradation ↓ LD accumulation
Nath et al. [34]	2016	DCD Porcine kidney	HMP	SCS	↑ lactate, glutamate, fumarate, aspartate, acetate
Zhang et al. [39]	2016	DCD Rabbit kidney	HMP	SCS	↓ apoptosis ↓ cleaved caspase-3 ↑ezrin ↑ PI3K/AKT pathway

ROS: reactive oxygen species; NOS: nitric-oxide synthase; BUN: blood urea nitrogen; Cr: creatinine, LDH: lactate dehydrogenase; LD: lactic acid; ATP: adenosine triphosphate

the cell membrane. ATP-dependent membrane pumps, especially Na/K+ ATPase, stop functioning without ATP with subsequent accumulation of intracellular K+ and extracellular Na+ [11]. In this environment, the re-establishment of blood flow induces inflammation, an increase in reactive oxygen species and the release of cytokines by white blood cells [37]. Reactive oxygen species mediate mitochondrial damage and activation of the apoptotic pathway [38]. HMP is effective in this setting by reducing the concentration of cytokines (interleukin 1b, interleukin 2 and TNF-a), apoptotic mechanism and caspase-3 expression by ezrin/AKT pathways [39].

Hypothermic oxygenated machine perfusion

Hypothermia does not completely interrupt cell metabolism. Therefore, the addition of oxygen during hypothermic perfusion could satisfy the remaining metabolic demand by replenishing ATP stores through aerobic metabolism, which prevents damaging processes such as cell swelling, apoptosis, necrosis and ischemia-reperfusion damage [22].

Actually, hypothermic oxygenated machine perfusion (HOPE) is performed using a machine perfusion solution (Belzer MPS® UW Machine Perfusion Solution University of Wisconsin or Celsior® – Institut George Lopez, IGL), with a partial pressure of oxygen (pO2) between 450 mmHg and 750 mmHg and a renal artery pressure of 25-30 mmHg. Several animal and preclinical studies were performed to investigate the role of oxygen administration in hypothermic conditions (Tab. II).

A large part of the studies has analyzed DCD animal models, a category of donors most susceptible to post-transplant complications, following the good results obtained on DCD donor liver transplants undergoing HOPE [40].

A study by Kron et al. compares various methods of storage for DCD kidney rodent models: cold storage, cold storage and normothermic perfusion, cold storage and HOPE and cold storage and hypothermic de-oxygenated perfusion (HNPE). HOPE-treated DCD kidneys showed better function after transplantation than cold-stored grafts. Moreover, this study showed several benefits, being HOPE demonstrated to prevent macrophage activation with less TLR-4 positive cells, less TNF-a release and to prevent endothelial cells activation after implantation [41].

In addition, HOPE treatment prevents mitochondrial ROS production, although the addition of oxygen could lead to an increase in the production of reactive oxygen species resulting in cellular damage. However, a study by Hoyer et al. demonstrated improved flow and lower resistance within the renal parenchyma with HMP at 100% O_2 reflecting vascular and endothelial function and integrity. Furthermore, cell integrity is also supported by lower LDH values, lower tubular dilatation and lower gGT values, markers of tubular damage in urine. In this study, after two hours of perfusion, graft function has been demonstrated to be significantly superior compared to HMP without oxygenation [42].

Moreover, a study by Patel et al. compared porcine kidney DCD models subjected to hypothermic oxygenated perfusion (HPM/95% O_2) or subjected to HPM aerated (HPM/air). This study showed that hypothermic perfusion at high oxygen concentrations (95% O2) increases the production of tricarboxylic acid cycle intermediates and thus ATP, improves flow dynamics at the renal cortical level and does not increase the production of reactive oxygen species [43].

In a trial by Thuiller et al., the positive results of oxygenated HMP were confirmed by the 3-month follow-up. Two weeks after transplantation, porcine DCD model treated with oxygenated HMP had lower creatinine values and three months after transplantation, renal fibrosis indices were lower than the cohort who was not treated with oxygen [44].

One of the problems of oxygen supply is the timing. In fact, reducing the time to a short period could add several benefits, especially logistic management [27].

Darius et al. [45] analyzed porcine DCD models who underwent autotransplantation after a 30 minutes warm ischemia time that were randomized to different perfusion strategies: 2 hours of oxygenation during HMP either at the start (n = 6), or end of the perfusion, for a total of 22 h HMP. Outcomes were compared to standard, nonoxygenated HMP and continuous oxygenated HMP. The aim was to determine the optimal conditions for hypothermic kidney storage in order to supply an effective oxygenation and to afford an easy logistic management during donation and grafts' transport.

All the grafts that underwent oxygenation had a better early graft function measured on creatinine levels if compared to the group with exclusive hypothermic perfusion. Two hours of O2 administration at the start of HMP was superior to end-ischemic O2 supply during HMP and equivalent to the continuous oxygenation group.

Metabolic profile achieved in the perfusate (reduction of lactates, succinate and FMN) and in renal tissues (low expression of Nrf2 and HIF-1a) suggests that O2 supply at the beginning of HMP could be an easier and better storage strategy to protect mitochondrion from free radicals damage and improve early graft function if compared to standard HMP and O2 supply at the end of HMP, achieving results that are comparable to continuous HOPE.

Such strategy could be interesting especially for DCD kidneys since it allows to correct oxygen debt due to warm ischemia, which is typical of these grafts, and facilitate organ transport.

Another important aspect of oxygenated hypothermic perfusion seems to be its action on the immune system. Innate and adaptive immunity are activated upon reperfusion when a superoxide burst from the mitochondrial respiratory chain induces tissue injury and damage associated molecular patterns [46].

Table II. Preclinical hypothermic oxygenated machine perfusion studies.

Study [ref]	Year	Model type	Storage study group	Storage control group	Outcomes
Kron et al. [41]	2016	DCD rodent kidney; non-DCD kidney in control group	Cold storage Cold storage + NP Cold storage + HOPE Cold storage + HNPE	SCS (15 minutes)	HOPE vs NP ↓ROS ↓ TLR-4 + cell, ↓TNF-a release ↓activation endothelial cells
Hoyer et al. [42]	2014	DCD porcine kidney	HMPnoox HMPair HMPox100%	/	HMPox100%: ↑ renale flow ↓renale resistance ↓ LDH ↓ tubular dilatation ↓ gGT
Patel et al. [43]	2019	DCD porcine kidney	HMP/O2 95%	HMP-air	HMP/O2 95%: ↑ TCA ↑ ATP ↑ flow dynamics
Thuillier et al. [44]	2013	DCD porcine kidney	KAnoO2 KA	/	KA: ↓ serum creatinine peak ↓ interstitial fibrosis (3 mo: post transplantation)
Kron et al. [47]	2019	Rodent kidney	Allogeneic untreated Allogeneic TAC Allogeneic HOPE	Syngeneic control	Allogeneic untreated: death by AR Allogeneic TAC: No rejection Allogeneic HOPE: No rejection ↓ fibrotic remodeling ↑ kidney function
Darius et al. [46]	2020	DCD porcine kidney	2h HOPE + 20h HMP 20h HMP + 2h HMPO2	22h HMP 22h HOPE	early graft function 22h-HOPE > 2hHOPE+20hHMP > 20hHMP+2hHOPE > 22h HMP Renal flow 22h HOPE > 22h HMP 2h HOPE + 20h HMP > 20h HMP + 2h HMPO2 > 22h HMP Renal resistance 22h HOPE > 22h HMP 2h HOPE + 20h HMP ≈ 20h HMP + 2h HMPO2 > 22h HMP [Succinate] HMP > 20h HMP + 2h HMPO2 > 22h HOPE > 2h HOPE + 20h HMP [Lactate] HMP > 20h HMP + 2h HMPO2 > 22h HOPE > 2h HOPE + 20h HMP

NP: normothermic perfusion; HNPE: hypothermic de-oxygenated perfused; TLR-4: Toll-Like receptor-4; TNF-a: tumor necrosis factor-alfa; HMPnoox: HMP without oxygenation; HMPair: oxygenated HMP with air; HMPox100%: 100% oxygen gGT:gamma-glutamyl-transferase; TCA: tricarboxylic acid cycle; KAnoO2: Kidney Assist non-oxygenated MP; AR: acute rejection

A study by Kron et al. demonstrated the beneficial effects of HOPE on the immune response after allogeneic transplantation in murine models. In fact, in kidney recipients treated with HOPE without any immunosuppression, a lower immune response was observed, as assessed by a lower release of cytokines, T-cell activation and macrophages. In addition, these grafts showed better function and less fibrosis, resulting in significantly improved survival of the recipients, compared to untreated allogeneic controls (no HOPE, no immunosuppression) [47].

CLINICAL STUDIES

The 2009 study by Moers et al. opened a new chapter for the perfusion machine clinical utilization. In this randomized controlled trial, 672 transplants were randomly assigned to HPM or SCS and analyzed: HPM was demonstrated to reduce the risk of DGF (primary endpoint). In addition, a subgroup analysis including kidneys from EC-DBD demonstrated a significantly reduced risk of DGF and higher 1-year graft survival in machine-perfused kidneys compared with cold-stored kidneys [48].

After this study and with the increasing use of marginal grafts, several meta-analyses of randomised trials comparing HPM with SCS were performed (Tab. III).

O'Callaghan et al. carried out a meta-analysis of eighteen studies (7 RCTs and 11 non-RCTs) comparing hypothermic machine perfusion and static cold storage prior to 2013. Grafts undergoing HMP showed a reduction in the incidence of DGF, no differences in PNF and graft survival at one year, with no difference in the type of donor: DBDs, DCDs and ECDs [49].

A review by Tingle et al. showed that HMP reduces the risk of delayed graft function when compared to SCS. This benefit is significant in both donation following circulatory death and donation following brain death grafts and was demonstrated to be stronger for DCD grafts: in fact, the number of perfusions required to prevent one episode of DGF was 7.26 and 13.60 in DCD and DBD grafts,

Table III. Clinical studies. HPM *vs* SCS

O'Callaghan et al. Systematic review and meta-analysis of hypothermic machine perfusion *versus* static cold storage of kidney allografts on transplant outcomes (2013) [49].				
Study	**Year**	**Study design**	**Donor type**	**Outcomes**
Halloran et al.	1985	RCT	DBD,DCD	HMP ↓ DGF vs SCS No difference PNF, AR, long-term renal function or patient survival
Mozes et al.	1985	RCT	DBD	
Heil et al.	1987	RCT	DBD	
Danielewicz et al.	1996	RCT	DBD,DCD	
Van der Vliet et al.	2001	RCT	DCD	
Moers et al.	2008	RCT	DBD,DCD	
Watson et al.	2010	RCT	DCD	
Tingle et al. Hypothermic machine perfusion is superior to static cold storage in deceased donor kidney transplantation: a meta-analysis (2020) [50].				
Study	**Year**	**Study design**	**Donor type**	**Outcomes**
Alijani et al.	1985	Quasi-randomized	DBD	HMP ↓ DGF *vs* SCS in DBD and DCD HMP improves graft survival 1 and 3 years in DCD e DBD graft
Halloran et al.	1985	RCT	/	
Mozes et al.	1985	RCT	DBD	
Heil et al.	1987	RCT	DBD	
Merion et al.	1990	Quasi-randomized	DBD	
Veller et al.	1994	Unclear	DBD	
Matsuno et al.	1994	/	DCD	
Kwiatkowski et al.	1999	RCT	DCD	
Van der Vliet et al.	2001	RCT	DCD	
Moers et al.	2009	RCT	DBD,DCD	
Watson et al.	2010	RCT	DCD	
Chen et al.	2014	RCT	DCD	
Zhong et al.	2017	RCT	DCD	
Tedesco-Silva	2017	RCT	DBD	

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Table III. *continues*

Peng et al. Hypothermic machine perfusion versus static cold storage in deceased donor kidney transplantation: a systematic review and meta-analysis of randomized controlled trials (2019) [51].				
Study	**Year**	**Study design**	**Donor type**	**Outcomes**
Mozes et al.	1985	RCT	DBD	HMP ↓ DGF *vs* SCS HMP improve graft survival at 3 years No difference in: PNF, graft renal function, duration of DGF, AR, postoperative hospital stay and patient survival
Heil et al.	1987	RCT	DBD	
Halloran et al.	1987	RCT	/	
Jaffers et al.	1989	RCT	DBD	
Robert et al.	1990	RCT	/	
Matsuno et al.	1994	/	DCD	
Van der Vliet et al.	2001	RCT	DCD	
Kwiatkowski et al.	2009	RCT	DBD	
Moers et al.	2009	RCT	DBD,DCD	
Watson et al.	2010	RCT	DCD	
Tedesco-Silva et al.	2017	RCT	DBD	
Wang et al.	2017	RCT	DCD	
Zhong et al.	2017	RCT	DCD	
Bellini et al. Machine perfusion for abdominal organ preservation: a systematic review of kidney and liver human grafts (2019) [52].				
Study	**Year**	**Study design**	**Donor type**	**Outcomes**
Amaduzzi et al.	2011	RCT	DCD	HMP ↓ PNF and DGF ↑ one-year transplant survival No difference in serum creatinine or post-transplant eGFR
Bellini et al.	2019	Retrospective	DBD, DCD	
Dion et al.	2015	Retrospective	DBD, DCD, ECD	
Forde et al.	2012	Retrospective	DBD, ECD	
Forde et al.	2016	Retrospective	ECD	
Gallinat et al.	2012	RCT	DBD-DCD	
Gallinat et al.	2015	RCT	ECD	
Gallinat et al.	2017	Prospective	DBD	
Guy et al.	2015	Prospective	DCD, ECD	
Jochmans et al.	2010	RCT	DBD,DCD	
Kox et al.	2018	RCT	DBD,DCD,ECD	
Kuo et al.	2011	Retrospective	DCD,DBD	
Merion et al.	1990	RCT	DBD	
Moers et al.	2009	RCT	DBD/DCD	
Moustafellos et al.	2007	Retrospective	DCD	
Paul	2008	RCT	ECD	
Plata-Munoz	2010	Retrospective	DCD	
Sedigh	2013	Retrospective	ECD	
Tedesco-Silva et al.	2017	RCT	DBD	
Wang et al.	2017	RCT	DCD	
Yao et al.	2016	Prospective	DCD	
Yuan et al.	2014	Prospective	DCD	

RCT: randomized clinical trial; HMP: Hypothermic machine perfusion; SCS: static cold storage; DGF: delayed graft function; PNF: primary non- function; AR: acute rejection; ECD: expanded criteria donors

respectively. The study also showed strong evidence that HMP also improves graft survival in both DBD and DCD grafts, at both 1 and 3 years [50]. Comparable results in terms of DGF were also evaluated in the reviews by Peng et al. [51] and Bellini et al. [52], which also showed prolonged 1-year and 3-years survival. On the other hand, there is discordance on the effect of HMP for what concerns PNF rates in those studies [49,51-52].

HMP can be used in various fashions. The aforementioned studies examined continuous HPM, in which perfusion begins immediately after organ retrieval and ends at the time of transplantation; an alternative method is to

start perfusion upon arrival of the organ at the transplant centre, after an initial SCS period. One of the main disadvantages of continuous HMP is the necessity to transport the perfusion machine to the organ retrieval site, which could be logistically unfavorable in case of remote periferich hospitals. On the other hand, end-ischemic HMP simplifies retrieval logistics, as transportation of the organs is performed under static cold storage. Moreover, the retrieval surgical staff may come from a different center than the transplant one, and could not be familiar with machine function and settings. A study by Gallinat et al. showed that this second strategy was also superior to SCS [53]. Forty-three pairs of kidneys from the same donor were evaluated, one of which underwent SCS alone and the other underwent SCS plus end-ischemic HMP (E-HMP). E-HMP showed better results in terms of PNF (0 *vs* 9.3%, p = 0.048) and DGF, which resulted as the strongest independent risk factor for 1-year graft survival (RR 38.2, p < 0.001). These results are surprising if we consider that the cold ischaemia time was longer for kidneys reconditioned by e-HMP *vs* only-SCS (13,4 hours *vs* 12,1 hours). Furthermore, this method increased the organ acceptance rate of ECD kidneys, as 10% of the SCS- preserved kidneys cohort were discarded by other centers.
Compared with SCS, HMP is expensive. However, looking at the cost-benefit ratio, HPM is advantageous because it reduces the hospitalization time after transplantation and the number of patients undergoing post-transplant dialysis [50].

For what concerns HOPE, there are few clinical studies that have evaluated the addition of oxygen to perfusion (Tab. IV).
A doubleblind paired multicentre RCT by Jochmans et al. published on Lancet in November 2020, was designed to evaluate if the addition of oxygen to hypothermic perfusion, gives additional benefits on graft from DCD donor aged 50 years or older [54]. The study population was chosen because it represents the fastest growing source of donor kidneys and kidneys donated after circulatory death are more susceptible to the ischaemiareperfusion damage. The results demonstrated a reduction in severe complications (Clavien-Dindo grade ⩾ IIIb) post-transplant, an improvement in graft survival at 12 months post-transplant, and a reduction in the relative risk of acute rejection in patients treated with oxygenated hypothermic perfusion compared to the control group undergoing HMP alone. The reduction of organ rejection rates is mostly notable, as it contributes to reduce long-term graft survival producing scarring and fibrosis of the kidney tissue. Moreover, the reduction of rejection risk granted through oxygenated perfusion may imply consequences on short-term functional outcomes: in the study, in fact, more than a third (36%) of transplant failures in the HMP group occurred 3 months after transplantation, indicating that immunological factors were most likely at play [54]. Finally, these results are consistent with previous animal studies [41,47].
Contrary to end-ischemic HMP, contrasting results emerge from the literature looking at the combination of SCS

Table IV. Clinical studies. Hypothermic oxygenated machine perfusion.

Study	Year	Study design	Donor type	Outcomes
Jochmans et al. [54]	2020	Randomised, doubleblind, paired, controlled HMPO2 *vs* HMP	DCD	HMPO2: ↓ post-transplant complications ↑ graft survival at 12 months post-transplant ↓ relative risk of AR in patients treated with oxygenated
Husen et al. [55]	2021	Prospective, randomized, parallel group, participant-blinded, controlled, multicenter SCS alone *vs* SCS + end-HMPO2	DBD-ECD	No differences
Ravaioli et al. [56]	2020	Prospective interventional arm with a retrospective case-controlled arm, pilot non-randomized SCS (SCS-K) *vs* SCS+HOPE (HOPE-K)	DBD-ECD	HOPE ↓ DGF

HMPO2: oxygenated hypothermic machine perfusion; AR: acute rejection; SCS: static cold storage

followed by short-term HOPE. In a prospective randomized multicenter trial by Husen et al., kidneys from expanded criteria donors were randomized to either SCS alone or SCS followed by end-ischemic HOPE [55]. There was no difference between the two groups either for the primary end-point (graft survival at 1 year) or for the secondary end-points, which included delayed graft function, primary non-function, acute rejection, estimated glomerular filtration rate and patient survival. The fact that the mean perfusion time was 4.67 hours, with a minimum of two hours, may suggest that a longer or earlier period of oxygen exposure should be employed to obtain clinically relevant results. However, no clinical benefits were noted in kidneys that were perfused for a longer period or after a shorter period of SCS either.

A clinical trial by Ravaioli et al. showed quite different outcomes [56]. Each patient included in the study group was transplanted with an ECD graft, trated in the pre-implantation phase with HOPE. Patients' outcomes in the HOPE (HOPE-K) group were compared to the ones of matched controls, whose organs were preserved by SCS (SCS-K group). The study population showed better outcomes with only 20% DGF rate compared to 40% in the control group; notably, all DGF events in the HOPE-K group occurred when the perfusion time was less than 2 hours. Moreover, comparing K-HOPE patients who received a graft undergoing perfusion treatment for more than 2 hours with grafts with shorter perfusion times, the DGF rate was significantly lower (0 *vs* 40%, p = 0.04). In the study no graft failure was registered.

Another interesting evidence concerning the benefits of hypothermic oxygen perfusion comes from a case report by the same group [57]. Marginal kidneys from a 78-year-old DBD with normal renal function were declined by Italian transplant centers due to bioptic Karpinski's score (right kidney: 6; left kidney:7). Both kidneys were recovered and preserved by HOPE and their use for transplantation was re-evaluated using perfusion parameters. During perfusion, the left kidney progressively increased its flow by 34% and the right kidney by 50%, thus the kidneys were considered eligible for double-kidney transplant, which was performed successfully. The recipient serum creatinine values at one month and three months after transplantation were in the normal range. The results of this study suggested that HOPE could be a useful tool for re-evaluating and improving the organ quality of marginal kidneys discarded due to histological score, but with adequate pre-donation renal function.

FUTURE PERSPECTIVES

The benefits of oxygenated and non-oxygenated hypothermic perfusion over standard preservation methods for marginal grafts are well known from preclinical and clinical data. A key question during organ preservation is whether it is possible to predict the likelihood of a kidney graft function recovery by assessing certain parameters on perfusate. The most analyzed markers are lactate dehydrogenase (LDH), glutathione S-transferase (GST), alanine aminopeptidase, N-acetyl-b-D-glucosaminidase (NAG), and heart-type fatty acid-binding protein (H-FABP). In particular, GST levels are closely related to warm ischaemic time (WIT) and are also an independent risk factor for DGF [58]. Another marker, urinary neutrophilgelatinase-associated lipocalin (NGAL), results in higher concentrations in grafts that showed lower eGFR levels at subsequent follow-ups [56]. Recent interest was also raised by perfusate concentration of extracellular histones and microRNA-21. Extracellular histones are involved in numerous pathophysiological processes such as sepsis, thrombosis, inflammation, and vascular dysfunction but have not yet been studied in the process of kidney injury and organ viability in a clinical setting of organ donation and transplantation. In a study performed on 390 kidneys from DCD donors, their level correlated with PNF, DGF and graft failure one year after transplantation [59]. Pump parameters, such as renal resistance values and perfusion flow indexes registered during hypothermic organ perfusion, appear to be promising parameters to predict graft function [60-62].

Particularly a study by Bissolati et al. shown how resistance values during perfusion can be even more effective than pre-implant histology to predict PNF, DGF rate and postoperative creatinine trend [60].

A recent study evaluated the relationships between pre-implantation biopsy scores and renal parameters during perfusion, finding a positive association between global histologic score, resistance values at start and resistance reduction rate. Nevertheless, histologic score was independently associated with 6-month eGFR [63].

Combined evaluation of biomarkers alone or as conjugate biomarkers, together with pump parameters and histology could allow a safer and effective use of organs otherwise discarded.

One potential tool that can be used in perfusate analysis is metabolomic activity assessment through nuclear magnetic resonance (NMR) spectroscopy. In a study by Guy et al. [64] nuclear magnetic resonance spectroscopy has been used to examine the metabolomic profile of HMP perfusate from human cadaveric kidneys awaiting transplantation and to identify possible discriminators between the profiles of kidneys with delayed graft function and immediate graft function (IGF). The perfusates were evaluated at different times during perfusion. Grafts that developed DGF had lower glucose levels both at the beginning (45 minutes) and at the end of perfusion (4 hours). A difference in inosine, leucine and gluconate levels between the DGF group and the IGF group was also demonstrated [64].

In addition to the ability to predict graft function, another possible application of machine perfusion is the graft reconditioning to improve short- or long-term outcomes or to increase the available organ pool. In this setting, nutrients, therapeutic gases, mesenchymal stromal cells, gene therapies, and nanoparticles could be delivered to effectively repair an extended criteria organ during the preservation period and prior to implantation.

Cell therapy is one of the most widely used reconditioning strategies. Mesenchymal stromal cells (MSC) are multipotent cells derived from bone marrow, adipose tissue but also from other tissues such as the umbilical cord. They have similar properties to stem cells, so once they reach the target organ, they can differentiate and interact with various pathophysiological processes [65]. In addition to their ability to differentiate, they can transport organelles to damaged cells [66] or secrete growth factors, cytokines, and extracellular vesicles (EV), acting directly on other cells or modifying the surrounding microenvironment [67-70]. MSCs also exhibit anti-inflammatory and immune-regulatory properties as demonstrated in a clinical trial by Erpicum et al. [71]. In this study, ten deceased donor kidney recipients received bone marrow MSC infusion on 3 ± 2 post-operative days and were compared with 10 case-controls. On post-operative day 7, the estimated glomerular filtration rate (eGFR) in the MSC-treated recipients was higher than in the control cases, but no difference in eGFR at 1 year was found. Furthermore, MSC-treated recipients showed an increased percentage of regulatory T-cells and improved early allograft function.

A successful way to deliver MSCs seems to be intra-arterial graft administration, which allows to prolong the survival and contact of cells *in-situ*, and to avoid the dispersion of cells from the target organ [72]. Supplementation of perfusate with MSC during HMP has been assessed in pre-clinical e clinical studies. A study by Gregorini et al. assessed if the administration of MSC-derived EVs or MSCs during HMP protects DCD murine kidneys from ischemic damage. The underlying pathogenetic mechanisms were also analyzed. Kidneys were retrieved after 20 minutes of warm ischemia and perfused for 4 h with Belzer Solution (BS) or BS supplemented with MSC or EV. Renal damage was quantified by histology and renal gene expression. Significantly less global ischemic damage was recorded in kidneys subjected to MSC/EV, quantified as fewer histological lesions such as bubble formation, tubular necrosis, tubular lumen obstruction and a reduction in the global renal damage score. Furthermore, up-regulation of genes encoding for membrane ion transporters, higher pyruvate concentration and lower glucose concentration in the perfusion fluid was recorded, suggesting higher energy activity [20].

The same group performed a study on kidneys from DBD-ECD donors deemed unsuitable for transplantation to evaluate if hypothermic oxygenated perfusion (HOPE) with or without MSC-derived EV and subsequent normothermic reperfusion (NR) with artificial blood composed of bovine hemoglobin (HBOC) could prevent reperfusion injury [73]. In fact, conditioning with HOPE+EV and NR arrest the ischemic damage, prevents reoxygenation-dependent injury, and preserves tissue integrity. Indeed, kidneys only subjected to HOPE showed lower levels of COX-IV1, a component of COX, the last enzyme in the mitochondrial electron transport chain, which drives oxidative phosphorylation and is degraded under hypoxic conditions. The mitochondrial alteration was also confirmed by observation under an electron microscope. The increased vitality of the kidneys subjected to HOPE+EV was demonstrated by the IPT (tubular cell proliferation index), higher VEGF and HGF levels, higher glucose and lower lactate concentrations in the perfusate, showing that gluconeogenesis system in HOPE+EV group was preserved. This study suggests that EV delivery during HOPE can be considered a new organ preservation strategy for increasing the donor pool and improving transplant outcome.

Another opportunity offered by organ perfusion could be gene therapy. One of the most promising approaches would seem to be siRNAs (short-interfering RNAs) that induce degradation of homologous mRNA transcripts and block expression of the targeted gene [74]. Pre-clinical studies have shown their effectiveness in silencing genes linked to ischemia-reperfusion damage [75], but the problem of target organ specificity and short half-life when administered systemically remains. Administration of these during perfusion could be an advantage in terms of specificity and efficiency [76].

CONCLUSIONS

Hypothermic perfusion reduces the incidence of DGF in ECD-DBD and DCD, which are more susceptible to this type of complication. Further investigations are needed to assess long-term organ function.

Currently oxygenation of perfusate remains controversial; further clinical studies are needed to clarify the effect of different oxygenation parameters.

In addition, further research should be carried out to investigate other aspects such as whether machine parameters and perfusate analysis can predict graft function, and whether the addition of cells and cellular elements can recondition a graft to improve its function and consequently increase the donor pool.

Conflict of interest statement

The Authors declare no conflict of interest.

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