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

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CHAPTER 9

Cytokine adsorption to mitigate ischemia-reperfusion injury during ex-situ normothermic machine perfusion of porcine livers: A pilot study

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Manuscript in preparation

ABSTRACT

Background: Donor organ shortage has led to the utilization of extended criteria donor (ECD) livers, such as donation after circulatory death livers (DCD). ECD livers have a higher risk of developing post-operative complications because of their impaired tolerance to ischemia-reperfusion injury. *Ex situ* machine perfusion is a preservation modality that allows the implementation of repair strategies to minimize ischemia-reperfusion injury and improve quality and function. In this pilot study, the safety and efficacy of cytokine adsorption in preventing ischemia-reperfusion injury during ex-situ normothermic machine perfusion of donor livers is investigated.

Methods: Six porcine livers donated after circulatory death (DCD) were randomized for 3 hours of normothermic machine perfusion with the addition of a CytoSorb adsorber (n=3), or without (n=3), followed by 2.5 hours of warm reperfusion with autologous whole blood, to mimic in situ reperfusion during liver transplantation. Using ELISA, cytokine levels were measured in perfusate samples taken during NMP. In order to assess graft viability during reperfusion, perfusate and bile samples were collected, and hepatobiliary- cell function and injury were assessed.

Results: No immediate or post-perfusion complications attributable to the CytoSorb adsorber were observed. However, no differences in cytokine levels in the perfusion solution between the two groups at all time-points throughout NMP were seen. Moreover, cytokine levels in post-adsorber perfusate samples were similar to the cytokine levels in the pre-adsorber perfusate samples in the CytoSorb group. Graft function was adequate and similar in both groups during *ex-situ* warm reperfusion.

Conclusion: Cytokine adsorption during end-ischemic *ex situ* normothermic machine perfusion of DCD livers is safe and feasible. However, effective removal of cytokines during NMP was not observed.

INTRODUCTION

The access to liver transplantation continues to be hindered by the ever-growing shortage of suitable donor organs for transplantation[1]. A solution to remedy this problem is the increasing utilization of extended criteria donor (ECD) organs such as donor organs retrieved after circulatory death. The use of these marginal donor livers has brought forth the advantage of expanding the current donor pool. In fact, approximately 50% of all donor livers available for transplantation in the Netherlands are currently derived from donation after circulatory death (DCD)[2]. Nevertheless, these ECD livers, particularly DCD livers, have shown to poorly tolerate ischemia and thus incur greater ischemia-reperfusion (IR) injury as compared to the more optimal donor livers derived from brain dead donors (DBD). As a result, a significant proportion of ECD livers develop primary non-function, early allograft dysfunction and post-transplant cholangiopathy[3,4].

There are currently limited treatment options available to minimize hepatic IR injury during liver transplantation. Significant efforts have been made to limit both warm and cold ischemia times and more recently, optimize the preservation of ECD livers in ways that limit ischemia and minimize IR injury. One such example is the preservation of (ECD) donor livers using *ex situ* normothermic machine perfusion (NMP). NMP involves the provision of a continuous circulation of oxygen, nutrients, and other (metabolic) substrates to the donor organ at normal core body temperature prior to implantation. This preservation modality minimizes ischemia, avoids further depletion of ATP stores and curbs the inflammatory response elicited by the release of injurious pro-inflammatory cytokines. Nevertheless, experimental studies on IR injury during NMP illustrated that oxidative tissue injury and activation of an inflammatory immune response, albeit lower in comparison to static cold stored livers, still occurs[5,6]. Moreover, inherent to the model of *ex situ* NMP of an isolated liver, the pro-inflammatory cytokines and danger associated molecular patterns (DAMPs) that may be released upon reperfusion remain in the circulating perfusate. This potentially perpetuates IR injury and in turn, possibly counteracts the beneficial and protective effect of NMP.

Nonetheless, an attractive advantage of (normothermic) MP is the opportunity to apply repair strategies to potentially improve graft quality during perfusion. Therefore, in this study, we aimed to determine whether the addition of a cytokine adsorber during NMP of porcine DCD livers could effectively remove injurious cytokines and DAMPs from the circulating perfusate, thereby mitigating IR injury and improving graft function.

METHODS

Study groups

This study was performed using a porcine DCD liver model with livers obtained from a local slaughterhouse. Prior to procurement, livers were randomized to undergo end-ischemic *ex situ* NMP with or without the addition of a cytokine adsorber (CytoSorb 300, CytoSorbents$_{TM}$ Europe GmbH, Berlin, Germany). Randomization was performed by opening a sealed envelope containing the group assignment (intervention vs. control). For this pilot study, six livers were included. All six livers underwent an initial period of static cold storage (cold ischemia time) for three hours, followed by three hours of NMP with addition of the cytokine adsorber to the NMP circuit in the intervention group (n=3). Thereafter, all six livers underwent 2.5 hours of *ex situ* NMP using heparinized autologous whole blood to mimic *in vivo* reperfusion so as to evaluate graft function and viability.

Liver procurement

Livers from 5-month old white landrace pigs weighing between 100 and 120 kg were retrieved after circulatory death. Two liters of blood from the respective pigs were collected in 25,000 IU of heparin (Heparin LEO 5000 IU/ml, LEO Pharmaceutical Products, Denmark). This blood was stored at room temperature until priming of the machine perfusion device for *ex situ* reperfusion.

All thoracic and abdominal organs were excised *en-bloc* and the liver was isolated during the back table procedure. The portal vein was cannulated, using a 24 Fr cannula (Organ Assist, Groningen, the Netherlands). Cold flush via the portal vein was performed with 1 L of 0.9% saline (NaCl) with the addition of 25000 IU of heparin (Heparin LEO 5000 IU/ml, LEO Pharmaceutical Products, Denmark), immediately followed by 3 L of PumpProtect® / Belzer MPS® UW Machine Perfusion Solution (Carnamedica, Warsaw, Poland). Donor warm ischemia time, defined as the time of circulatory arrest until the time of hepatic cold flush via the portal vein, was limited to 30 minutes or less. After cannulation of the supratruncal abdominal aorta, the arterial tree was flushed with approximately 200 mL of PumpProtect® / Belzer MPS® UW Machine Perfusion and the open side branches were clipped. The cystic duct was ligated and the common bile duct was cannulated with an 8 Fr Meredith silicon catheter (Organ Assist, Groningen, the Netherlands) for bile collection during NMP and *ex situ* reperfusion.

Perfusion solution during ex situ NMP

The perfusion solution during NMP was an acellular, plasma-free perfusion solution containing a mixture of a bovine hemoglobin-based oxygen carrier, HBOC-201 (Hemopure, HbO2 Therapeutics LLC), Gelofusine and was fortified with nutrients, calcium, trace elements and antibiotics, as reported in detail previously[7]. The total volume of perfusion fluid was 2280 mL to which 10,000 IE of heparin were added.

CytoSorb cytokine adsorber

The CytoSorb® cartridge (CytoSorb 300, CytoSorbentsTM Europe GmbH, Berlin, Germany) is filled with CytoSorbents' proprietary hemocompatible, porous polymer beads. The dimensions of the pores in each bead are specifically designed to allow blood cells to pass through or around the beads whilst much smaller particles such as electrolytes simply pass through the pores on the beads surface. CytoSorb® has been optimized to broadly remove many cytokines, toxins and other inflammatory mediators weighing within the 5-60 kDa

molecular weight range, as most cytokines typically weigh. Adsorption of appropriately sized substances occurs by capturing and trapping these substances inside the beads' pores and channels via pore capture and surface adsorption. These substances are thus permanently removed from blood/ circulating fluid.

Normothermic machine perfusion

NMP was performed using the Liver Assist (Organ Assist, Groningen, the Netherlands). The temperature of the perfusion fluid at the start of NMP was kept at 20 °C to avoid a sudden large temperature shift following the cold ischemic period. The temperature was gradually increased within the first 30 minutes, to 37 °C (Table 1). Upon reaching 37 °C, all livers were perfused for 3 hours. Therefore, liver perfusion was performed for a total of 3.5 hours. Portal vein pressure was initially set at 5 mmHg and during the first half-hour increased in a step-wise manner along with the temperature increments, eventually reaching a set pressure of 11 mmHg at 37 °C. Similarly, the arterial pressure was initially set at 25 mmHg and eventually reached a maximum set pressure of 70 mmHg (Table 1). The circuit was supplied with 1L/min Carbogen (95% oxygen, 5& carbon dioxide). For the livers assigned to the intervention group, a CytoSorb adsorber/filter (CytoSorb 300, CytoSorbentsTM Europe GmbH, Berlin, Germany) was incorporated to the circuit. The filter was added in series to the circuit on the hepatic artery side of the device (Figure 1), with a mean flow of 500 mL/min through the filter.

Sampling:

Perfusate gas analyses were carried out at hourly intervals by using a handheld i-STAT device (Abbott Point of Care Inc., Princeton, NJ USA). When necessary, perfusate pH was corrected by administration of bolus doses of 10 - 20 mL of sodium bicarbonate ($NaHCO3^-$). In order to determine cytokine levels in the perfusate during NMP, samples were taken from two different sample points in the NMP circuit: before the CytoSorb cartridge (pre-adsorber) and after the CytoSorb cartridge (post-adsorber). Following collection, the samples were

immediately centrifuged at 2000g at 18 °C for 15 minutes, snap-frozen, and stored at -80 °C until analysis.

Bile was collected at hourly intervals in Eppendorf tubes under mineral oil to prevent interference with atmospheric air. Total bile production was determined gravimetrically and biliary pH, bicarbonate and glucose were measured every hour, using the handheld i-STAT device. The remaining bile was snap frozen for future analysis. Immediately after termination of NMP, the livers were flushed with 2 L of 0.9% NaCl at room temperature;1.5 L and 0.5 L through the portal vein and the hepatic artery, respectively. Thereafter, the livers were connected to a second device primed with autologous whole blood to mimic *in situ* reperfusion.

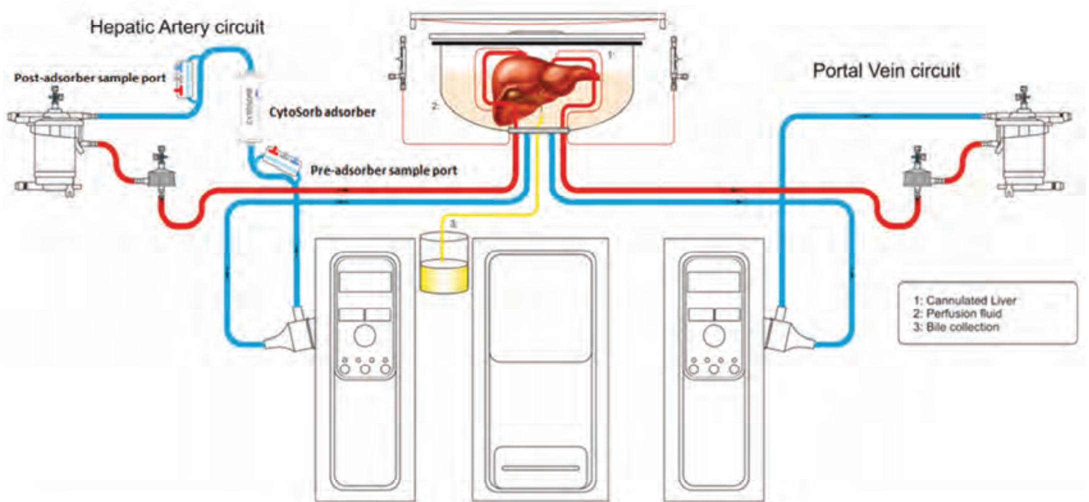

Figure 1: Schematic diagram of the Liver Asssit circuit during NMP showing the incorporation of the Cytokine Adsorber and the pre- and post-adsorber sample ports.

Ex situ warm reperfusion

All six livers underwent 2.5 hours of *ex situ* warm reperfusion with autologous whole blood. A second Liver Assist device was primed with the 2 L of heparinized whole blood collected during organ procurement. The temperature was set at 39 °C (physiological porcine core body temperature). Portal and arterial perfusion pressures were set to 11 and 70 mmHg, respectively. Livers were oxygenated with 1L/min of Carbogen (95% oxygen, 5% carbon dioxide), resulting in a PaO2 > 500 kPa.). Blood and bile gas analyses were performed at 30 min intervals. When necessary, blood pH was corrected with addition of bolus doses of 10 – 20 mL of sodium bicarbonate. Blood and bile samples were handled similarly to those taken during NMP, as described in the previous section.

Analysis of cytokine release and inflammatory response

Activation of the inflammatory response results in the subsequent release of pro- and anti-inflammatory cytokines. Previous studies investigating IR injury in liver transplantation have done so by measuring plasma levels of the inflammatory cytokines IL-1β, IL-6 and TNF-α[5,8]. Therefore, in order to determine the efficacy of cytokine filtration by the CytoSorb adsorber during end-ischemic *ex situ* NMP, concentrations IL-1β, IL-6 and TNF-α were measured in the perfusate using ELISA (Biotechne, Abingdon, UK). All ELISAs were performed according to the manufacturers' instructions.

Graft function and injury analyses

In additionally, to the point of care blood and bile gas analysis, plasma alanine aminotransferase (ALT) levels, a clinically established marker of hepatocyte injury, were measured using a routine biochemistry laboratory analyzer. Bile duct injury was assessed by determining lactate dehydrogenase (LDH) concentration; a marker of biliary epithelial cell death, in the collected bile samples using a routine biochemical method.

Statistical analyses

Given the small sample size within each group (n=3), values are presented as median and range. Statistical analyses to determine differences between the groups for this preliminary report were not performed.

RESULTS

Ischemia times

Donor warm ischemia time for livers in both groups was limited to 30 minutes or less, with the exception of one liver. Due to logistical reasons at the slaughterhouse, the hepatectomy for this was slightly delayed and flush was performed 32 minutes after circulatory arrest. Median warm ischemia time in the control group was 30 (30 – 32) minutes whereas the median warm ischemia time in the CytoSorb group was 30 (28 - 30) minutes. Median cold ischemia time (defined as the time from portal flush until connection of the liver to the NMP device) varied slightly between the groups; 183 (182 – 194) minutes in the control group vs. 187 (184 – 210) minutes in the CytoSorb group.

Perfusion characteristics and graft function during ex situ NMP

The median duration of time to reach 37 °C in the control group was 32 (31 – 37) minutes compared to 38 (33 – 44) minutes in the CytoSorb group. Despite following strict protocol, some livers took longer than 30 minutes to reach 37 °C. This may have been due to the larger size of these livers.

Portal and arterial flows steadily increased in both groups and remained stable after the first hour of NMP. Portal vein flow was slightly elevated in the control group whereas no visible differences were seen in arterial flow between the two groups (Figure 2A, B*)*. Upon reaching 37 °C, the pH was adjusted with the addition of 20 mL of NaHCO3-. Hereafter, the pH

remained stable and within physiological range in both groups throughout NMP (Figure 2C). All livers in both groups cleared lactate with no visible difference in the rate of clearance between groups (Figure 2D). Upon rewarming, glucose levels in the perfusate in both groups were seen to gradually increase but stabilized after 2 hours of NMP (Figure 2E).

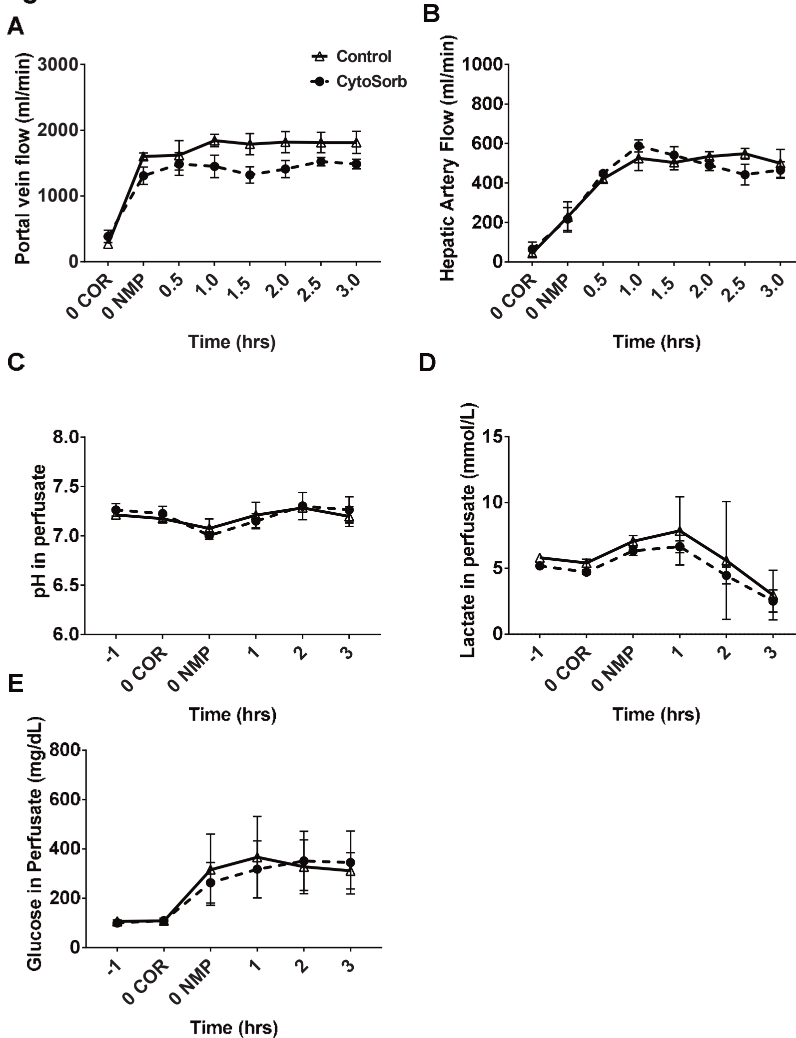

Figure 2: Comparison of machine perfusion characteristics and hepatocellular function during NMP between CytoSorb and control groups.

A: Portal vein flow (ml/min); B: Hepatic artery flow (ml/min); C: pH in the perfusate; D: Lactate clearance during NMP (mmol/L); E: Glucose release (mg/dL)

Cytokine concentrations in perfusate during ex situ NMP

Perfusate levels of all three cytokines demonstrated a steady increase during the three hours of NMP with no visible differences between the two groups at each time point throughout NMP (Figure 3). Given the use of a non-plasma based perfusion solution during NMP, the concentrations measured in the perfusate represented de-novo production and subsequent release. Median IL-1 β levels in the perfusate in both groups were comparable at the end of NMP; 1204 (218 – 2078) pg/mL in the control group compared to 1883 (1629 – 3243) pg/mL in the CytoSorb group (Figure 3A). Similarly, no differences were seen in IL-6 and TNF-α levels with the median concentrations of IL-6 and TNF-α at the end of NMP; 2804 (2324 – 3078) pg/mL and 1868 (126 – 6112) pg/mL in the control group compared to 2830 (2291 – 3008) pg/mL and 4807 (1097 – 7753) pg/mL in the CytoSorb group, respectively (Figures 3 C, E).

In order to assess the efficacy of the CytoSorb in the adsorption of cytokines during NMP, perfusate samples were taken from sample ports placed before the CytoSorb adsorber and after the CytoSorb adsorber (Figure 1). Figure 3B demonstrates the concentration of IL-1β measured in the perfusate samples before and after the CytoSorb adsorber during the 3 hours of NMP. At each time point, no visible difference is observed in the concentration of IL-1β in the post-adsorber perfusate as compared to the pre-adsorber perfusate. Similar to IL-1β, IL-6 and TNF-α levels in all three livers did not show a decrease in perfusate samples after adsorption in comparison to the pre-adsorber samples throughout NMP (Figure 3D,F).

Figure 3

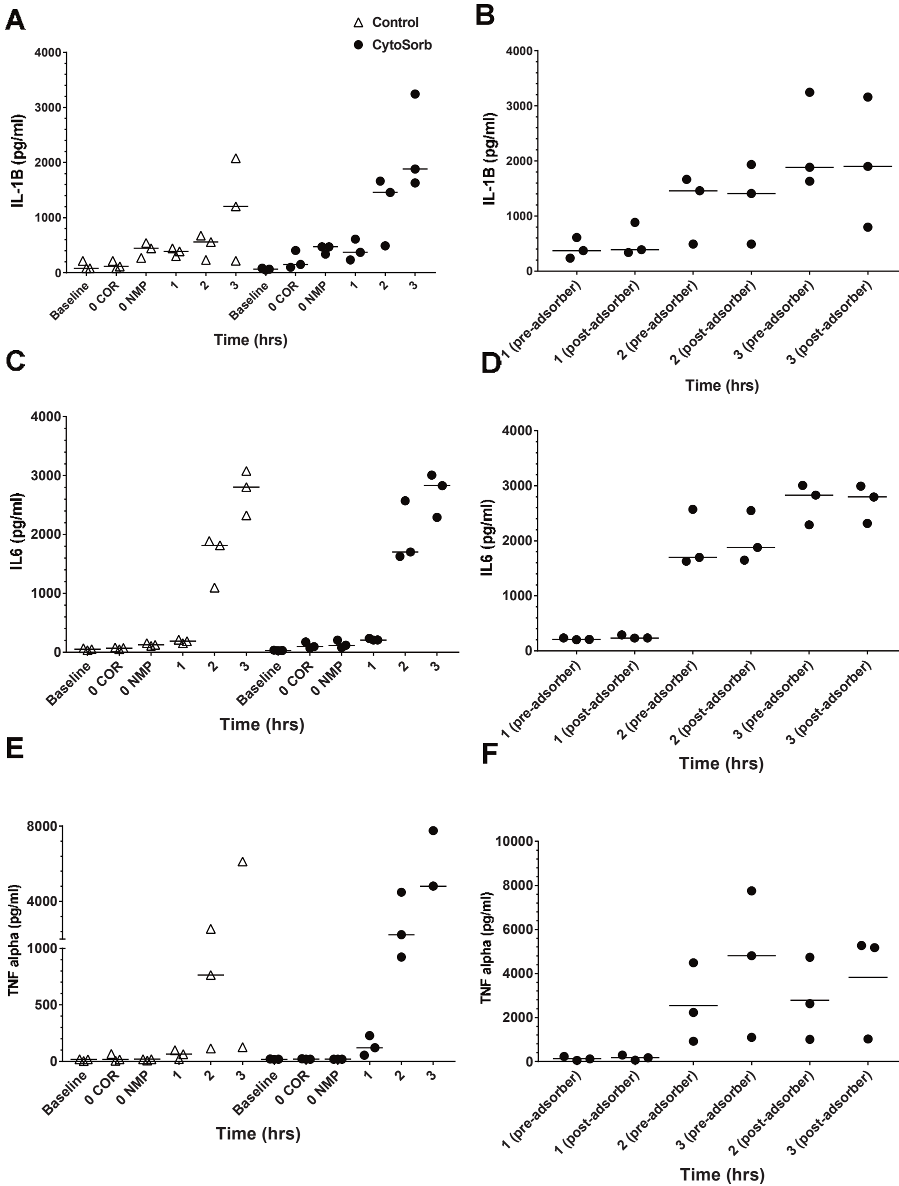

Figure 3: Cytokine levels in perfusate and comparison of pre-and post- adsorber samples during NMP. *A: Concentration of IL-1β in perfusate (pg/ml). No difference in IL-1β concentration is observed between the groups at all time-points during NMP; B: Comparison of IL-1β levels in pre- and post-adsorber perfusate samples. C: Concentration of IL-6 in perfusate (pg/ml). Similarly no difference in the concentration of IL-6 can be seen between the groups at all time-points during NMP; D: Comparison of IL-6 levels in pre- and post- adsorber samples E: Concentration of TNF-α in perfusate (pg/ml). F: Comparison of TNF-α levels in pre- and post-adsorber perfusate samples. Similar results can be seen for TNF-α.*

Liver graft and bile duct viability during mimicked in situ reperfusion

Portal vein flows in both groups steadily increased and stabilized after 30 minutes. Median portal vein flows at the end of reperfusion were 1210 (860 – 1780) mL/min in the control group and 1250 (940 – 1830) mL/min in the CytoSorb group (Figure 4A). Median hepatic artery flows were slightly higher in the CytoSorb group, as compared to control group (350 mL/min vs. 265 mL/min, respectively) (Figure 4B). This difference was due to the development of a dissecting hepatic artery aneurysm during warm reperfusion in one of the control livers. Therefore, pressures had to be kept low to prevent total rupture of the vessel. The other two livers in this group maintained hepatic artery flows >300 mL/min.

Perfusate pH remained stable and within the physiological range in both groups (Figure 4C). Two of the three control livers required an adjustment of the pH during the first hour of reperfusion whereas only one of the three CytoSorb livers required this adjustment. The total amount of sodium bicarbonate administered was therefore 40 mL for the control group vs. 20 ml in the CytoSorb group. Livers in both groups cleared lactate sufficiently with no visible difference between the groups. The lactate levels in the CytoSorb group appear lower than those in the control group at the end of reperfusion, however, this is attributable to lower levels at baseline (Figure 4D). Similar to the pattern demonstrated during NMP, plasma glucose levels gradually increase during reperfusion, with control livers showing a greater increase than that seen in CytoSorb livers (Figure 4E). To give an indication of hepatocellular injury, plasma ALT levels were measured. Figure 4F shows a gradual increase in ALT levels in the CytoSorb group, with a median level of 132 (63 – 166) U/L at the end of reperfusion. ALT levels in the control group remained stable overall, with a median level of 90 (52 – 123) U/L at the end of reperfusion.

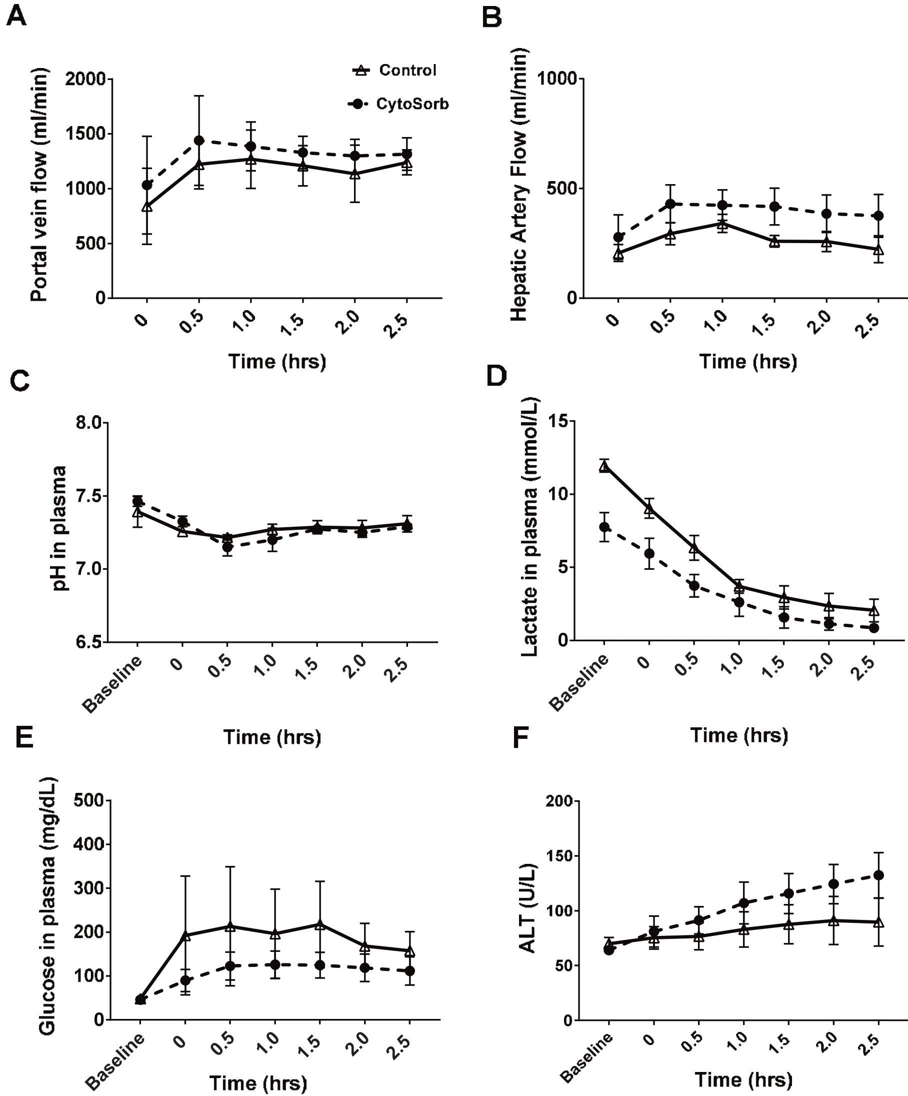

Figure 4: Comparison of machine perfusion characteristics and hepatocellular function and injury during ex situ reperfusion between CytoSorb and control groups.
A: Portal vein flow (ml/min); B: Hepatic artery flow (ml/min); C: pH in the perfusate; D: Lactate clearance during NMP(mmol/L); E: Glucose release (mg/dL); F: ALT release (U/L)

During reperfusion, all the livers in both groups produced substantial volumes of bile. Control livers had slightly higher cumulative bile production compared to the livers in the CytoSorb group (Figure 5A). This difference however, was minimal and not likely to be significant. Bile pH and the concentrations of biliary bicarbonate remained generally stable, exhibiting no visible differences between the groups (Figures 5B,C). As a marker of bile duct injury, biliary LDH was measured. Figure 5D shows a steady increase in biliary LDH levels in both groups, however no visible difference can be seen between the groups.

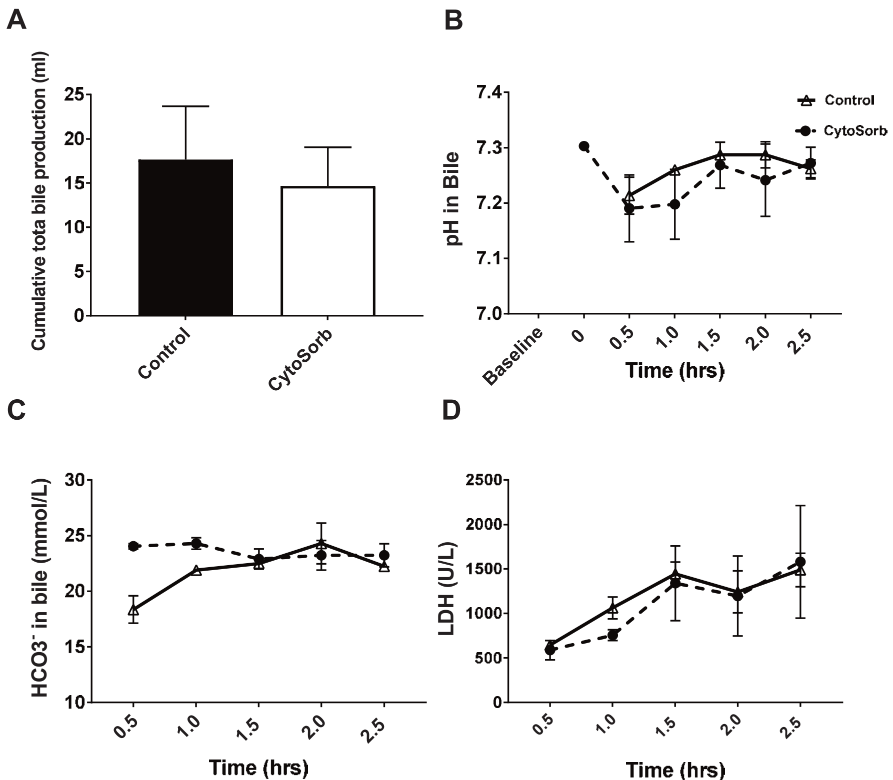

Figure 5: Cumulative bile production and cholangiocellular injury and function during reperfusion.
A: Comparison of cumulative bile production between groups. Livers in both groups produced similar amounts of bile (ml). B: pH in bile. pH in bile was the same in both groups, slightly less alkaline than physiological bile pH; C: Concentration of biliary bicarbonate (mmol/L). Minimal differences between the groups were observed in the bile; D: Concentration of LDH in the bile (U/L). Similarly, no differences were seen in the injury marker, LDH in the bile.

DISCUSSION

The results of this pilot study demonstrate that cytokine adsorption during *ex situ* machine preservation of (porcine) DCD livers is safe and feasible as no immediate or post-perfusion complications attributable to the adsorber were observed. However, the addition of the CytoSorb® adsorber to the perfusion circuit did not result in a decrease of circulating cytokines in the intervention group, and graft quality and function were comparable between the intervention (CytoSorb) and control groups.

With scarcity in the availability of optimal donor organs for transplantation, utilization of DCD organs in the past two decades has markedly increased[9]. The downfall of DCD organ transplantation however, is the increased risk of developing of post-transplant complications, many of which are due to IR injury[4,10]. *Ex situ* machine perfusion is a preservation modality that minimizes ischemia in donor organs during the preservation phase which results in less IR injury upon reperfusion. Nevertheless, given that absolute prevention of ischemia is currently difficult to achieve in daily clinical practice, strategies to further mitigate ischemia-related injury are necessary to improve graft quality and function in DCD liver grafts.

The removal of cytokines using hemoadsorption has been advocated in the management of severe inflammatory-driven disease states such as sepsis and severe systemic inflammatory response syndrome (SIRS)[11]. Several studies have reported effective reduction of circulating cytokines during extracorporeal treatments such as extracorporeal membrane oxygenation (ECMO) or renal replacement therapy (RRT), leading to improved morbidity and survival in patients admitted on intensive care units[12,13]. During transplantation, the reestablishment of blood flow during reperfusion results in the release of pro-inflammatory cytokines, activation of neutrophils and adhesion molecules on the sinusoidal endothelium leading to downstream tissue damage[14,15].Therefore, the incorporation of cytokine adsorption during *ex situ* machine preservation in an effort to capture and remove circulating pro-inflammatory mediators to minimize the extent of IR injury is currently being explored. The efficacy of cytokine

adsorption and the subsequent effect on graft function however, is currently inconclusive. Iskender et al reported that porcine DCD lungs that underwent 12 hours of ex vivo lung perfusion (EVLP) with the CytoSorb adsorber, following a cold ischemia period of 24 hours, showed a significant reduction in cytokines during perfusion as well as significantly less edema and neutrophil infiltration[16]. A plausible explanation for the contrasting findings of this study and our pilot study could be the fact that the lungs in this study underwent significantly longer cold ischemia times (>20 hours) compared to the livers in our study. Therefore, the lungs likely endured greater ischemic injury which would result in a higher inflammatory response and a greater release of cytokines during machine perfusion. Moreover, adsorption of cytokines by the CytoSorb is primarily concentration-dependent; the higher the concentration, the higher the adsorption rate. Therefore, the efficacy of the CytoSorb in the reduction of cytokines and the significant difference in cytokine levels between the two groups in the study by Iskender et al, may have been due to the higher concentrations of cytokines released as a result of longer cold ischemia times. In fact, this notion was strengthened by Kakishita et al who reported no improvement in lung physiology during EVLP of lungs perfused for 12 hours with the CytoSorb after ischemic period of less than 90 minutes. They concluded that the lack of improvement in lung function was due to the absence of significant lung injury as a result of short ischemia times[17].

Interestingly, a recent study by Hosgood et al, in which NMP was performed on porcine kidneys for six hours using the CytoSorb adsorber after a cold ischemia time of 22 hours showed no significant difference in cytokine levels between the CytoSorb and control groups. Moreover, cytokine adsorption was not associated with improved kidney graft function[18]. Therefore, the suggestion that the lack of efficacy of the CytoSorb to remove cytokines during machine perfusion is due to the absence of significant ischemic injury in organs undergoing shorter periods of ischemia is disputed by Hosgood et al. These paradoxical findings highlight the need for further investigation. In current clinical practice, cold ischemia time for donor livers deemed to be viable for transplantation is ideally limited

to 8 hours. Therefore, it is critical that future studies establish appropriate cold ischemia times that reflect cold ischemia times in current clinical practice in their experimental model so as to induce injury that is comparable to the injury incurred by donor livers in current clinical practice. That way, the efficacy of the CytoSorb can be assessed and it can be determined if the CytoSorb does indeed provide additional protection against IR injury.

Furthermore, Hosgood et al observed an upregulation of the majority cytokines occurring only after 3 hours of NMP. The livers in our study underwent only 3 hours of perfusion so this too, may possibly explain why we did not observe effective cytokine adsorption in our cohort. The concentrations of the cytokines in the perfusate during the 3 hours of NMP were likely too low to generate a sufficient driving force to stimulate adsorption across the CytoSorb membrane.

To our knowledge, this is the first study to describe cytokine adsorption during NMP of (porcine) donor livers. This pilot study serves as a proof-of-concept in which we were able to confirm the safety and feasibility of cytokine adsorption during NMP of donor livers. Nevertheless, despite the promise of cytokine adsorption as a strategy to minimize IR injury and improve graft function, valid conclusions on the efficacy of this intervention remain to be drawn. The few published reports on cytokine adsorption have helped us recognize that the short ischemia times as well as the short perfusion times in our experimental design may have limited this study from achieving the primary end-point. Therefore, the next phase of our research is to revise our current experimental model by prolonging ischemia and perfusion times so as to determine whether or not a greater inflammatory response occurs, and whether this results in effective cytokine adsorption during NMP. We then aim to assess whether the adsorption of cytokines leads to significantly reduced IR injury and improved graft function of the liver grafts.

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