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REVIEW

Therapeutics administered during *ex vivo* liver machine perfusion: An overview

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Abstract

Although the use of extended criteria donors has increased the pool of available livers for transplant, it has also introduced the need to develop improved methods of protection against ischemia-reperfusion injury (IRI), as these "marginal" organs are particularly vulnerable to IRI during the process of procurement, preservation, surgery, and post-transplantation. In this review, we explore the current basic science research investigating therapeutics administered during *ex vivo* liver machine perfusion aimed at mitigating the effects of IRI in the liver transplantation process. These various categories of therapeutics are utilized during the perfusion process and include invoking the RNA interference pathway, utilizing defatting cocktails, and administering classes of agents such as vasodilators, anti-inflammatory drugs, human liver stem cell-derived extracellular vesicles, and δ-opioid agonists in order to reduce the damage of IRI. *Ex vivo* machine perfusion is an attractive alternative to static cold storage due to its ability to continuously perfuse the organ, effectively deliver substrates and oxygen required for cellular metabolism, therapeutically administer pharmacological or cytoprotective agents, and continuously monitor organ viability during perfusion. The use of administered therapeutics during machine liver perfusion has demonstrated promising results in basic science studies. While novel therapeutic approaches to combat IRI are being developed through basic science research, their use in clinical medicine and treatment in patients for liver transplantation has yet to be explored.

Key words: Therapeutics; Liver transplantation; *Ex vivo* machine perfusion; Ischemia reperfusion injury; Organ preservation; Extended criteria donors

Core tip: The use of extended criteria donors has increased the donor pool of available livers for transplant but has also introduced other hurdles in protecting these vulnerable

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organs against ischemia-reperfusion injury (IRI). Current basic science research is aimed at mitigating the effects of IRI during the transplantation process by administering therapeutics during *ex vivo* liver machine perfusion. Of interest include therapeutics aimed at invoking the RNA interference pathway, utilizing defatting cocktails, and administering classes of agents such as vasodilators and anti-inflammatory drugs to reduce the damage of IRI following liver procurement and transplantation for ultimate preservation of the organ.

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INTRODUCTION

The overall increasing success of liver transplantation over the last several years has unfortunately introduced one of the most significant hurdles to date - longer waiting lists and increased mortality while on the waiting list. In an effort to combat the organ shortage, transplant centers have extended the criteria for donors often considered for transplantation. Common categories of extended criteria donors (ECDs) now being included in the context of the donor liver pool include donation after cardiac death (DCD), hepatic steatosis, donors of advanced age, organs that have experienced prolonged normothermic and cold storage, and donors with an increased infectious risk. The inclusion of ECD in the donor pool has increased access to previously deemed un-transplantable organs by 77% while reducing the mortality of those on the waitlist by over 50%[1].

While inclusion of ECDs has positively impacted the pool of livers available for transplant, the new criteria has also highlighted the need for improved methods to ameliorate ischemia-reperfusion injury (IRI) in these less than optimal organs due to a weakened defense against ischemia-reperfusion injury during the transplantation process[2]. Ischemia-reperfusion injury occurs when blood supply to an organ is inhibited and then later restored, with this process resulting in oxidative damage, cell death, and generation of reactive oxygen species (ROS)[3]. The hepatic molecular pathways involved in IRI are complex with liver sinusoidal endothelial cells and hepatocytes as the initial targets for cell death as a result of ATP depletion. Neutrophils and macrophages then accumulate in the liver leading to ROS generation while hepatic stellate cells then become activated to aid in recovery, ultimately leading to fibrosis of the allograft[4-6].

Targeting specific candidates implicated in hepatic IRI therefore becomes challenging due to the complex molecular pathways that become activated. Some of the activated pathways and molecules include the complement cascade, the innate immune response and toll-like receptors (TLRs), CD4 T lymphocytes, inflammatory cytokines propagating the post-inflammatory response, nuclear factor κB (NF-κB) leading to production of TNF-α, adhesion molecules, apoptotic pathway activation, and ROS production and release[7,8]. As it will be discussed, basic science research focused on hepatic IRI has attempted to target many key mediators implicated in the IRI cascade. Most studies rely on using a combination of therapies that block multiple, perhaps redundant, reperfusion injury pathways in order to achieve a significant reduction in injury and overall improvement in graft function[9].

There currently exists no established clinical therapies to avoid IRI, and the main method implemented to reduce IRI relies on limiting the cold preservation period and re-warming of the organ[2]. Other methods of graft protection prior to transplantation include immunosuppressive agents and modulation of the immune response. Immunosuppressive therapies, particularly in the setting of kidney transplantation, has proven to be advantageous when the donor is treated prior to graft procurement or when the graft is directly treated during perfusion or cold storage[10].

While static cold storage (SCS) remains the gold standard for liver preservation, *ex vivo* machine perfusion (MP) preservation of the liver is gaining attention from both basic scientists and transplant surgeons alike. *Ex vivo* MP is an attractive alternative to SCS due to its ability to continuously perfuse the organ microcirculation, effectively delivery substrates and oxygen required for cellular metabolism, therapeutically administer pharmacological or cytoprotective agents, and continuously monitor organ

viability during perfusion[2].

CURRENT *EX VIVO* LIVER MP CLINICAL TRIALS

Several recent liver transplantation clinical studies demonstrate the logistical feasibility and safety of MP in the hospital setting. Ravikumar *et al*[11] published the first Phase 1 normothermic perfusion trial of 20 patients who underwent NMP liver transplantations demonstrating decreased AST levels compared with controls during the first 7 d and a 95% one-year patient survival rate in the NMP group. Although there was no statistical difference in the primary outcome, this study reported the first use of NMP as logistically feasible and safe for use in the clinic[11]. Selzner *et al*[12] report the use of normothermic *ex vivo* liver perfusion in 10 human liver grafts using an albumin-based Steen solution with comparable outcomes to traditional SCS post-liver transplantation. In addition, Czigany *et al*[13] report an ongoing open-label, phase 2 randomized controlled trial using HMP in liver transplantation from ECDs although primary and secondary endpoints and extent of IRI have not yet been published.

Another recent advancement in human liver MP involved the first randomized controlled trial of 220 liver transplantations performed by Nasralla *et al*[14] at normothermic preservation conditions and demonstrated a 50% lower level of graft damage compared to the traditional cold static method of preservation in addition to 50% fewer discarded organs in the normothermic machine perfused group. This trial describes the novel expansion of normothermic human liver MP from experimental bench studies to introduction into clinical practice and demonstrates its benefit over the traditional cold method of preservation. While a larger study is needed to determine the impact of NMP on liver graft and patient survival, preliminary results indicate an exciting future for normothermic liver preservation.

Although the purpose of this review is not to highlight every current clinical trial involving *ex vivo* liver MP to date, it is worth noting that the number of both prospective and retrospective studies investigating the role of HMP *vs* SCS in human liver transplantation is increasing but still remains limited. These studies have been conducted in a variety of countries including the United States, Switzerland, the Netherlands, and the United Kingdom[15]. The clinical use of *ex vivo* liver MP may be viewed as a limitation due to it being in its infancy as a standard therapy in liver transplantation. However, the basic science advances that will be highlighted in this review continue to expand the applications of *ex vivo* liver MP closer to its acceptance as a more reliable, efficacious method of organ preservation for liver transplantation.

BASIC SCIENCE *EX VIVO* LIVER MP THERAPEUTICS

In this paper, we review basic science advances made in the area of therapeutics administered specifically during *ex vivo* liver machine preservation transplantation models (Table 1) and their importance in extending the donor criteria for liver transplantation in a clinical setting. While therapeutics for this review were only considered in the context of the liver, administered therapeutics during MP of additional organs such as the lungs, heart, and kidneys are currently being explored in an effort to reduce IRI during transplantation and increase the available organs suitable for transplantation. Therefore, the impact of therapeutics administered during MP to mitigate IRI during and post-organ transplantation holds tremendous potential to increase the donor pools of many transplantable organs while simultaneously reducing the waiting time for those hoping to gain a second chance at life.

RNA interference and its therapeutic role in the liver during ex vivo MP

One of the most recent basic science advances in liver MP therapeutics includes utilization of the RNA interference (RNAi) pathway to silence specific genes implicated in IRI. RNAi selectively silences genes by the RNA-induced silencing complex (RISC) upon hybridization with the target mRNA, subsequently leading to degradation of the mRNA by Argonaute, an RNase H enzyme. If there are mismatches between the RNA complex and the target mRNA, silencing can occur at the post-transcriptional level leading to translational repression or exonucleolytic degradation[16].

The overall RNAi mechanism contains several unique regulatory RNA molecules including microRNA (miRNA), small interfering RNA (siRNA), and short-hairpin RNA (shRNA). There have been only a handful of reports of the RNAi pathway being implemented in the context of animal liver transplantation. For example, Li *et al*[17]

Table 1 Major categories of *ex vivo* machine perfusion therapeutics in liver transplantation

Category	Agents
RNAi pathway	siRNA
	Anti-sense oligonucleotide (Miravirsen)
Defatting cocktail	Variable
Vasodilators	Prostacyclin
	BQ123 and Verapamil
	Prostaglandin E1
Others	Anti-inflammatory agents
	Human liver stem cells extracellular vesicles
	δ-opioid agonist (Enkephalin)
	NLRP3 inflammasome inhibitor mcc950

RNAi: Ribonucleic acid interference; siRNA: Small interfering ribonucleic acid; NLRP3: Nucleotide-binding domain leucine-rich repeat containing family pyrin domain containing 3.

employed a rat liver transplantation model to study the effects of Fas siRNA on IRI. Hydrodynamic injection of 200 nmol/kg Fas siRNA transfection of the penile vein was performed 48 h before liver procurement. Measurements from the recipient rats demonstrated reduced ALT levels, decreased apoptotic index levels, and reduced Fas mRNA and protein levels 24 h after blood reperfusion.

Contreras *et al*[18] delivered caspase-8 or caspase-3 siRNA in an *in vivo* C57BL/6 mouse model *via* the portal vein by high-volume injection 1 hour before induction of ischemia for 90 min. Results demonstrated a reduction in caspase-8 and caspase-3 gene expression of greater than 60% following siRNA injection. In addition, siRNA-treated mice showed improved survival for greater than 30 d when treated with caspase-8 siRNA (30%) and caspase-3 siRNA (50%) compared to controls where all of the mice died within five days after being subjected to total liver ischemia.

In addition, Wu *et al*[19] targeted interleukin-1 receptor-associated kinase-4 using shRNA (IRAK-4-shRNA) in a rat liver transplantation model to prevent IRI. IRAK-4 is implicated in the downstream signaling pathways of lipopolysaccharide (LPS) activation of IRI in addition to its role in Toll-like receptor (TLR) and IL-1R mediated innate immune responses and was therefore selected as an ideal candidate for shRNA targeting to prevent IRI[19-23]. In this study, rat liver grafts were perfused *via* the portal vein with a plasmid expressing IRAK-4-shRNA for 4 min during the cold ischemia time and then stored in University of Wisconsin (UW) perfusion solution for a period of 6 h prior to transplantation. Post-liver transplantation results indicated improved liver function, preserved tissue architecture, decreased IRAK-4 mRNA and protein levels, decreased NF-kB, TNFa, IL-6, and IL-1B levels over a period of 180 minutes post-reperfusion[19].

While these previous studies demonstrated the utilization of the RNAi pathway to selectively target genes implicated in IRI by introducing siRNA or shRNA using hydrodynamic injection, our group most recently reported the first use of *ex vivo* liver MP as a method of siRNA delivery prior to rat liver transplantation[24]. In this study, siRNA targeting the Fas receptor was added directly to the perfusion solution and MP of the liver was maintained at either hypothermic (4 °C) or normothermic (37 °C) conditions using a closed loop perfusion circuit[24]. The Fas siRNA construct was conjugated to invivofectamine lipid nanoparticles and the siRNA-lipid complexes were perfused *via* the portal vein for 4 h. Confocal imaging studies revealed Fas siRNA distribution throughout the liver sinusoids and central veins in both the hypothermic and normothermic conditions[24].

This study demonstrated for the first time siRNA uptake and distribution in the liver following *ex vivo* MP at hypothermic and normothermic conditions. While future studies will examine the effects of siRNA uptake and delivery using MP in a rat transplant model, this report highlights the exciting use of RNAi in the context of *ex vivo* liver preservation.

In addition, our group utilized a similar *ex vivo* normothermic machine preservation system as aforementioned to silence the p53 tumor suppressor gene in a rat liver damage model. Rats were injected with p53 siRNA conjugated to invivofectamine prior to initiation of liver damage[25]. To induce liver damage, the liver hilum was clamped for 15 minutes. Confocal microscopy studies revealed p53 siRNA uptake into the machine perfused liver with reduced levels of the inflammatory cytokines IL-1, IL-6, and TNFα compared with controls[25].

The utilization of the RNAi pathway in the context of machine preservation of the liver is a new concept with promising therapeutic value. Although administering therapeutic siRNA to human donors before procurement is clinically feasible, the cost of therapy and potential for side effects increase[26]. The therapeutic dosage of siRNA delivery to the donor liver could be efficiently delivered with little to no side effects to other organs as the siRNA therapeutic concentration would be based upon the weight of the donor liver rather than the weight of the whole donor at the time of procurement[26]. In addition, preliminary studies demonstrate the uptake of siRNA in both hypothermic and normothermic temperatures in a rat model suggesting that perfusion temperatures have little to no effect on uptake while future studies will be aimed at addressing the effect of temperature on siRNA efficacy in silencing its target in a transplant model. Therefore, inclusion of siRNA in the MP solution for liver transplant models demonstrates promising and exciting results while also holding tremendous therapeutic value for future use in the operating room.

It is also worth noting that anti-sense oligonucleotide (ASO) therapies have also recently been used against hepatitis C virus (HCV) infection. Mir-122 is a known miRNA of the liver and is a target of miravirsen, which is currently in Phase 2 clinical trials for treatment of HCV[27]. Miravirsen has been shown to nearly completely eradicate HCV presence in cell culture. In addition, miravirsen was shown to sequester miR-122 (a necessary agent for HCV infection) in pig liver without inducing harmful effects[28]. Miravirsen is a recent exciting advancement in liver-targeted therapies as it could significantly increase the donor pool by allowing the transplantation of HCV^+ livers.

Although the concept of gene silencing using RNAi in liver transplantation MP models is in its earliest stages of development and optimization, it holds exciting potential as a future therapeutic to combat the damage caused by IRI as a result of liver transplantation.

Defatting cocktails to reduce steatosis during MP of the liver

The increasing prevalence of obesity and metabolic syndrome defined as insulin resistance, hyperlipidemia, hypertension, and hyperglycemia, have contributed to potential donors developing hepatic steatosis. Hepatic steatosis is defined as having intrahepatic triacylglycerol (TAG) of at least 5% of the total liver weight or 5% of hepatocytes containing lipid vacuoles without a patient history of secondary contributing factors including viral infection, excess alcohol intake, or drug treatments[29]. Reports indicate that 33% of the United States adult population has nonalcoholic hepatic steatosis[30].

Potential donors with hepatic steatosis are now included in the ECD donor pool, however this criterion has implications in post-transplant outcomes. An analysis of the Scientific Registry of Transplant Recipients reported that liver allografts with greater than 30% macrovesicular steatosis were independently predictive of reduced 1-year graft survival[31].

Furthermore, steatotic livers are particularly susceptible to IRI, increasing the risk of postoperative morbidity and mortality after liver surgeries and liver transplantations[32]. Recent basic science evidence also provides support that steatosis exacerbates the effects of IRI. Chu *et al*[33] demonstrated in a rat model of liver steatosis that the steatotic-IRI livers had elevated ALT levels, evidence of histological injury, and impaired mitochondrial complex-1 function after partial hepatic normothermic ischemia compared to the lean liver controls. Liss *et al*[34] provided evidence in a murine model of hepatic IRI that steatosis increases plasma ALT, inflammatory cytokine levels such as TNF-α and IL-6, and necroptosis markers RIPK1, RIPK3, and MLKL compared with low-fat diet controls.

Gehrau *et al*[35] explored the effect of IRI on immune response pathways in human graft biopsies classified based upon the degree of graft steatosis. The results showed that compared with non-steatotic control grafts, the steatotic grafts had significant post-transplant innate immune response activation of IL-6, IL-8, and IL-10, macrophage production of nitric oxide (NO) and ROS, and neutrophil and leukocyte recruitment around the sites of hepatocyte lipid accumulation[35]. Ramachandran *et al*[36] also demonstrated that NFκB P65 is associated with the inflammatory pathway implicated in IRI and necrosis in rat steatotic liver transplantation. Multidrug donor preconditioning of steatotic rat liver grafts has also been reported to abolish the IRI inflammatory mechanism while preventing an increase in parenchymal cell death following cold storage and reperfusion[37].

A systematic review examining the animal model experimental studies investigating hepatic steatosis and IRI found that livers with > 30% macrovesicular steatosis were associated with a lower graft and recipient survival rate as a result of the effects of IRI[38].

More recently, liver MP has been investigated as a method of steatotic liver

preservation and has shown promising results. Bessems *et al*[39] compared the traditional method of cold storage *vs* hypothermic MP for rat donor steatotic liver preservation. After 24 h of either hypothermic cold storage or MP, results demonstrated reduced levels of AST and LDH and increased bile production, ammonia clearance, urea production, and ATP levels after MP *vs* cold storage.

Vairetti *et al*[40] examined the effects of rat liver preservation using MP at 20 °C in steatotic livers compared to the SCS method of preservation. Results demonstrated that the adenosine triphosphate/adenosine diphosphate ratio and bile production were higher and oxidative stress and biliary enzymes were lower in the machine preservation-treated steatotic livers compared with the SCS method of preservation[40]. Additionally, there was a 2-fold increase in TNF α levels and caspase-3 activity in the SCS steatotic livers compared with the machine perfused livers[40]. These findings suggest that MP at 20 °C improves rat steatotic liver preservation compared with the SCS method.

Subnormothermic machine preservation has also been investigated in the context of macrosteatotic rat livers and has shown to reduce parenchymal ALT, mitochondrial glutamate dehydrogenase release while protecting against steatotic-induced sinusoidal microvascular alterations and preserving mitochondrial structure[41]. Thus, implementation of defatting protocols seeks to efficiently decrease the proportion of macrosteatotic hepatocytes while ensuring viability and functionality in the remaining hepatocytes[42].

In an effort to mitigate the detrimental effects of hepatic steatosis on transplantation outcomes, several animal studies have investigated the role of defatting protocols to reduce the intrahepatic TAG content prior to transplantation. These protocols may span a period of days to weeks and rely on a change in diet to alter the fat content in livers prior to transplantation.

The concept of defatting steatotic livers holds significant therapeutic and clinical potential as defatting in humans has shown to decrease steatosis. For example, one studied investigated the implementation of a protein-rich (1000 kcal/d) diet, exercise (600 kcal/d), and the lipid-lower drug bezafibrate (400 mg/d) for 2-8 wk in 11 candidates for living-donor liver transplantation[43]. Results demonstrated significantly improved body weight, BMI, and steatosis allowing for the transplantation of 7 of the treated liver grafts to recipients[43]. Post-transplant tests demonstrated liver functioning with no significant differences in measured functional parameters[43]. Additionally, a study of 120 consecutive living donors with non-alcoholic fatty liver disease of ≥ 30% or an estimated donor-recipient weight ratio of < 0.8 demonstrated that following diet and exercise modifications leading to ≥ 10% total cholesterol reduction and ≥ 5% weight reduction, an improvement in steatosis of ≥ 20% was seen in the 120 donors[44].

The aforementioned studies demonstrate the feasibility of reducing fat content using established defatting protocols that are reliant on a diet change prior to liver transplantation, however they do not reflect a viable approach for liver grafts that are procured and intended for transplant, typically requiring a time frame of less than 12 h[45,46]. While the use of defatting protocols to reduce steatosis in donor livers for transplantation has provided initial promising results for expanding the liver donor pool, further experimental animal studies investigating the use of *ex vivo* perfusion of donor livers to reduce steatosis remains limited.

Jamieson *et al*[47] demonstrated in a porcine model that agents involved in peroxisome proliferation for lipid export, visfatin to reduce triglyceride (TG) levels, and forskolin to stimulate oxidation of lipids and ketogenesis decreased hepatocyte TG levels by 31% in 48 h of normothermic MP. Periportal hepatocytes were "defatted" compared to hepatocytes near the perivenous region with an overall increase in bile production[47]. Additionally, Nagrath *et al*[48] utilized a perfusate medium supplemented with defatting agents (forskolin, GW7647, hypericin, scoparone, visfatin, and GW501516) on steatotic livers from obese Zucker rats. Following *ex vivo* normothermic perfusion for 180 min with the defatting perfusate medium, the TG content decreased by 65% and produced elevated bile levels compared to the control perfusion[48].

Liu *et al*[49] reported the use of subnormothermic (20 °C) MP supplemented with a defatting cocktail for 6 h in obese Zucker rats. Results demonstrated a significant increase in very low density lipoprotein (VLDL) and TG content in the perfusate in groups with and without the defatting cocktail. Additionally, the oxygen uptake rate, VLDL and TG secretion, and venous resistance were also similar in both groups[49]. This study demonstrates the process of lipid export during subnormothermic MP.

In another study, the addition of carvedilol, a beta- and alpha-adrenergic blocking agent, used commonly in the setting of ischemic heart disorders and hypertension, to UW solution prevented rat hepatic injury associated with IRI in both steatotic and non-steatotic livers after 2 h of normothermic perfusion[50-52].

As basic science methods aimed at reducing hepatic steatosis prior to transplantation are constantly being explored and optimized, clinical studies investigating the role of defatting cocktails in MP is relatively limited. Since the field of liver transplantation has only recently established MP as the superior preservation method compared to SCS, we expect more clinical studies focused on the addition of therapeutic agents to the perfusate, such as defatting cocktails, in the near future.

Boteon *et al*[53] has recently utilized pharmacological agents in the setting of *ex situ* normothermic MP to enhance lipid metabolism in steatotic human donor livers discarded for transplantation. Using a previously published cocktail of drugs by Nagrath *et al*[48] and supplemented with L-carnitine, Boteon *et al*[53] added this defatting cocktail to the perfusate at normothermic conditions (37 °C). Results showed that within 6 h of NMP supplemented with the pharmacologic defatting cocktail, the steatotic livers had enhanced lipid metabolism with decreased TG content, decreased vascular resistance of the portal vein with increased flow, decreased lactate levels, and decreased tissue expression of markers implicated in IRI such as CD14 and CD11b and decreased cytokine profiles of TNF-α and IL1β[53]. Most notably, following pharmacologic NMP treatment, all 5 treated livers were considered transplantable based upon viability criteria established by the authors[48,53].

This particular study highlights the potential power of therapeutics administered during MP to salvage previously deemed untransplantable livers while addressing the liver donor pool shortage. To our knowledge, there exists no clinical trial to date using defatting protocols with NMP in the setting of human liver transplantation, as the pharmacological defatting agents used at the bench have not yet been approved for use in humans in a clinical setting[54]. While Boteon *et al*[55] report cytotoxicity results of the defatting cocktail in the setting of primary human hepatocytes on non-parenchymal liver cells, an exciting future for approved pharmacologic defatting therapeutics for human liver perfusion and transplantation surely exists.

Vasodilator administration during liver machine preservation

While IRI induces damage at the cellular level as highlighted, microvasculature is also disrupted. IRI insult affects endothelial cells by disrupting the normal barrier function, vascular tone, and expression of adhesion molecules[56]. Nitric oxide is reduced during reperfusion and can therefore no longer control vasodilation during this period[56]. Additionally, capillaries become occluded due to the activation of inflammatory and coagulation cascades[56]. Therefore, several labs have examined therapeutic agents in MP to preserve the microvascular integrity of the liver.

In a study designed to address the susceptibility of uncontrolled non-heart-beating donors to warm IRI during liver transplantation, the authors devised a new rat model technique for liver grafts using short oxygenated warm *ex vivo* perfusion (SOWP) and prostaglandin E1 (PGE1)[57,58]. PGE1 has been shown to have vasodilative and hepatoprotective effects such as reducing hepatocytic degeneration, central and portal ICAM-1 expression, central and sinusoidal VCAM-1 expression, central and portal P-selectin expression, and portal and sinusoidal E-selectin expression in the context of reperfusion[57]. Results from the SOWP and PGE1 study showed increased total bile production during reperfusion to the same level as the heart-beating donor grafts[58]. Additionally, PGE1 supplementation to the SOWP buffer decreased AST, ALT, and TNFα levels following 1 h of reperfusion[58]. Necrosis and apoptosis were examined *via* histology and TUNEL staining and demonstrated significantly reduced levels following PGE1 treatment with SOWP[58]. This study revealed that SOWP and PGE1 treatment before cold preservation improves the functioning of liver grafts following warm IRI[58].

In a similar SOWP study, Maida *et al*[59] performed rat liver transplantations following a 6 h cold preservation period in order to determine the *in vivo* effects of SOWP supplemented with PGE1 in DCD rats. Results indicated that in the PGE1-treated SOWP groups, serum liver enzymes, cellular damage, and intercellular adhesion molecule 1 levels were significantly decreased compared to the control group[59].

Nassar *et al*[60] investigated the role of a prostacyclin analog, epoprostenol sodium, in a pig DCD model during NMP for 10 h after 60 min of warm ischemia time and demonstrated lower levels of AST, ALT, LDH, increased bile production, and preserved hepatic architecture compared with the control groups. Echeverri *et al*[61] studied the safety and efficacy of BQ123 (endothelin1 antagonist), epoprostenol (prostacyclin analogue), and verapamil (calcium channel antagonist) in a pig transplantation model using normothermic *ex vivo* liver MP. Livers treated with BQ123 and verapamil demonstrated increased hepatic artery flow and reduced hepatocyte injury compared with controls[61].

Addition of vasodilators to the perfusate solution during MP in animal transplantation models demonstrates significant therapeutic potential. While IRI

incites damage to the microvasculature, vasodilators such as PGE1 impart protective effects on the vasculature such as preserving adhesion and selectin expression for maintenance of vascular integrity.

Bae *et al*[62] have also explored the use of α-tocopherol with Vasosol in a DCD rodent model as an additive in the perfusate during HMP to reduce inflammatory and apoptotic markers implicated in reperfusion injury. Addition of α-tocopherol to the HMP solution reduced ALT levels during reperfusion and also reduced levels of inflammatory cytokines IL-6, TNF-α, and MCP-1, and caspase 3/7 as a result of reducing cytochrome C mRNA levels[62].

Clinical trials investigating the role of vasodilator additives to machine perfusate also highlight the importance of therapeutics in the human liver transplantation setting. The first human clinical trial using HMP for liver transplantation was completed by Guarrera *et al*[63] and compared HMP-preserved livers in 20 adults with a SCS matched group[45,63,64]. A Vasosol solution was used as the HMP perfusate which included added antioxidants, metabolic substrates, and vasodilators including nitroglycerin and prostaglandin E1[45,63]. Results indicated significantly reduced peak levels of AST, ALT, total bilirubin, and serum creatinine in the HMP preserved group. Molecular analysis from this clinical study revealed that HMP attenuated expression of inflammatory cytokines, oxidation markers, adhesion molecules and chemokines, and apoptosis and CD68 positive macrophages compared with the SCS group[64]. The overall early graft dysfunction rates were 5% in the HMP groups compared with 25% in the control group, and the mean hospital stay was also shorter in the HMP group compared with the SCS group suggesting that HMP with vasodilator additives in liver transplantation is a safe and future method of liver perfusion and preservation[45].

OTHER THERAPEUTICS IMPLEMENTED IN LIVER MP

Additional classes of therapeutics used in the setting of liver MP include anti-inflammatory agents, human liver stem cell-derived extracellular vesicles (HLSC-EV), and more recently, the δ-opioid agonist, enkephalin. While these therapeutics will not be discussed extensively in this review, they are summarized in Table 2 and are important to mention given their potential therapeutic role in the liver transplantation field.

Goldaracena *et al*[9] reported improved liver function and lower inflammation in a pig transplantation model using anti-inflammatory agents (alprostadil, n-acetylcysteine, carbon monoxide, sevoflurane) added to perfusate in NMP. Rigo *et al*[65] demonstrated, for the first time, reuptake of HLSC-EV in *ex vivo* rat liver perfusion. Treated livers demonstrated reduced necrosis and apoptosis, lower hypoxia-induced markers, and superior liver function (lower AST and LDH).

Recently, Beal *et al*[66] demonstrated the efficacy of enkephalin, a δ-opioid agonist, to reduce oxidative stress in a rat *ex vivo* perfusion model. Treated livers had lower AST and malondialdehyde levels, in addition to higher ATP and glutathione levels in the perfusate. Yu *et al*[67] also most recently reported that the selective NLRP3 inflammasome inhibitor mcc950 added to the perfusate of an HMP system and intravenously injected in a pig liver transplantation model demonstrated improved outcomes in DCD organs compared with controls.

CONCLUSION

The use of administered therapeutics during machine liver perfusion has demonstrated promising results in basic science studies and initial clinical reports indicate their safety and efficacy in human liver transplantation. While novel therapeutic approaches to combat IRI are being developed through basic science research, their use in clinical medicine and treatment in patients for liver transplantation has yet to be fully explored. The number of human clinical trials investigating the use of liver MP is increasing, however the information obtained from these trials remains limited until additional robust studies are performed. A proposed summary of the advantages and limitations of the aforementioned MP therapeutics is offered in Table 3. While the therapies mentioned are relatively recent advances in the field of *ex vivo* liver MP, potential future directions are also included in Table 3 to indicate areas of further exploration. It is also worth noting that in terms of future considerations, the aforementioned studies in this review commonly employed multiple therapeutic agents in the perfusion solution, and several studies highlighted the need to delineate which agents out of the administered cocktail

Table 2 Other major therapeutic additives in models of *ex vivo* liver machine perfusion

Ref.	*Ex vivo* perfusion type	Therapeutic	Problem	Animal	Model	Samplesize	*Ex vivo* perfusiontime (h)	Outcomes
Goldaracena *et al*[9], 2016	SNMP	Anti-inflammatory agents (Alprostadil, n-acetylcysteine, carbon monoxide, sevoflurane)	IRI	Pig	Transplantation	5	4	During EVLP: lower AST, TNF-α, IL-6 Lower HA levels, β-galactosidase and higher IL-10 (nonsignificant) After transplantation: Lower bilirubin, lower IL-6, lower cleaved caspase 3 staining, intact sinusoidal endothelial cell lining Lower AST, TNF-α, HA, ALP and higher IL-10 (nonsignificant)
Rigo *et al*[65], 2018	NMP	HLSC-EV	IRI	Rat	EVLP	9	4	HLSC-EV uptake in treated livers Reduced necrosis and apoptosis on histology, lower Suzuki tissue injury score, lower apoptosis, lower AST and LDH, lower HIF-1α & TGF-β1 (hypoxia induced markers) NMP had low hematocrit to induce hypoxia
Beal *et al*[66], 2019	NMP	Enkephalin (δ-opioid agonist)	IRI	Rat	EVLP	6	4	10 μmol/L determined to be optimal concentration in an *in vitro* model: Lower ALT and MDA; better preservation of structural architecture; decreased caspase-3 expression; decreased TUNEL staining; decreased phosphorylation of p38 and JNK; increased expression of p-Akt, PI3K, p-Bad and Bcl-2

Yu *et al*[67], 2019	HMP	NLRP3 Inflammasome Inhibitor mcc950	IRI	Pig	Transplantation	6	2	All reduced in HMP-postop group with added mcc950 in perfusate and IV administration of mcc950 After transplantation: TNF-α, IL-1β, β-galactosidase, post-reperfusion serum ALT and AST, MDA, apoptosis staining, caspase-1 levels

SNMP: Subnormothermic machine perfusion; NMP: Normothermic machine perfusion; HMP: Hypothermic machine perfusion; IRI: Ischemia-reperfusion injury; EVLP: *Ex vivo* liver perfusion; AST: Aspartate aminotransferase; HA: Hyaluronic acid; ALP: Alkaline phosphatase; HLSC-EV: Human liver stem cells-derived extracellular vesicles; LDH: Lactate dehydrogenase; ALT: Alanine aminotransferase; MDA: Malondialdehyde; IV: Intravenous.

indeed conferred the most protective effect on the liver during *ex vivo* perfusion. MP therapeutics in liver transplantation therefore has tremendous potential to increase the liver donor pool and decrease the waiting time for lifesaving organs.

Table 3 Summary of proposed advantages and limitations of liver machine perfusion therapeutics

Therapeutic	Advantages	Limitations/future considerations
RNAi pathway	Selectively targets and silences/degrades specific genes	Most beneficial/effective siRNA target against liver IRI in transplantation remains to be identified
	Mechanism of siRNA silencing pathway is generally understood	Potential for administration of multiple siRNA constructs each with a different target
	Organ-specific uptake	Requires design of siRNA against target mRNA and validation of target silencing
	Permits imaging studies visualizing tissue uptake and distribution	
Defatting cocktails	Ability to restore liver function by defatting	Steatotic livers are already predisposed to IRI[32]
	Aimed at enhancing natural lipid metabolism *via* lipid export, reduction of triglyceride levels, and stimulation of lipid oxidation and ketogenesis	Mechanisms of glucose and lipid control in liver remain poorly defined[69]
		Undefined consensus for quantifying degree of steatosis[70]
		Need for perfusate exchange protocol as secreted triglycerides recirculate causing further increase in lipid deposition[47]
		Kinetics of defatting may surpass average timeframe of liver transplantation[48]
Vasodilators	Focused on improving intrinsic function of liver to improve blood flow *via* smooth muscle relaxation and vasodilation	Effects of vasodilators in marginal grafts remains unclear[61]
	Act to increase arterial flow and decrease post-sinusoidal resistance[71]	Combination of agents does not allow for specific identification of most beneficial agent(s)
Anti-inflammatory agents	Some agents also act as vasodilators[68,72,73]	Mechanisms of anti-inflammatory agents remains unexplored in context of *ex vivo* liver perfusion[9]
	Act to scavenge free radicals to prevent IRI[74]	Combination of agents does not allow for determination of which specific agents were beneficial[9]
	Ability to protect other cell types such as endothelial cells[75,76]	
HLSC-EV	Regenerative and hepatoprotective properties[77,78]	Unknown mechanism of hypoxic protection[65]
	Diverse differentiating capabilities[77]	Timing of EV uptake during NMP currently unknown[65]
	Contain mRNA and miRNA subsets that modulate activity of target cells[79]	
	May serve as option for liver diseases without need for stem cells transplantation[65]	
δ-opioid agonist (Enkephalin)	Protects against oxidative stress[66]	Unknown therapeutic role in setting of post-perfusion liver transplant model with measured outcomes of graft function[66]
	Prevention of mitochondrial dysfunction *via* opioid receptor signaling[66]	
	Protection against IRI by slowing cellular metabolism[80,81]	Unknown role in cold ischemia conditions for liver transplant models[66]
NLRP3 inflammasome inhibitor (mcc950)	Blocks NLRP3-inflammsome activation preventing inflammatory liver damage[82,83]	mcc950 half-life is 3.27 h, while NLRP3 inflammasome activation lasts for several days after reperfusion[84,85]
	Reduces apoptosis post liver transplantation[67]	Additional mcc950 inhibition studies involving *in vitro* and *in vivo* models needed[67]

HLSC-EV: Human liver stem cells extracellular vesicles; NLRP3: Nucleotide-binding domain leucine-rich repeat containing family pyrin domain containing 3.

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