Hemostatic system activation and reperfusion injury in liver machine preservation and transplantation of extended criteria donor livers

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CHAPTER 8

Hypothermic machine perfusion in liver transplantation

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ABSTRACT

Dynamic preservation strategies are a promising option to improve graft quality before transplantation, and to extend preservation time for either logistic or treatment reasons. In contrast to normothermic oxygenated perfusion, which intends to mimic physiological conditions in the human body, with subsequent clinical application for up to 24 hours, hypothermic perfusion is mainly used for a relatively short period with protection of mitochondria and subsequent reduction of oxidative injury upon implantation. The results from two randomized controlled trials, where recruitment has finished are expected this year. Both ex situ perfusion techniques are increasingly applied in clinical transplantation including recent reports on viability assessment, which could open the door for an increased liver utilization in the future.

INTRODUCTION

Machine perfusion before organ transplantation is a hot topic as many organs are declined due to a lack of means to ensure graft quality, for example steatotic grafts, or livers donated after circulatory death (DCD)(1). The utilization of such “marginal” livers varies highly between centers, and depends on donation rates, risk strategies, and surgical experience(2). The decision to decline a donor liver is frequently based on “gut feeling” instead of on objective parameters(3). Machine perfusion concepts offer the advantage to test organ function before transplantation, and to optimize metabolic deficiencies. However, despite numerous efforts in this field during the last 20 years, it remains unclear which perfusion procedures and *ex-situ* viability tests are most effective and easy to implement into the complex scenario of liver transplantation. This review aims to summarize current clinical applications, highlights underlying mechanisms and new biomarkers to assess viability during hypothermic machine perfusion (HMP) of livers. Additionally, we describe the injury to the biliary tree throughout the process of liver donation, preservation and transplantation, and show the protective effect of HMP.

1. Clinical Studies Evaluating Hypothermic Liver Perfusion

1.1 Basis and Techniques:

The first prospective clinical trial evaluating *ex situ* hypothermic machine perfusion for the preservation of human livers was published in 2010 by Guarrera and colleagues from Columbia University Medical Center, US. The group utilized hypothermic Vasosol solution circulated via both portal and hepatic artery cannulation (HMP)(Figure 1), which was allowed to equilibrate with ambient air to maintain oxygen tension. Comparing this technique to static cold storage (SCS), they noted significant differences in post-operative liver and renal function, lower post-operative complications, and decreased markers of inflammation and cellular injury, favouring HMP[3,4]. Performed in parallel, trials in Europe evaluating hypothermic machine perfusion have shown similar benefits. In addition, as hypoxia and

mitochondrial energy depletion have been implicated in IRI, groups have added oxygenation to the perfusion circuits for optimal organ preservation(5,6). Two techniques of hypothermic ex situ machine perfusion currently predominate in European transplant centers, both utilizing stationary continuous oxygenation circuits applied during recipient hepatectomy post-SCS:hypothermic oxygenated perfusion via portal vein alone (HOPE) and dual portal vein and hepatic artery hypothermic oxygenated perfusion (D-HOPE)(Figure 1). Together, HMP, HOPE and D-HOPE are undergoing active study, especially for preservation of ECD and DCD grafts (which have reduced ischemic tolerance), in efforts to expand the donor organ pool.

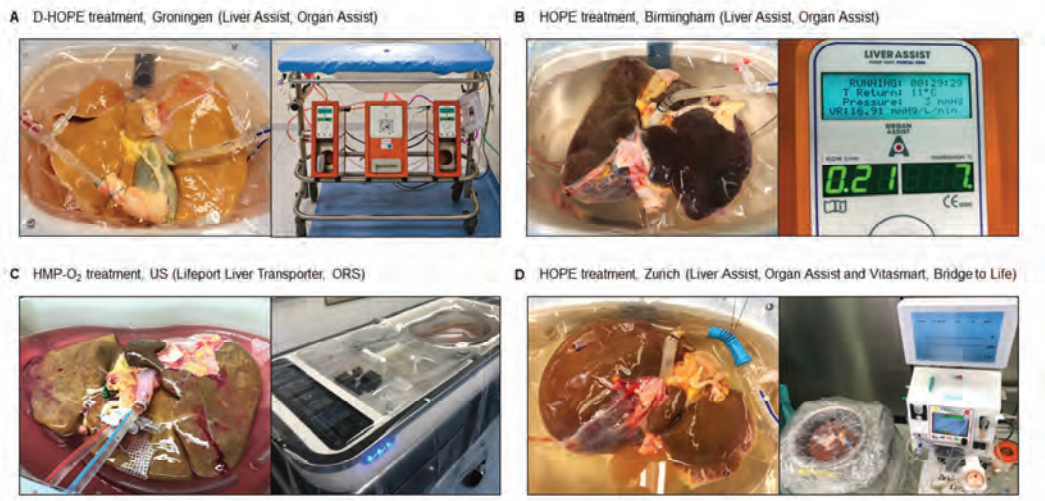

Figure 1: Hypothermic machine perfusion of human donor liver grafts- current techniques and devices. *Clinical examples of hypothermic oxygenated perfusion of human livers performed at the four different transplant centres. Current available devices in clinical use including technical variations are shown.*

1.2 Published Trials

Guarrera et al. published the first US-trial of HMP (n=31) vs. SCS (n=30) for "orphan" ECD livers, declined by other centers in their region. HMP resulted in lower serum markers of liver and kidney damage, shorter hospital stays (13.6 vs. 20 days, p=0.001), and lower biliary complications (p=0.001)(7). Dutkowski and colleagues, from the University Hospital Zurich,

Switzerland, published the first results evaluating HOPE of DCD grafts. They utilized low pressure continuous oxygenated perfusion via portal vein, and compared patients receiving donation after brain death (DBD; n=8) vs. DCD (n=8). All organs underwent SCS, followed by 1-2 hours of HOPE for the DCD cohort prior to transplantation. Post-operatively patients faired similarly with respect to graft function, peak liver function enzymes, and kidney function; ICU and overall hospital stay, as well as 3- and 6-month biliary complications also did not differ with significance. This showcased the protective effects of HOPE as DCD livers, organs classically associated with a propensity for preservation injury and post-operative complications, performed similarly to DBD organs (8).

Applying this technique to a larger patient cohort, the same group compared 25 HOPE-treated DCD grafts vs. 50 matched DCD grafts undergoing SCS. HOPE-treatment resulted in significantly lower post-transplant ALT, decreased biliary complications, and increased 1-year graft survival. When comparing the treatment arm with conventionally stored DBD livers, the group again showed similar patient outcomes, underscoring the protective effect, even when applied for a short period pre-transplant(9). Most recently, in a larger series with longer follow-up, Schlegel and colleagues compared HOPE-treated DCD liver transplants (n=50) to matched untreated DCD grafts (n=50) to conventionally stored DBD liver transplants (n=50)(10). Results again favoured perfusion; even with extended warm ischemia times - the injury phase most implicated in IRI, Schlegel et al. observed 5-year graft survival of 94% in HOPE-treated DCD liver transplants vs. 78% in untreated DCD grafts (p=0.024)(10).

Dual hypothermic oxygenated perfusion (D-HOPE), has been applied to DCD liver grafts by Porte and colleagues at the University Medical Center Groningen, Netherlands(11,12). They compared end-ischemic D-HOPE (n=10) vs. conventionally stored DCD grafts at the same center (n=20). Six month and 1-year graft survival was 100% in the study arm vs 80% and 67% for the controls (p=0.052). Peak post-transplant liver and biliary function labs improved with D-HOPE, an effect which persisted up to 30 days post-operatively. Perfused grafts also had increased ATP, perhaps reflecting more effective oxygen utilization by hepatocytes(11).

D-HOPE preservation of DBD grafts has also been evaluated (13,14). Patrono et al. have recently published a series using dual-perfusion HOPE (n=25) for DBD grafts from older donors, with greater steatosis, or ischemia time >10hrs. D-HOPE resulted in significantly lower incidence of stage 2-3 acute kidney injury as well as lower severe post-reperfusion syndrome. Other post-operative outcomes, including rates of biliary complications, were similar between groups(15).

1.3 Ongoing Debates and Future Directions

The promising results with hypothermic machine perfusion in liver transplantation have prompted further study of these techniques both in the US and Europe. Completed and ongoing randomized controlled trials are currently investigating the application to ECD, DCD and DBD grafts (DBD and ECD grafts: RCTN15527114; portal vein perfusion only; liver assist device, organ assist); DCD – D-HOPE: NCT02584283; portal vein and hepatic artery; liver assist device, organ assist); DBD and ECD organs, dual hypothermic liver perfusion: NCT03484455 (organ recovery system)). Oxygenation of perfusate is now utilized by all groups, and a portable oxygen “pre-charged” HMP pump (HMP-O2) is currently in trial in the US. Protocol variations between centers such as target flows, pressures, O2 delivery mechanisms and route of perfusion (PV alone vs Dual) are actively debated and tested. Combined clinical protocols including normothermic regional perfusion with cold storage and endischemic HOPE or D-HOPE or controlled oxygenated rewarming (COR) are currently explored with promising results (16–18). Furthermore, markers of liver injury are being studied in real time, evaluating graft function throughout perfusion, with hopes to not only improve preservation technique but also select for optimal organs prior to transplant.

2. Underlying mechanism and viability testing

2.1 Normothermic and hypothermic re-oxygenation after ischemia

Recent work has shown that metabolic changes during warm and cold ischemia occur at similar ranges in different species, including mouse, pig, and human(19). For example, warm

ischemia causes a dramatic decrease in ATP/ADP-ratio in various tissues, while rapid achievement of hypothermia significantly delays the loss of adenine nucleotides, underlining the importance of organ cooling(19,20). Additionally, accumulation of succinate during ischemia has been determined for several organs, including liver, brain, kidney, and hearts in various species(19,20). Such selective increase of succinate instead of other citric acid cycle metabolites during ischemia triggers a rapid production of mitochondrial reactive oxygen species (ROS) at complex-I during reoxygenation (Figure 2). The mechanism of ROS release relates to dissociation of reduced flavin-mononucleotide (FMNH2) at complex-I, which is directly oxidized within the mitochondrial matrix to FMN, superoxide anions, and hydrogen peroxide in the presence of oxygen(21,22). Consecutively, complex-I suffers oxidative injury at a special subunit, called the Cys39 residue of the Q-site (ND3-subunit). Such pre-injured complex-I, which lacks FMN is not able to perform the physiological NADH-oxidation. The complex-I dysfunction lead to subsequent, reduced efficiency of the entire respiratory chain with subsequent impairedadenosine triphosphate (ATP)-production.

Based on this, any machine perfusion of ischemic organs with an oxygenated perfusate induces mitochondrial oxidative stress to various extent, depending on the amount of accumulated succinate(19). Of note, mitochondrial ROS production occurs within the first minutes of reintroduction of oxygen to ischemic tissues, and further initiates an opening of the mitochondrial membrane pore with consecutive release of mitochondrial DNA together with other DAMPs and multiple cytokines(23–25). Accordingly, a release of signaling proteins has been recently confirmed during end-ischemic normothermic perfusion of several organs(6,26–29).

A logical primary target of perfusion strategies is therefore the prevention of mitochondrial succinate accumulation, or to decrease mitochondrial ROS formation(19). Interestingly, mitochondria appear more resistant to FMNH2 oxidation and FMN loss from complex-I during cold compared to warm oxygenated reperfusion (Figure 2)(21). Likewise, mitochondria are more effective at uploading cellular ATP at hypothermic temperatures,

when consumptive processes are significantly reduced(6,30). Hypothermic oxygenated perfusion (HOPE) of livers or kidneys after ischemia protects therefore, first, from significant mitochondrial ROS-release, and, secondly, provides uploaded cellular energy reserves before implantation(24,31). Both effects depend, however, on the amount of accumulating metabolites during ischemia. Of note, the changes in mitochondrial metabolism during HOPE are detectable by perfusate analysis during perfusion(24,32). A similar central role of attenuating mitochondria derived oxidative injury and metabolic reprogramming has been recognized in other biological and medical fields, including aging and cancer development(33–35). Further downstream to the protection from such initial injury has significant consequences where HMP improves microcirculation and perfusion quality, removes waste products and provides a generally reduced inflammatory environment.

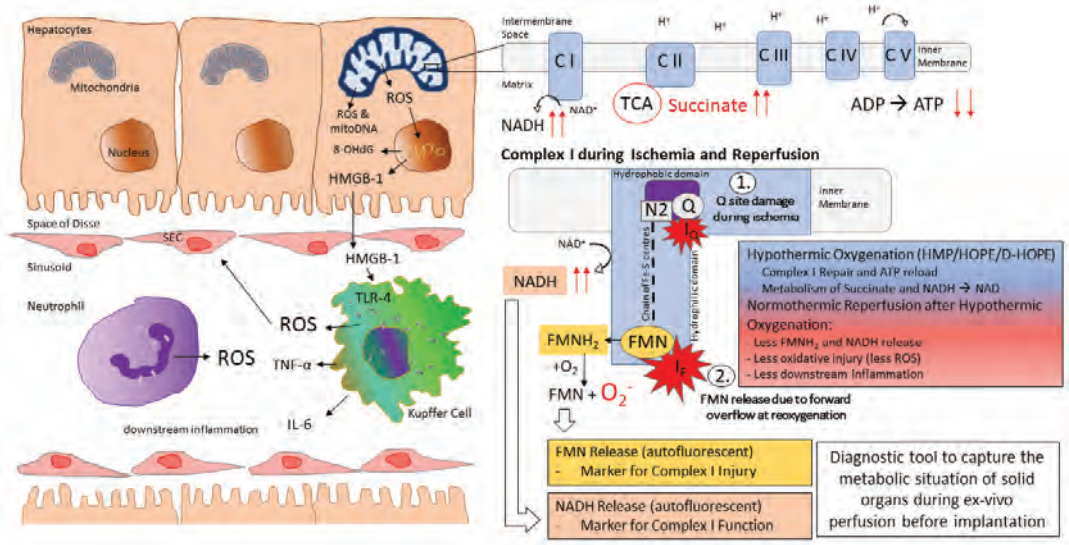

Figure 2: Protective mechanism and viability assessment through hypothermic oxygenated perfusion. *This chart presents the underlying mechanisms of liver injury during warm and cold ischemia, which subsequently becomes evident at oxygenated reperfusion under normothermic conditions. Initial ROS and FMNH2 release from complex 1 present the instigators of the entire reperfusion injury cascade with downstream DAMPs and cytokine release with increasing inflammation throughout continuous normothermic reperfusion in-situ after graft implantation or ex-situ on a perfusion device. End-ischemic cold oxygenated perfusion has been shown to protect mitochondria from this initial injury induces a repair of complex 1 with subsequent improved function of the respiratory chain, which lead to recharging of ATP at complex V and triggers metabolism of succinate and other metabolites, which accumulate during warm and cold ischemia. When livers become rewarmed at implantation or during normothermic perfusion, the injury is significantly less, based on such improved mitochondrial function during previous hypothermic oxygenation. Additionally,*

the entire liver metabolism can be captured by fluorometric analysis of mitochondrial function (NADH) and injury ($FMNH_2$) using the auto-fluorescent properties of such two molecules, representing complex I behavior during reoxygenation in the cold. Importantly, quantification of $FMNH_2$ and NADH predicts liver function and further outcomes after transplantation and therefore guides surgeons to decide, if a high-risk liver is metabolically "good enough" to become utilized for transplantation or not.

2.2 Viability assessment during hypothermic liver perfusion

Normothermic liver or kidney perfusion at near physiologic conditions appears logical to determine visible signs of organ function. Yet, the current set of parameters used to determine viability during ex-situ normothermic liver perfusion failed to predict function or irreversible injury after implantation(3,36,37). For example, lactate clearance, bile production or liver enzyme release were identified to be only weak predictors. In addition, bile glucose or pH have been suggested to be more informative for post-transplant biliary injury, however validation of this data set is required(38). Recent work has shown, that the metabolic status of organs can also be monitored during HMP. Particularly, mitochondrial injury and function can be assessed by measuring perfusate flavin mononucleotide (FMN), released from complex-I (Figure 2)(21). Current data suggest, that perfusate analysis during HOPE is predictive for later graft function(39). These results are in clear contrast with the low predictive value of conventional perfusate parameters, including liver transaminases or perfusate lactate levels, which repeatedly failed to recognize impaired liver function after implantation(36). Future perfusate analysis should therefore target on real-time monitoring of the mitochondrial metabolism to enable accurate prediction of oxidative stress and subsequent downstream inflammation upon transplantation(40). The combination of mitochondrial metabolites including FMN, NADH, succinate, and purine metabolites, may allow future detailed assessment of mitochondrial function before implantation.

FMN and NADH - testing is currently done during the first 30-45 min of HMP or HOPE perfusion. FMN is an auto-fluorescent molecule, released from mitochondria complex I during reoxygenation. A few microliters of perfusate are obtained during perfusion and FMN is quantified in a microplate reader (Spectroscope) at a certain wave length. Results are

available within a few minutes. In extended criteria donor livers and particularly in DCD donors, we currently follow the reported threshold of 10,000 A.U. to accept or not a certain graft.

3. The impact of hypothermic machine perfusion on biliary complications after liver transplantation

One of the major problems currently faced in liver transplantation is the development of biliary complications. Biliary complications occur in up to 30% of liver transplant recipients, which result in a mortality rate ranging from 6% to 12.5% (41,42). In the literature, three distinct types of biliary complications have been described; biliary leakage, anastomotic strictures (AS) and non-anastomotic biliary strictures (NAS), also known as ischemic-type biliary lesions (ITBL). The occurrence of biliary complications affect patients' long-term survival, result in an increased rate of re-transplantation and significantly impact the quality of life and cost of care(43–46).

As several studies have reported, DCD liver grafts are particularly more susceptible to developing NAS. The exact aetiology of NAS is yet to be fully understood but factors such as the duration of warm and cold ischemia are recognized as critical predictors for the development of NAS (47,48). NAS is frequently recurrent and curative treatment is often challenging and unsuccessful (49,50). Therefore, preventing the development of NAS and other biliary complications by optimizing the preservation of donor liver grafts to prevent injury to the biliary tree prior to implantation is necessary.

3.1 Effects of hypothermic machine perfusion (HMP) on post-transplant non-anastomotic strictures

Eight clinical studies specifically comparing the effect on injury and graft function of HMP to SCS have been performed. Despite a few differences in the HMP protocols (single vs. dual perfusion, active oxygenation vs. no active oxygenation), each of these studies aimed to investigate whether or not HMP provided a beneficial effect in the protection of the biliary tree

and hence reduce the incidence of developing NAS. All but two studies reported a lower incidence of biliary complications in liver grafts that underwent HMP as compared to SCS (Table 1). A recently performed meta-analysis of 6 of the 8 studies confirmed these findings as a significantly lower incidence of biliary complications occurred in HMP-treated livers compared to SCS (OR:0.47,95%CI:0.28=0.76,P=0.003)(51).

Table 1: Overview of clinical HMP studies outlining the study design, the main study endpoints and the incidence of biliary complications

Author	Study period	Study design	Cohort HMP/Control (n)	Model	Device	Perfusion duration (hours)	Route of perfusion	Study end-points	Incidence of biliary complications
Guarrera et al.	2004 - 2008	CS	20/20	DBD	Medtronic	3-7	PV + HA	Incidence of PNF, EAD, 1- year graft and patient survival	2 (HMP) vs. 4 (control)
Guarrera et al.	2007 -2012	CS	31/30	ECD-DBD	Medtronic	4-7	PV + HA	Incidence of PNF, EAD, vascular and biliary complication, 1-year graft and patient survival	4 (HMP) vs. 13 (control) P=0.016
Dutkowski et al	Not available	CS	8/8	DCD	ECOPS (Organ Assist)	1-2	PV	Graft function, EAD, biliary complications, 1-year graft and patient survival	2 (HOPE) vs. 2 (DBD controls)
Dutkowski et al.	2005 -2014	CS	25/50/50	DCD (+DBD control)	Liver Assist	1-2	PV	Graft function, EAD, biliary complications, graft and patient survival	20% HOPE DCD vs. 46% DCD control (p=0.03) 20% HOPE DCD vs. 24% DBD control (p=ns)
Van Rijn et al	2008 - 2014	CS	10/20	DCD	Liver Assist	2	PV + HA	Graft function, biliary complications, 1-year graft and patient survival	Ischemic cholangiopathy: 10% (DHOPE) vs. 35% (control) P=0.10
Schlegel et al	2012 - 2017	CS	50/50/50	DCD	Liver Assist	1-2	PV	Graft function, post-transplant complications, 1-year graft and patient survival	NAS: 8% HOPE DCD vs. 22% DCD control (p= 0.09) AS: 24% HOPE DCD vs. 18% DCD control (p=0.62)

Patrono et al	2016 -2018	CS	25/25	ECD-DBD	Liver Assist	2-3	PV + HA	Post-reperfusion syndrome, EAD, biliary complications, 6-month graft and patient survival	8% (HMP) vs. 8% (control) P=ns

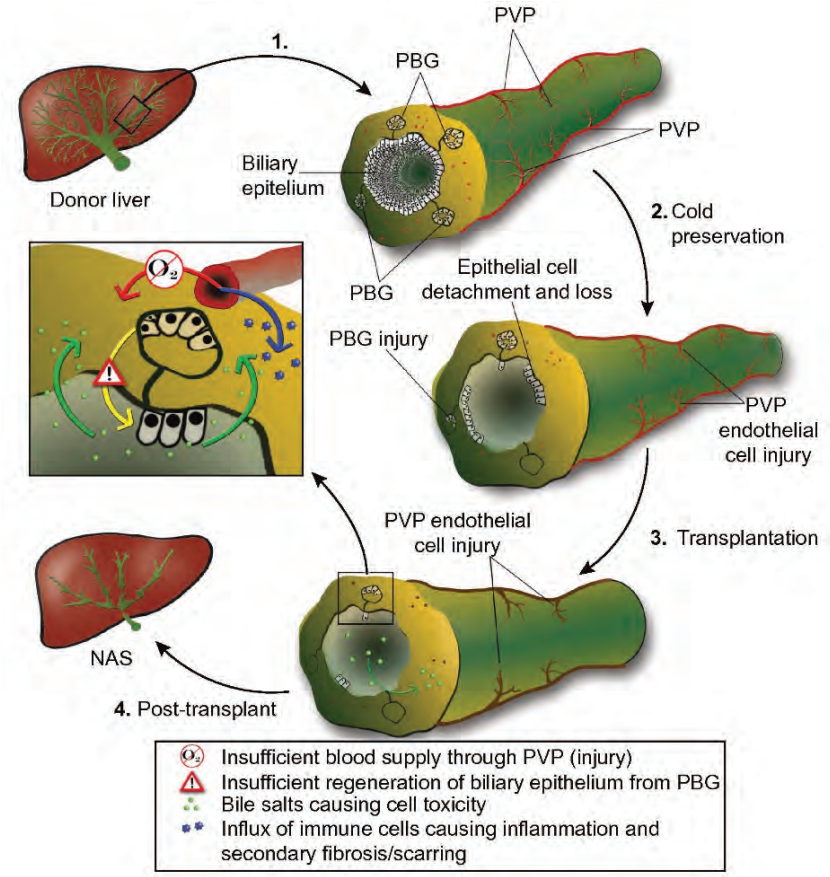

Figure 3*: Proposed multifactorial pathogenesis of the development of NAS. *For DCD livers, the initial ischemic insult occurs during the agonal phase and eventual circulatory arrest (warm ischemia), thereafter static cold preservation (cold ischemia) in both DCD and DBD livers results in injury to critical components of the bile duct; the peribiliary vascular plexus, peribiliary glands and biliary epithelium. Histological analyses have shown detachment and loss of biliary epithelial cells following a period of ischemia. However, this alone does not culminate in the development of NAS. Severe injury to the PBGs and PVP has also been associated with the development of NAS[58]. After transplantation, as a result of IR injury, particularly to the endothelium of the PVP, an insufficient blood supply through the PVP may lead to secondary ischemia of the biliary luminal epithelium and the PBG, thus limiting regeneration of the biliary epithelium. Moreover, diffusion of bile salts toxicity through the epithelium to the PBGs as well as influx of activated immune cells may cause additional damage to the bile ducts resulting in secondary fibrosis and scarring.* Figure was obtained from Weeder et al[59]. Machine perfusion in liver transplantation as a tool to prevent non-anastomotic biliary strictures: Rationale, current evidence and future directions. Journal of Hepatology, Volume 63, Issue1,July2015,Pages265–275. Link to formal publication: https://doi.org/10.1016/j.jhep.2015.03.008*

3.2 Proposed mechanisms of the protective effects of HMP on the biliary tree

The exact aetiology of NAS remains elusive. However, one of the main mechanisms described to be responsible is ischemia-reperfusion injury (IRI) (Figure 3). Even though cooling livers to 0–4°C during SCS significantly reduces the need for oxygen, cellular metabolism never reaches complete cessation(52). This results in intracellular depletion of ATP, cell swelling due to diminished Na/K-ATPase activity and subsequent electrolyte shifts. Upon re-oxygenation, formation of toxic radical oxygen species and activation of the immune system leads to cell death which further exacerbates injury(53). It has been shown that bile duct epithelial cells are more susceptible to IRI and exhibit more cell death than hepatocytes(52,54). Therefore, several transplant programs aim to limit CITs and anastomosis times in order to minimize ischemic injury. Nevertheless, IRI remains inevitable in the transplantation process unless ischemia is completely eliminated. HMP however, enables preservation of grafts whilst minimizing ischemia and as pre-clinical animal studies have demonstrated, leads to a significantly decreased release of liver enzymes, pro-inflammatory markers and markedly less cell necrosis of the peribiliary arterioles in DCD livers treated with HMP prior to transplantation[12,13].

Interestingly, Guarrera and his team have reported a lower incidence of biliary complications in HMP-treated liver grafts despite performing HMP without active oxygenation(7). They attribute this to better continuous flushing and circulation of adequate oxygen (from ambient air), ATP-substrates and vasodilators to the peribiliary vascular microcirculation. In contrast, all other groups performing clinical HMP implement active oxygenation. However, the main difference between these groups is whether the livers are perfused through the portal-vein alone, or through both portal vein and hepatic artery. As described in detail in section 2 of this paper, active oxygenation results in the metabolization of succinate (known to trigger mitochondrial dysfunction) without the concomitant release of injurious reactive oxygen species which normally occurs upon re-oxygenation at normothermic temperatures, thus mitigating IRI.

No clear consensus has been reached yet on which (HMP) methodology is superior in regards to optimal preservation of the biliary tree. The results of current and previously concluded RCT's (NCT01317342;NCT02584283;NCT03484455) will bring forth much awaited answers.

4. Summary and Future Aspects

Hypothermic oxygenated liver perfusion has been demonstrated to improve graft survival and reduce complications in various clinical scenarios. The results of the five currently ongoing randomized controlled trials, are awaited. Through mitochondrial reprogramming, cold organ oxygenation improves complex-I-V function and recharges ATP and subsequently prepares the organs for normothermic reperfusion during transplantation. Based on such cellular protection, the utilisation of marginal livers has been increased when HMP was applied and the biliary tree is protected from significant injury and complications after transplantation.

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