EXTENDED CRITERIA GRAFTS AND EMERGING THERAPEUTICS STRATEGY IN LIVER TRANSPLANTATION. THE UNSTABLE BALANCE BETWEEN DAMAGE AND REPAIR

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Abbreviations

ECD extended criteria donors

LT liver transplantation

HCC hepatocellular carcinoma

ICU intensive care unit

BMI body mass index

DCD donors after cardiac death

IRI ischemic reperfusion injury IRI

EAD early allograft dysfunction

PNF primary nonfunction

ITBL ischemic-type biliary lesion

MP machine perfusion

SCS static cold storage

HMP hypothermic machine perfusion

NMP normothermic machine perfusion

DCs dendritic cells

SEC Sinusoidal endothelial cells

KCs Kupffer cells

HSCs human stellate cells

ATP adenosine triphosphate

LPCs liver progenitor cells

TNF-α tumor necrosis alpha

NF-κB nuclear factor κB

HGF hepatocyte growth factor

EGF epidermal growth factor

TGF-β transforming growth factor beta

WIT warm ischemic time

CIT cold ischemia time

IC ischemic cholangiopathy

ROS reactive oxygen species

DAMPs danger-associated molecular patterns

ICU intensive care unit

HCV Hepatitis C Virus

DBD donors after brain death

NRP normothermic regional perfusion

cDCD controlled DCD

HOPE hypothermic oxygenated machine perfusion

D-HOPE dual-hypothermic oxygenated machine perfusion

MSCs mesenchymal stromal cells

EVs extracellular vesicles

BMMSCs bone marrow mesenchymal stem cells

HO-1 heme oxygenase

HLSC human liver stem-like cells

NPs nanoparticles

CNPs cerium-oxide nanoparticles

siRNA small interfering RNA

GalNAc N-acetylgalactosamine

CONFLICTS OF INTEREST

The authors disclosed no conflicts of interest

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ABSTRACT

Due to increasing demand for donor organs, “extended criteria” donors (ECD) are increasingly considered for liver transplantation (LT), including elderly donors and donors after cardiac death (DCD). The grafts of this subgroup of donors share a major risk to develop significant features of ischemic reperfusion injury (IRI), that may eventually lead to graft failure.

Ex-situ machine perfusion (MP) technology has gained much interest in LT, because represents both a useful tool for improving graft quality before transplantation and a platform for the delivery of therapeutics directly to the organ.

In this review, we survey ongoing clinical evidences supporting the use of elderly and DCD donors in LT, and the underlying mechanistic aspects of liver aging and IRI that influence graft quality and transplant outcome. Finally, we highlight evidences in the field of new therapeutics to test in MP in the context of recent findings of basic and translational research.

Keywords: liver transplantation, elderly donors, donors after cardiac death, IRI, aging, machine perfusion, nanotherapeutics

Key points

- Due to the shortage of organs available for transplantation "extended criteria" donors, as elderly donors and donors after cardiac death (DCD), currently represent an important resource in organ transplantation
- Liver aging and warm ischemia time of DCD donors contribute to liver vulnerability and increase the risk of severe ischemia-reperfusion injury (IRI) that can lead to poor graft outcome after transplantation.
- Ex-situ machine perfusion (MP) counteracts IRI and graft dysfunction before transplantation.
- Ex-situ MP may act as a platform to test new therapeutic strategies, as stem cells and nanoparticles.

1. INTRODUCTION

Liver transplantation (LT) is the "standard-of-care" treatment for selected patients with acute and chronic liver failure or hepatocellular carcinoma (HCC). The results of LT improved dramatically over the past 15 years [1] due to advances in surgical techniques, organ preservation, donor and recipient management and immunosuppression regimens. Nevertheless, the number of patients awaiting a LT far exceeds the available grafts and the waiting list mortality remains high. The use of so called marginal or "extended criteria" donors (ECD) has been proposed recently to improve the availability of donor allografts and reduce waiting list mortality [2]. Even if a worldwide definition doesn't exist, Eurotransplant defines ECD livers those grafts with one or more of the following criteria: donor age>65 years, intensive care unit (ICU) stay with ventilation>7 days, body mass index (BMI)>30, macro-steatosis>40%, serum sodium>165 mmol/l, SGPT>105 U/l, SGOT>90 U/l, serum bilirubine>3 mg/dl, donors after cardiac death (DCD) or euthanasia donor [3].

All ECD liver grafts share the common denominator of being more vulnerable in comparison to standard grafts to ischemia-reperfusion injury (IRI), hence carrying an increased risk of post-LT early allograft dysfunction (EAD), primary nonfunction (PNF) and ischemic-type biliary complications (ITBL). The key steps of IRI occur at several timepoints in LT management and involve multiple cells types that induce acute and chronic downstream graft injury acting via an interconnected network of molecular pathways [4].

Static cold storage (SCS) is the standard method for organ preservation and is characterized by prolonged hypothermic ischemic storage that increase susceptibility to IRI and allograft-related complications [5]. Ex-situ machine perfusion (MP) is a preservation method developed to protect organs from the detrimental effects of IRI, facilitate the repair/regeneration of ECD grafts in order to expand the donor pool and improve graft function after LT. Potential beneficial protective mechanisms of MP to improve outcome of marginal grafts have been demonstrated for both hypothermic (HMP) and normothermic machine perfusion (NMP) during preclinical and pilot clinical studies [6, 7]. Graft treatment during ex-situ MP is a very attractive approach to further improve its quality and the potentiality of using MP as a platform to objectively assess new therapeutics is emerging. Approaches to maximize long-term organ survival by combined use of MP and therapeutical agents are currently being investigated. More recently the use of stem cells, stem cell-derived extracellular vesicles (EVs) and nanoparticles represents a challenge in the bench-to-bedside research process [8].

This narrative review address on issues related to the use of ECD, focusing on elderly and DCD donors in LT, integrating clinical evidences and biological aspects such as liver aging and IRI. We highlight novel trends and future perspectives in the field of MP and on the clinical application of new therapeutic strategies, such as stem cells and nanotechnology in the context of recent findings of basic and translational research.

2. THE INFLUENCE OF DONOR AGE AND ISCHEMIA REPERFUSION INJURY ON LIVER TRANSPLANTATION

2.1 Mechanisms of liver ageing: morphology, metabolism and regeneration

The liver aging is driven by interacting factors as metabolic alterations, oxidative DNA damage, impaired regeneration, mitochondrial dysfunction, shortening of telomeres, and other processes that might determine the higher propensity of elderly donors graft to develop irreversible lesions and have negative impact on its survival [9-14].

Healthy aged livers exhibit morphological and metabolic changes, as volume reduction, changes in hepatic parenchyma, arteriolar walls thickening, brown aspect due to lipofuscin

aggregates, derived from the oxidation of lipids and proteins, and decrease in liver blood flow (Fig. 1) [15]. Aging affects liver synthetic function in terms of proteins and clotting factors but not of liver enzymes, which maintain relatively normal metabolic functions under physiological conditions [16]. Elderly livers are characterized by an increased lipid accumulation that promotes lipotoxicity and steatosis, a decreased gluconeogenesis and mitochondrial metabolism and by moderate age-related changes in biliary function [17]. The aging process is also globally driven by an unbalanced stimulation and response of the immune system. Monocytes/macrophages, natural-killer cells, regulatory T-cells and peripheral B-cells have decreased functions and aged dendritic cells (DCs) show defects in antigen presentation and T-cell activation.

Aging-related changes occur across multiple liver cell types. Sinusoidal endothelial cells (SEC) show a decrease in markers involved in organ regeneration, homeostasis and metabolism [18]. Hepatic macrophages (Kupffer cells, KCs) display phenotypic and functional modifications, as a proinflammatory phenotype, reduced autophagic activity and changes in phagocytic activity [10, 18, 19]. Aged hepatic stellate cells (HSCs) harbor activation markers, exhibiting a pro-contractile and proliferative phenotype, and show increased intercellular accumulation of lipids, that result exacerbated in presence of liver injury [14, 18, 20]}.

Senescent hepatocytes increase in size, show polyploidy, a state connected to restricted proliferation [21], lipid droplet accumulation, decreased mitochondrial metabolism, lower adenosine triphosphate (ATP) content and increased production of reactive oxygen species (ROS). As result of these alterations, hepatocytes show reduced metabolic and regenerative activities and are more susceptible to IRI [11]. Nevertheless, senescent hepatocytes, as wells as cholangiocytes, do not show telomere attrition, a widely recognized marker of cell senescence, as instead found in KCs and HSCs [12]. This difference might explain the preservation of peculiar features of hepatocyte and hepatic function despite organ atrophy in aging.

A clinically significant age-related change is a marked decline in the rate of hepatic regeneration that should be considered when transplanting an elderly organ. Although molecular mechanisms are still partially unknown in humans, the decrease in cell cycle and autophagy, the compromised differentiation of liver progenitor cells (LPCs), and the increase in apoptosis seem to play key roles. The process of liver regeneration involves multiple cellular processes and a complex interaction between cytokines and growth factors. Although hepatocytes rarely divide under normal circumstances, they can switch from quiescent to proliferative phenotype immediately upon hepatectomy, contributing to liver regeneration. Cytokines, such as tumor necrosis factor alpha, interleukin 6, and transcription nuclear factor kappa B (NF-κB) act in the first phase of liver regeneration switching the hepatocyte cell cycle from G0 to G1 [22, 23]. Cycle progression is then

driven by growth factors, such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF) [24]. Hepatocyte proliferative response to EGF is decreased in older animals compared to young animals, suggesting that aging impairs the responsiveness of the cells to growth factors [25]. Then, hepatocyte proliferation terminates under the regulation of transforming growth factor beta (TGF-β) and interleukin-1 (alpha and beta) [23].

Although the contribution of LPCs to liver regeneration is still uncertain some studies showed that the differentiation of LPCs is involved in the regeneration of animal livers [26]. LPCs are progenitor cells residing in the canal of Hering that can differentiate into hepatocytes and cholangiocytes in order to support liver regeneration [27,So, 2020 #238]. The ability of LPCs to respond to hepatic injury declines with age, as demonstrated in old mice, where LPCs failed to respond to the injury, resulting in impaired liver regeneration [9].

Autophagy is a cell pro-survival response that plays an important role in liver homeostasis and regeneration providing energy to hepatocytes derived by recycling damaged intracellular macromolecules generated in stress conditions, such as during hepatectomy [28]. Autophagy is mediated by lipid bilayer structures called autophagosomes, which sequester cytoplasmic materials and fuse with lysosomes to deliver their cargo that will be digested by lysosomal enzymes for recycling [13, 28]. Aging is characterized by a decrease in the number and function of autophagosomes and lipofuscin accumulation [29]. The accumulation of lipofuscin in lysosomes of senescent cells determine insufficiency of lysosomal degradative capacity of the intracellular macromolecules, compromising autophagy process. Lipofuscin deposition also hampers autophagic mitochondrial turnover, promoting the accumulation of senescent mitochondria, characterized by inefficient ATP production and oxidative stress that, in turn, trigger mitochondrial and lysosomal pro-apoptotic pathways and cell death [30,Xu, 2020 #90]. Since a reduction in autophagy is associated to aggravated IRI in aging livers, improving autophagy through pharmacological intervention could be an effective strategy to protect hepatocytes during and after IRI and promote regeneration in senescent livers.

2.2 Ischemic-reperfusion injury in elderly and DCD grafts

IRI is a crucial phenomenon characterized by an interplay of processes such as inflammation, autophagy, apoptosis and cell death [31, 32], and is responsible for transplant dysfunction and failure. Donor age, prolonged ischemia time and organ recovery capacity have a relevant contribution to the severity of IRI in LT and clinical reports suggest a synergistic effect between increasing donor age and prolonged ischemia on PNF, EAD and ITBL.

To distinguish between ischemic phase and reperfusion is fundamental to understand the mechanism of IRI (Fig.2). Ischemia determines the transition from aerobic to anaerobic metabolism with consequent depletion of ATP deposits in hepatocytes, SECs and KCs and the consequent tissue hypoxia and cell damage [4]. Older livers have less functional reserves, due to smaller volumes, less total blood flow and regenerative potential compared to younger livers, resulting more susceptible to ischemic injury [33]. Moreover, they have fewer functional mitochondria which are more susceptible to ATP depletion during cold storage and subsequent warm reperfusion. According to the surgical practice, ischemic injury can occur during the warm ischemia time (WIT) or the cold ischemia time (CIT) [4]. WIT is particularly prolonged in DCD donors [34, 35]. During WIT the liver graft maintains a normal ex-situ metabolic degree but, in absence of oxygen and nutrients, the anaerobic activity leads to the downfall of ATP and lactic acid accumulation, resulting in local tissue acidosis, damage of membranous sodium-potassium ATPases pump and following intracellular calcium influx, with subsequent cell swelling and detachment of ribosomes [36, 37]. Moreover, blood stasis and damaged SECs increase the risk of intravascular thrombosis [38, 39]. Study results offer conflicting evidence regarding microthrombi formation in DCD livers. An animal model demonstrated the evidence of microthrombi formation in the peribiliary vascular plexus [40]. A human study confirmed the presence of peribiliary vascular microthrombi in DCD and donors after brain death (DBD) liver grafts, but without correlation to the development of ITBL [41]. In contrast, Verhoeven et al. [42] reported no evidence of increased microthrombi formation in human DCD liver grafts. Additionally, the use of tissue plasminogen activator in a prospective randomized controlled failed to demonstrate a protective effect for ITBL [43]. Rapid cooling during procurement is beneficial, as it slows the metabolic rate and decreases mitochondrial oxidative stress upon reperfusion [44], and it still represents the gold standard for organ preservation [45]. However, in the context of DCD donation, this paradigm should be revised. In fact, the introduction of normothermic regional perfusion (NRP) worked as a perfusion bridge between asystole and procurement, provided a means of assessing liver function before transplantation, and allowed to switch an urgent procedure in an unhurried donor operation, minimizing the risk of graft injury during the procurement. Moreover, numerous evidences exist that NRP enables organ repair, converting circulatory arrest into a period of ischemic preconditioning and preparing the liver graft for the subsequent cold storage period [46]. After prolonged CIT, the allograft is metabolically impaired and its outcome depends on its ability to recover from the ischemic injury [47, 48]. Recently, the UK DCD risk score included CIT as a predictor of graft loss and PNF after DCD LT [49].

The reperfusion occurs under normothermic conditions, either following organ implantation or ex-situ on a perfusion device [4, 50]. As above-mentioned, the reperfusion phase exacerbates the initial injury, leading to massive release of ROS from enzymatic sources such as the xanthine-oxidase system, uncoupled nitric-oxide synthase (NOS) system, NADPH-oxidase system and mitochondrial electron transport chain. The mitochondrial injury causes further energetic depletion and triggers cell death by ROS and death-associated molecular patterns (DAMPs). DAMPs activate both KCs and DCs through reaction with Toll-like receptors (TLRs), provoking a sustained inflammation with release of cytokines and chemokines and recruitment and activation of additional neutrophil leukocytes and monocytes [51, 52].

Severe IRI can cause cell dysfunction with different death mechanisms such as, apoptosis, necrosis and autophagy, according to the triggering pathways activated by the IRI process [29, 31]. Recent studies on inflammation and IRI elucidated the characteristics of necroptosis, a form of cell death that shared both properties of apoptosis and necrosis, characterized by the disruption of the plasma membrane, lysis of a swollen cell and release of DAMPs, exacerbating tissue damage with subsequent EAD and PNF [52, 53]. The role of autophagy has not been clarified yet in LT. While some studies have shown a defective autophagy in liver following IRI [54-56] an increase has been observed in others, with beneficial [57, 58] or detrimental effects [59, 60]. The protective properties of autophagy might be important in restoring cellular homeostasis and function in marginal grafts in which autophagy is impaired. However, an excessive autophagy will prompt cell death by apoptosis and necrosis. A recent study in a murine model demonstrated that hypothermic-oxygenated machine perfusion (HOPE) could attenuate DCD liver injury by increasing autophagy levels [61]. Further studies should examine whether the stimulation or the inhibition of autophagy influence graft recovery, so to improve transplant outcomes when ECD grafts are used.

3. THE SYNERGISTIC EFFECT OF AGEING AND ISCHEMIC DAMAGE IN ELDERLY AND DCD GRAFTS IN LIVER TRANSPLANTATION. THE CLINICAL EXPERIENCE.

3.1 Elderly grafts in liver transplantation

The use of grafts from older donors in LT is growing in response to the substantial gap between supply and demand. Since elderly grafts can be associated with increased risk of complications such as EAD and ITBL [62], an exhaustive assessment of donor and recipient characteristics is mandatory. In fact, many donor variables have been identified as associated with increased risk of graft failure and mortality: [63, 64] donor age>60 years, DCD donor type, prolonged WIT, partial

grafts, CIT>10 hours, ICU stay>5 days, reduced donor height [65], severe graft steatosis [66] and positive donor HCV- and HBV-status [67].

An analysis of 11 studies including 30,691 LT cases, showed that using grafts from older donors significantly increased the pooled risks of graft failure by about 66%, and that older donors had a significant effect on post-LT prognosis, showing a linear trend, with increments in graft failure of about 12% per 10-years increase in donor age. [68]. An analysis from the UK database on DCD [49] showed that properly selected donors (age<60, BMI<28, functional-WIT<20 minutes, CIT<6 hours) and appropriate donor-to-recipient match (primary transplant, recipient MELD<26, recipient age<60) grant a 5-years graft survival around 80%. A more recent analysis using UNOS data demonstrated that, despite the mortality risk for DCD LTs decreased over time, DCD grafts still suffer a higher discard rate and lower survival than DBD grafts. [69].

From the first successful transplant of an 86-year-old liver graft [70], studies showed favorable overall long-term results using octogenarian and even nonagenarian donors when proper donor evaluation and donor-to-recipient matching are assured [71-77]. A retrospective cohort study performed on the Eurotransplant database highlighted a linear association between donor age and graft failure and that the increased risk of graft failure is associated to prolonged CIT [48]. These findings were confirmed by two other studies [74, 78]. Indeed, shortening CIT represents an efficient strategy to improve outcome [48, 79].

Another worrisome aspect about the use of very old graft is the potential risk for vascular complications. After a survey within UNOS Registry found the use of old donors (>70 years) was associated with an incidence of hepatic artery thrombosis of 3.2%, which corresponded to a 66% increased risk compared with the population of donors<40 years [80], other papers reported a low rate of vascular complications when using very old grafts [81]. At back table, careful assessment of vessels quality and patency, and a low threshold for discarding liver grafts in the presence of occlusive atherosclerosis are crucial for reducing the incidence of vascular complications with older livers [82]. Vascular quality is even more important in the context of ex-situ perfusion, in which vessels manipulation and cannulation may promote arterial damage or dissection.

The rate of biliary complications after LT, which include a wide spectrum of functional and anatomical abnormalities, varies from 10 to 40% and may lead to graft loss and patient death [83-85]. Previous series highlighted that donor age is an independent risk factor for ITBL [77, 86, 87]. The incidence of ITBL ranges from 3.9% to 25% according to donor, transplant, graft, and recipient characteristics [87]. Although great variability in incidence rates may partly be accounted for by different definitions of ITBL, higher percentage are reported when older and DCD grafts are used

[86]. ITBL may ultimately lead to graft loss and patient death with estimated 10-year graft failure rates of 20%- 50%.

Three mechanisms are credited to be involved in ITBL: IRI [88], bile salts [89] and immune-mediated mechanisms [90].

IRI of the epithelial lining of the bile ducts has long been viewed as the main determinant of the development of ITBL. Clinical studies have demonstrated that extensive injury to and loss of the biliary epithelium can be found in over 90% of livers transplant [41, 91, 92], but only a minority of these livers develop ITBL. This suggest that insufficient regeneration of the biliary epithelium, rather than the initial amount of injury determines post-transplant cholangiopathy [41]. Regeneration and repair of biliary epithelium may result from proliferation of mature cholangiocytes aligning the bile duct lumen or from proliferation and migration of epithelial cells from the peribiliary glands (PBG). The deeper parts of PBG of extrahepatic and intrahepatic bile ducts have been identified as niches of multipotent stem/progenitor cells. [93]. Interestingly, histological injury of the deep, extramural PBG of the donor bile duct at the time of transplantation has been identified as an risk factor for the development of ITBL [41]. suggesting that the regenerative capacity of bile ducts rather than the initial amount of biliary epithelial injury determines whether a liver develops post-transplant cholangiopathy.

3.2 DCD, age and liver transplantation

DCD donors represent a specific type of ECD for whom death is declared on cardiopulmonary criteria rather than cessation of whole brain function. DCD liver grafts are increasingly used to overcome the general organ shortage despite increased risks of graft failure and ITBL remain critical concerns [35]. DCDs are classified into four categories as part of the Maastricht criteria. Most donors worldwide are controlled DCD Category-III donors. However, even uncontrolled DCD Category-II donors are used in clinical practice, especially after the introduction of NRP [94] and ex-situ MP. LT from DCD grafts is associated with an increased incidence of PNF (3-7%) and ITBL (2-40%). In addition, up to 10% of the recipients die due to graft related causes with an additional 10% of patients being re-listed for transplantation within a year of the initial transplant [95-97]. Graft quality assessment is notoriously difficult and heavily relies on surgeon's experience. As a result, only a small percentage of DCD livers are used.

The influence of donor age (namely>60 years) on the results of DCD-LT is debatable. In UK DCD donors account for 30-40% of the whole pool [98, 99]. Limitations of risk factors, advances in graft preservation, and immunosuppression significantly improved clinical outcomes [100]. Recently, it

has been demonstrated that with appropriate recipient selection and limitations of donor risk factors, DCD livers yield outcomes similar to DBD [98].

During the last years a significant shift of median donor age in DCD cohort towards 70 years was noted. De Vera et al. [101] found that only transplantation of donors aged>60 years (RR=5.61;p=0.05) was an independent predictor of biliary complications (DCD vs DBD median donor age and biliary complications rate were 37 vs 39 years (p=0.23) and 25% vs. 13% (p<0.001) respectively), while De Oliveira et al. [102] showed that donors>60 years did not reach statistical significance as possible causes of biliary complications (DCD vs DBD median donor age and ITBL rate were 49 vs 41 years, p=0.0002 and 2.5% vs. 0%, p<0.005, respectively) and Schlegel et al. [49] did not find a significant difference in graft loss related to DCD donor age (median donor age 49 years). Cascales-Campos et al. [103] compared, in a monocentric series, LT results between DCD and DBD donors and the influence of donor age: no differences in terms of post-LT complications were found between DCD and DBD donors>70 years. Graft and patient overall survival, PNF, hepatic artery thrombosis, ITBL, biliary complications and rate of re-LT were similar between the two groups (DCD vs DBD median donor age and biliary complications rate were 65.3 vs 68.4 years (p=0.78) and 6% vs. 1% (p<0.21), respectively). Ruiz et al. [100], in a recent single-center experience transplanted 13 livers from controlled DCD (cDCD) donors>65 years achieving the same perioperative and midterm outcome as younger donors (no cases of ITBL and no graft loss observed over a medium follow-up period of 19 months). The authors justified these favourable results by using NRP. NRP preservation seems to limit arteriolar and biliary necrosis reducing post-operative biliary complications. A recently published observational cohort study on cDCD showed that the use of post-mortem NRP in cDCD LT reduces post-operative biliary complications (OR 0.14;p<0.001), ITBL (OR 0.11;p=0.008) and graft loss (HR 0.39;p=0.008), and allows LT even from cDCD donors of advanced age [99].

4. STRATEGIES TO EXPAND THE POOL OF AVAILABLE GRAFTS USING ELDERLY AND DCD LIVERS: CURRENT AND FUTURE APPLICATIONS OF MACHINE PERFUSION TECHNOLOGY

4.1 Machines perfusion technology

Several strategies have been introduced to increase donor pool and improve outcomes when ECD are used. Compared to SCS, MP is a dynamic preservation approach that offers the opportunity to resuscitate grafts repairing putative cell injury and assess organ viability before implantation. Based on temperature, oxygenation and type of perfusion, we distinguish the following type of MP

technology: HMP (perfusate solution temperature ranges from 4-11°C), HOPE (perfusate solution is actively oxygenated), dual-hypothermic oxygenated machine perfusion (D-HOPE) (graft is perfused from both portal vein and hepatic artery), NMP (liver is perfused using a blood-based solution at a temperature ranging from 32-38°C).

Hypothermic perfusion is based on the concept that ischemic livers, treated by cold oxygenated perfusion, are characterized by significant less mitochondrial ROS-release with subsequent less downstream inflammation and provides uploaded cellular energy reserves before implantation [104]. Guarrera et al. were the first to report the use of HMP in 2010 [105] and in 2015[106] they published a series of 31 "orphan" livers successfully transplanted after HMP reconditioning. Then, Dutkowski et al. [107] showed fewer biliary complications rate and a better 1-year graft survival comparing DCD livers undergoing HOPE versus SCS matched controls. Good 5-years graft survival of hyphotermically perfused DCD livers was recently reported by Schlegel et al. [108] underlying the potential role of HOPE in marginal organs reconditioning. Similarly, D-HOPE showed excellent graft survival and reduced biliary complications rate[109, 110].

During NMP, grafts are preserved under quasi-physiological conditions allowing the restoration of cellular metabolism and replenishment of ATP homeostasis. Its role in human setting was first documented by Ravikumar et al. [111] in a non-randomized prospective trial reporting no differences with SCS controls and demonstrating the safety and feasibility of this preservation technology. Afterwards, Nasralla et al. [112] performed the first NMP vs SCS randomized trial demonstrating improvements of NMP in terms of reduced post-LT transaminases peak and increased organ utilization (50% fewer discarded organs). Positive effects on marginal grafts have been reported by Watson et al. [113] in an observational study of 47 discarded livers, in which 22 were successfully transplanted after NPM, while Ghinolfi et al. in a pilot, prospective randomized trials comparing NMP to SCS in very old grafts, showed no difference in terms of clinical or biochemical outcomes [6].

Despite numerous studies in this field, it is still unclear which perfusion procedures are most effective in the complex scenario of LT. Results of most important clinical studies using MP technology are summarized in Table 1.

4.2 Machine perfusion: a platform for organ therapeutics.

MP has the further potential to act as a platform for testing the effects of therapeutic agents directly on the organ, thus avoiding many of the limitations associated with the systemic administration. New approaches to maximize long-term organ survival by combined use of MP and therapeutical agents are currently being investigated and include use of stem cells, stem cell-derived extracellular

vesicles (EVs) and nanoparticles for the targeted delivery of drugs or genetic material to the donor organ (Table 2).

In the pursuit of transplantation, cell therapy involves EVs and many cell types, such as mesenchymal stromal cells (MSC), DCs, regulatory T-cells and B-cells, that because of anti-inflammatory and pro-regenerative activities can contribute to the reduction of IRI. However, the use of cell therapeutics showed some limitations due to limited half-life in the body after intravenously administration and non-specific targeting to an organ after transplant. Currently, preclinical studies have highlighted as stem cells and cell-derived EVs administered during MP may have a potential therapeutic in the field of LT [114].

Bone marrow MSCs (BMMSCs) have highly immunomodulatory and regenerative capabilities and play an important role in the study of organ damage repair participating in the anti-inflammatory response, regulating transplantation immunity and improving IRI [115]. BMMSCs were assessed during NMP on DCD liver quality, studying the changes in donor liver microcirculation. The association of BMMSCs and NMP pre-transplantation improved DCD liver microcirculation and quality compared to NMP alone, reducing hepatocyte apoptosis and mitochondrial damage, inhibiting intrahepatic macrophage activation and restoring endothelial function [116]. Moreover, the same authors have also demonstrated that NMP combined with BMMSCs relieves oxidative stress injury inhibiting NF-κB pathway and improves mitochondrial function in rat DCD livers [117]. In another report, BMMSCs were modified with heme oxygenase (HO-1/BMMSCs), a potent cytoprotective enzyme that prolongs the survival time of BMMSCs, and perfused into DCD rat liver grafts by NMP. HO-1/BMMSCs combined with NMP exerted protective effects on DCD livers and significantly improved recipient prognosis [118].

EVs, small vesicles released by cells, play a pivotal role in cell-to-cell communication via the shuttling of cargo molecules (protein, RNA, DNA, miRNA etc.) characteristic of their parent cell [119]. EVs derived from human liver stem-like cells (HLSC), a population of stem-like cells resident in adult liver with regenerative properties [120], DCs [121] and from hepatocytes [122], play a therapeutic role to counteract IRI through mitochondrial autophagy, increasing immune inhibition, proliferation and regenerative processes [123]. Rat liver perfused with HLSC-EV showed lower levels of hypoxia inducible factor-1, a marker of hypoxic tissue injury, as well as markers of liver damage in comparison with controls, providing a rationale for a possible pharmacological intervention with MSC-EV during NMP [120].

Nanotechnology in LT management represents a new strategy to mitigate the inevitable effects of damage from IRI and recover marginal organs prior to transplantation. The nanoparticles (NPs) used for clinical use are composed by natural/organic materials (such as liposomes, polymers,

proteins, etc.) or inorganic materials (such as gold, iron oxide, quantum dots, etc.), but new and efficient material are emerging. The use of appropriately modified NPs able to recognize the target organ and carry therapeutic agents, represents advantages over systemic therapy, such as the use of lower dosages, reduced systemic side effects and localized and controlled drug delivery [124].

Immunosuppressive drugs are systemically administered to patients to prevent acute and chronic graft rejection, but can cause significant side effects. Lately, the research is focusing on the possible use of NPs as delivery vehicles for small-molecule immunosuppressive compound into both graft (ex-situ) and recipient [125]. Dependent on physiochemical properties, NPs can directly interact with the constituents of the immune response macrophages, antigen presenting cells, B-cells or T-cells, and exhibit an array of immunosuppressive effects. For example, metal-oxide nanoparticles can directly affect adaptive immune cells [126]. The study of Nadig et al. has demonstrated that micelle NP modified with targeting ligand for endothelial cells and containing rapamycin, antibiotic that inhibits T-cell effectors and protects the endothelium, confer local immunosuppressive effects and reduce oxidative stress in endothelial cells, without systemic side effects [127]. These results suggest the feasibility targeted drug delivery and their application to an allograft ex-vivo in a perfusion solution prior to implantation. The therapeutic potential for targeted nanomedicines delivered during ex-situ NMP has been showed in the study of Tietjen Gt et al., in that surface NP conjugation with an anti-CD31 antibody enhances targeting of NPs to graft ECs of human kidneys undergoing NMP [128].

Although the importance of inhibiting oxidative stress has been recognized in hepatic IRI there is currently no approved pharmacological intervention in clinical practice. It has been demonstrated that the use of antioxidant a-tocopherol during HMP in a DCD rodent model [129] improve liver graft preservation, limiting mitochondrial oxidative stress and related inflammatory mediators. The antioxidant molecules have characteristics that affect their use in clinical applications, including poor water solubility, short biological half-life as well as non-specific removal by the vascular endothelial and the mononuclear phagocytosis system. Encapsulations of antioxidants into NPs could represent a potential solution to these problems. The use of cerium-oxide nanoparticles, already known as the antioxidant and anti-inflammatory agents [130, 131], and NPs containing carnosic acid, a natural antioxidant, alleviated the symptoms of hepatic IRI by scavenging ROS and pro-inflammatory response in animal models of ischemic hepatic injury [132-134], suggesting their future use as prophylactic agent for the treatment of hepatic IRI in LT

Another strategy is delivery of genetic material against key receptors or enzymes implicated in IRI injury. However, studies into gene therapy within the MP platform is still in its early stage. An effective approach for attenuation of oxidative stress during IRI may be the antioxidative gene

delivery strategy aimed at increasing levels of antioxidant enzyme expression. Mice pretreated with NPs containing gene plasmid for superoxide-dismutase and catalase, key enzymes in ROS detoxification, provided elevated antioxidative enzyme activity as the result of the gene delivery in the liver and protection against hepatic I/R injury [135].

Besides, the use of small interfering RNA (siRNA) is a promising strategy to silence specific genes implicated in IRI, but their application in clinical setting is still limited due to non-specific absorption of all tissues, instability in the blood and rapid degradation by serum nucleases. To overcome this issue, siRNA is encapsulated in NP targeting specific tissue. A hepatocyte-specific delivery system was obtained using N-acetylgalactosamine (GalNAc) that recognize asialoglycoprotein receptors overexpressed in hepatocytes. Conjugation of GalNAc to fully chemically stabilized siRNAs enable efficient hepatocyte delivery both in vitro and in vivo [136]. Moreover, galactose-conjugated liposome nanoparticles containing a siRNA for Toll-like receptor 4, critical mediator of inflammation and organ injury, showed a therapeutic effect associated to inflammation and ROS decrease and an overall reduction of organ damage [137]. SiRNA delivery with machine perfusion may be particularly useful for improving the viability of marginal organs by alleviating IRI. SiRNAs added to the perfusate solution have been successfully delivered to rat liver grafts during NMP and HMP. In addition, lipid-based nanoparticles containing siRNA targeting apoptotic gene p53 were successfully uptaken by the rat liver during NMP, as demonstrated by confocal images. The uptake was also associated to reduced levels of the inflammatory cytokines compared with control [138]. Gillooly AR et al. applied siRNA to rat livers via ex vivo MP before transplantation, demonstrating as siRNA therapy during MP is a promising frontier to improve graft dysfunction. Lipid NP with a siRNA targeting Fas receptor, involved in apoptotic process, were added to perfusion solution during NMP and HMP, showing SiRNA-lipid NP uptake into the endothelium and central veins, with increased uptake in the HMP [139].

CONCLUSIONS

Several clinical studies produced encouraging results to expand the donor pool with elderly and DCD donors. These allografts are more vulnerable to IRI because suffer from aging-related morphological and functional alterations, and from prolonged warm and cold ischemia that contribute to poor graft outcome after LT. Ex-situ MP technology has been investigated to evaluate pre-transplant graft function and to improve the quality of marginal livers prior to transplantation. Moreover, MP may provide a unique opportunity as platform to test new therapeutics directly on graft before transplantation. Preclinical studies on animal models and discarded livers are ongoing to test the potential of stem cells and nanoparticles therapy to attenuate IRI in LT, promote

engraftment after transplantation and ultimately improve post-LT outcomes. Future efforts are required to reach the successful translation of promising new therapeutics to the clinical practice.

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TABLES

Table 1. Clinical studies using MP technology

Author, Center, Year	Type of study	Type of MP	*N*	Type of donors	Donor age (mean)	EAD* n, (%)	1-year patient survival (%)	1-year graft survival (%)	Biliary complication n, (%)	ITBL n, (%)
Ravikumar et al., Multicenter, 2016	CT, phase 1	NMP	20	16 DBD 4 DCD	58	3(15)	95	N.A.	3(15)	0
Selzner et al., Toronto (CA), 2016	CS	NMP	10	8 DBD 2 DCD	48	N.A.	N.A.	N.A.	0	0
Bral et al., Edmonton (CA) 2017	CS	NMP	9	6 DBD 3 DCD	56	5(55.5)	N.A.	N.A.	0	0
Nasralla et al., Multicenter, 2018	RS	NMP	121	87 DBD 34 DCD	56	12(10)	95.8	95	13(10,7)	7(5,7)
Bral et al., Edmonton (CA), 2019	CS	NMP	43	43 DBD 10 DCD	37 bb 40 lc	5(19) 6(35)	N.A.	N.A.	4(15) bb 4(24) lc	0 0
Ceresa et al, multicenter (UK), 2019	CS	NMP	31	23 DBD 8 DCD	58	4(13)	90	84	0	0
Ghinolfi et al, Pisa (IT), 2019	RS	NMP	10	DBD	81	2(20)	N.A.	N.A.	1(10)	0
Matton et al., Groningen (NL), 2019	CS	NMP	4	DCD	N.A.	N.A.	N.A.	N.A.	0	0
Mergental et al., Birmingham (UK), 2016	CS	NMP	5	1 DBD 4 DCD	49	N.A.	N.A.	N.A.	0	0
Watson et al., Cambridge (UK), 2017	CS	NMP	22	6 DBD 16 DCD	56	N.A.	92	83	4(18,1)	4(18,1)
Mergental et al., Birmingham (UK), 2020	CT, phase 2	NMP	22	12 DBD 10 DCD	56	7(31,8)	100	86.4	6(27,2)	4(18,1)
Raigani et al., Boston, 2020	CS	NMP	12	5 DBD 7 DCD	44	N.A.	N.A.	N.A.	N.A.	N.A.
Guarrera et al., New York (USA), 2015	CS	HMP	20	DBD	39	1(5)	90	90	2(10)	2(10)
Guarrera et al. New York (USA), 2015	CS	HMP	31	N.A.	57.5	6(19)	83.8	81	4(12,9)	0

De Carlis et al., Milan (IT), 2017	CS	HMP	2	DBD	18 and 65	N.A.	N.A.	0	0	0
Dutkowski et al., Zurich (CH), 2014	CS	HMP	8	DCD	54	N.A.	N.A.	N.A.	2(25)	0
Dutkowski et al., Multicenter, 2015	CS	HMP	25	DCD	54	5(20)	N.A.	90	5(20)	0
Kron et al., Zurich (CH), 2017	CS	HMP	6	5 DCD 1 DBD	65	N.A.	100	100	N.A.	0
van Rijn et al., Groningen (NL), 2017	CS	HMP	10	DCD	53	N.A.	100	100	N.A.	N.A.
Dondossola et al, Milan (IT), 2019	CS	HMP	6	DCD	51	2(33)	N.A.	N.A.	0	0
Schlegel et al., Multicenter, 2019	CS	HMP	50	DCD	57	N.A.	96	96	20(40)	4(8)
Patrono et al, Turin (IT), 2018	CS	HMP	25	DBD	70	8(32)	N.A.	N.A.	4(16)	2(8)
Rayar et al., Rennes (FR), 2020	CS	HMP	25	DBD	70	28	91	88	2(8)	N.A.
Muller et al., Zurich (CH), 2020	CS	HMP	93	DCD	61	N.A.	86	93	32(34,4)	8(8,6)
Ravaioli et al., Bologna (IT), 2020	RS	HMP	10	DBD	77.5	0	100	100	1(10)	N.A.
Patrono et al, Turin (IT), 2020	CS	HMP	50	47 DBD 3 DCD	69	11(22)	N.A.	N.A.	N.A.	N.A.
van Rijn et al., Multicenter 2021	RS	HMP	78	DCD	52	20(26)	92	NA	34 (43)	5(6)
van Leeuwen et al., Groni. (NL), 2019	PCT	HMP +NMP	11	DCD	63	N.A.	100	100	4(36,3)	1 (9)

MP: machine perfusion; EAD: Early Allograft Dysfunction, *from Olthoff KM et al., *Ann Surg.* 2015;262(3):465-475; ITBL: Ischemic-type biliary lesions; CT: clinical trial, NMP: Normothermic Machine Perfusion, DBD: donation after brain death; DCD: donation after circulatory death; N.A.: not available; CS: cohort studies; RS: randomized studies; HMP: Hypothermic Machine Perfusion; bb: back-to-base; lc: local; PCT: prospective clinical trial.

Table 2. Potential therapeutic strategies in MP

Stem cells and EV-derived stem cells	Effects	Model	Ref.
BMMSCs	Reduce hepatocyte apoptosis and mitochondrial damage, inhibit intrahepatic macrophage activation and restore endothelial function	DCD rat liver NMP	[116, 117]
HO-1/BMMSCs	Low transaminase levels, preserve liver morphology, and decrease proinflammatory cytokine levels. Effects mediated via HMGB1 expression and TLR4 pathway inhibition	DCD rat liver NMP	[118]
EV-HLSC	Reduce histological damage, apoptosis, RNA overexpression of hypoxia-inducible factor 1-alpha and transforming growth factor-beta 1	rat liver hypoxic NMP + EV-MSC	[120]
EV-DCs	Decrease liver IRI injury, inflammatory cytokines, improve liver function	Rat hepatic IRI	[121]
EV-hepatocytes	Increase hepatocyte proliferation in vitro and in vivo	In vitro hepatocytes and murine hepatic IRI	[122]
Nanoparticles	**Effects**	**Model**	**Ref.**
Micelle NP targeting ligand for endothelial cells and containing rapamycin	Immunosuppressive effects, reduce oxidative stress	In vitro endothelial cells	[128]
CNPs	Decrease of hepatocyte and several serum inflammatory markers Prevent hepatic IRI, decrease ROS generation and pro-inflammatory response	Male Sprague Dawley rats Mouse hepatic IRI	[132] [133]
Chitosan NPs containing carnosic acid	Reduce liver ischemia/reperfusion injury, show anti-oxidative, anti-apoptotic and anti-inflammatory properties	C57BL/6 mice hepatic IRI model	[134]
Galactose-	Decrease inflammation and ROS	Mouse warm hepatic IRI	[137]

conjugated liposome NPs with a siRNA for TLR4Toll-like receptor 4	generation Overall reduction of the injury area and organ damage		
Lipid-based NPs with a siRNA targeting p53	The uptake of siRNA reduced levels of the inflammatory cytokines compared with control	Rat liver NMP	[138]
Lipid NPs with a siRNA targeting Fas receptor	SiRNA-lipid NP uptake into the endothelium and central veins, with increased uptake in the HMP	Rat livers NMP and HMP	[139]

DCD: donors after cardiac death; IRI: ischemic reperfusion injury IRI; MP: machine perfusion; DCs: dendritic cells; ROS: reactive oxygen species; EVs extracellular vesicles; BMMSCs: bone marrow mesenchymal stem cells; HO-1: heme oxygenase; HLSC: human liver stem-like cells; NPs: nanoparticles; CNPs: cerium-oxide nanoparticles; TLR4: Toll-like receptor 4 siRNA small interfering RNA; GalNAc: N-acetylgalactosamine

LEGENDS TO FIGURES

Fig. 1 Liver aging mechanisms. During aging, a number of structural, metabolic and molecular alterations have been described. These alterations may result in impaired liver function.

Fig. 2 Different phases of IRI influencing graft quality and graft/recipient outcome**.**

During WIT the liver graft maintains a normal ex-situ metabolic degree but, in the absence of oxygen and nutrients, the anaerobic activity leads downfall of ATP and accumulation of lactic acid, resulting in local tissue acidosis, intracellular calcium influx, with subsequent cell swelling and detachment of ribosomes.

The ischemic phase is characterized by cell damage while the reperfusion phase is characterized by inflammation. However, these two processes are not completely disjointed because, during ischemia-induced cell damage, several molecules involved in inflammatory processes, can be released, exacerbating cell damage during the reperfusion phase. Contents and fragments of dead cells can act as death-associated molecular patterns (DAMPs), which can, in turn, promote further inflammation and cell death.

The reperfusion phase exacerbates the initial injury, leading to release of ROS that may accumulate in cells and overwhelm the hepatic antioxidant system, leading to inflammation, and cell damage.

Prolonged and severe IRI can cause cell dysfunction with different death mechanisms such as autophagy, apoptosis and necrosis according to the triggering pathways activated by the IRI process.