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

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CHAPTER 4

Production of physiologically relevant quantities of hemostatic proteins during normothermic machine perfusion of human livers

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ABSTRACT

Ex-situ normothermic machine perfusion (NMP) provides the opportunity to assess graft function and viability, particularly of sub-optimally functioning donor livers, prior to transplantation. During *ex-situ* NMP, donor livers usually resume normal metabolic and synthetic functions, such as hemostatic protein production. However, the quantities of these proteins produced are currently unknown. Six donor livers declined nationwide for transplantation underwent 6 hours of end-ischemic NMP using a heparinized plasma-free perfusion fluid. Concentrations of key pro-hemostatic proteins (Factors II, V, VII and X, fibrinogen and VWF), anti-coagulant proteins (protein C and antithrombin III) and fibrinolytic proteins (plasminogen and tissue-type plasminogen activator) were measured in perfusion fluid at regular intervals during NMP and compared with a plasma-based reference solution. Pro-coagulants showed an increase of 9-57% of the levels measured in the plasma reference solution whereas anticoagulant and fibrinolytic protein levels amounted to 41-71% and 18-116%, respectively. This study demonstrates the capability of donor livers perfused with a plasma-free perfusion fluid to produce substantial amounts of hemostatic proteins during a relatively short period of NMP. These results are influential in determining appropriate anticoagulation protocols to avoid activation of hemostasis throughout NMP.

INTRODUCTION

Ex-situ normothermic machine perfusion (NMP) is a novel technology being applied in both experimental[7,38,39] and clinical[14,40,41] settings in an effort to overcome ischemia-related preservation injury associated with static cold storage (SCS), as well as to improve the quality of sub-optimally functioning donor organs. These high-risk/extended-criteria donor (ECD) livers are increasingly being utilized to meet the on-going demand for donor organs[42]. Performed at 37℃ and under conditions closely resembling *in-vivo* physiology, NMP can be carried out for either the entire preservation period between procurement and implantation (preservation machine perfusion), or after a donor liver has undergone a period of traditional SCS (end-ischemic machine perfusion)[13] . NMP provides the ability to recondition and optimize the function of ECD livers[5,9,41] as well as grant the possibility to test the function and viability of the organ prior to implantation[23]. The *in-vivo* physiological conditions maintained during NMP necessitate the need for a perfusion fluid that mimics the composition of whole blood. To date, perfusion fluids used in NMP studies are composed of either plasma-based solutions[39] or plasma-free solutions consisting of colloids such as Gelofusine[14-16,43], Belzer solution[44], Steen solution[45], albumin[15,40] or crystalloids such as sterofundin[46]. The use of colloid or crystalloid solutions for NMP is becoming increasingly sought after as this avoids the use of human plasma which is scarce, costly and logistically challenging.

The liver synthesizes pro- and anti-coagulant proteins along with components of the fibrinolytic system. During *ex-situ* NMP, *in-vivo* graft reperfusion is mimicked and the donor livers resume normal metabolic and synthetic functions[15,47]. Livers are therefore, likely to synthesise hemostatic proteins during NMP. Indeed, it has been previously demonstrated that the international normalized ratio (INR) of a plasma-free perfusion solution decreases over time during 8 hours of NMP of discarded human livers, which likely indicates production of procoagulant proteins during perfusion[41]. However, the levels of procoagulant proteins produced were not quantified in this study and the INR values reported are likely an underestimation as the perfusion fluid contained substantial amounts of heparin, which are

known to interfere with INR measurements. To date, duration of NMP of donor livers in published data ranges from 3.5 to 24 hours[17,45,46]; Ravikumar et al. and Bral et al., even managed to successfully transplant a donor liver after 18.5 and 22.5 hours of NMP respectively[14,43]. Based on previously published data, it is likely that physiological amounts of hemostatic proteins can be produced during prolonged periods of NMP. Therefore, the objective of this study was to quantify the levels of individual hemostatic proteins during NMP of ECD donor livers with a plasma-free perfusion solution.

MATERIALS AND METHODS

Donor livers

Six human donor livers that were declined for transplantation by all three transplant centres in the Netherlands, and by all other centres within the Eurotransplant region, were included in this study. Liver procurement started after a five minute 'no touch' period following declaration of cardiac arrest and circulatory death in the case of donation after circulatory death (DCD). In the case of donation after brain death (DBD) liver procurement, 25,000 units of heparin were provided intravenously before cross clamping. The same dose of heparin was added to the preservation solution in the case of DCD liver procurements. All livers were retrieved using a standard surgical technique of *in situ* cooling and flush-out with ice cold preservation fluid (University of Wisconsin [UW]). Livers were subsequently packed, stored on ice and transported to our center. During this period, all 6 livers were preserved with ice cold UW preservation fluid. Back-table and machine perfusion procedures performed on these livers has been previously described in detail by Matton et al.[48] This study protocol was approved by the medical ethical committee of the UMCG.

Machine perfusion solution

The total volume of the perfusion solution amounted to 2202 ml to which 20,000 IE of unfractionated heparin was added. The perfusion solution consisted of 500 ml of the colloid Gelofusine (4% Gelofusine®, B Braun, Meslungen, Germany), albumin and saline. HBOC-201 (Hemopure®, HbO2 Therapeutics LCC. Souderton, PA, USA) was used as a substitute for packed human red blood cells during NMP and functioned as the oxygen carrier during NMP. HBOC-201 is a hemoglobin-based oxygen carrier solution derived from bovine blood. Successful use of this solution during NMP of human donor livers in an experimental setting has very recently been reported[40]. Further addition of vitamins, nutrients, antibiotics as well as adjustments to the pH, osmolality, colloid osmotic pressure were made to ensure physiological concentrations were maintained.[48]

End-ischemic *ex-situ* NMP

Based on prior experience[23,39], end-ischemic *ex-situ* NMP was performed at 37℃ using a pressure-controlled liver perfusion device (Organ Assist, Groningen, the Netherlands) for a duration of 6 hours as previously described[48].

Perfusion fluid samples were collected before the start and at every half hour of NMP and immediately kept on ice until end of NMP. These samples were then centrifuged for 5 min at 2700 rpm at 4ºC, transferred to aliquots and stored at-80ºC.

Plasma-based reference solution

A plasma-based reference solution using fresh frozen plasma (FFP) provided to us by the blood bank was made in order to make valid comparisons with the concentrations of the proteins measured in the plasma-free NMP perfusion solution. With exception of FFP, the composition of this fluid was identical to the plasma-free solution. Plasma-based reference solutions using 6 separate batches of FFP were generated.

Plasma levels of individual hemostatic proteins in the perfusate during NMP

Levels of hemostatic proteins were analysed in the plasma-free perfusate samples and the 6 plasma-based reference solutions by enzyme-linked immunosorbent assays (ELISA), as the presence of heparin within the solutions prohibited meaningful quantification of these analytes using clot-based assays.

Prothrombin was quantified using the Human Prothrombin total antigen assay ELISA kit (Molecular innovations, Inc, Novi, MI, USA). Perfusate levels of FVII, FV, FX, plasminogen, and protein C were quantified using ELISA kits from Abcam (Cambridge, UK). Antithrombin levels were quantified using the Antithrombin Human-Serpin duoset ELISA (R&D systems; Bio-Techne, Oxon, UK), and tissue plasminogen activator (tPA) levels using the ZYMUTEST TPA-AG (Brenntag, Dordrecht, the Netherlands), respectively. All ELISAs were performed according to manufacturer's instructions. VWF and fibrinogen antigen levels were determined with in-house ELISAs using commercially available polyclonal antibodies. (DAKO, Glostrup, Denmark)[49].

Statistics

Statistical analyses were performed using SPSS version 23 for Windows (SPSS Inc., Chicago, Ill, USA). Continuous variables were presented as medians and range and categorical variables were presented as absolute numbers and percentages.

Table 1. Donor liver characteristics

Type of donor	
DCD	**5**
DBD	**1**
Age (years)	65 [63 – 71]
Gender	
Male	3
Female	3
BMI	25 [23 - 31]
Cause of death	
Anoxia	2
CVA	2
Trauma	2
ET-Donor risk index[a]	3.0 [2.23 – 3.3]
Reason for rejection	
DCD + age >60	4
Steatosis	1
Other[b]	1
Serum ALT (IU/L)[c]	46 [12 – 106]
Total donor warm ischemia time[d] **(min)**	39 [25 – 56]
Cold ischemia time[e] **(min)**	482 [372 – 528]
Liver weight (kg)	1.67 [1.25 – 1.99]

Continuous variables are presented as median and range, categorical variables as absolute numbers.

[a] *ET-Donor risk index was calculated according to Braat et al*[50]
[b] *DCD age 57 in combination with out-of-hospital cardiac arrest*
[c] *Last known value before procurement*
[d] *Time between withdrawal of life support until the aortic cold flush in the donor (DCD only).*
[e] *Time between the donor aortic cold flush until the start of normothermic machine perfusion.*

RESULTS

Five of the six livers were from DCD donors. The primary reasons for rejecting these livers for transplantation were; DCD livers from donors older than 60 years of age, steatosis and uncontrolled cardiac arrest. Donor demographics and the procedural variables of the six donor livers that underwent end-ischemic NMP are summarized in Table 1.

Levels of hemostatic proteins in samples taken during NMP

No hemostatic proteins were detectable in the plasma-free perfusate samples prior to connection of the livers to the NMP device (*Figure 1*). During NMP, gradual increases in perfusate levels of all the proteins measured were observed.

During the six hour NMP period, perfusate levels of procoagulant factors II, V, VII and X as well as fibrinogen exhibited a gradual steady increase (*Figure 1A-E*) reaching up to 10%, 9%, 59%, 9% and 31% of the plasma-based reference solution, respectively (Table 2). Substantial amounts of VWF were released into the perfusate over time (*Figure 1F*), with levels at the end of NMP reaching 76% of levels seen in the plasma-based reference solution (Table 2).

The anticoagulant proteins antithrombin (ATIII) and particularly, protein C substantially increased over time (*Figure 1G-H)*, with perfusate levels of protein C exceeding the levels in the plasma-based reference solution in some livers *(*Table 2). At the end of NMP, median perfusate levels of ATIII and protein C were 41% and 71% of levels in the plasma-based reference solution, respectively (Table 2).

Perfusate levels of key fibrinolytic proteins plasminogen and tPA are presented in *Figures 1I-J.* Plasminogen gradually increased over time during NMP, amounting to close to 20% of normal plasma levels at the end of NMP (Table 2). Perfusate levels of tPA peaked at 30 minutes and the levels at the end of NMP, remained higher compared to the tPA levels in the plasma-based reference solution (Table 2; *Figure 1I-J*).

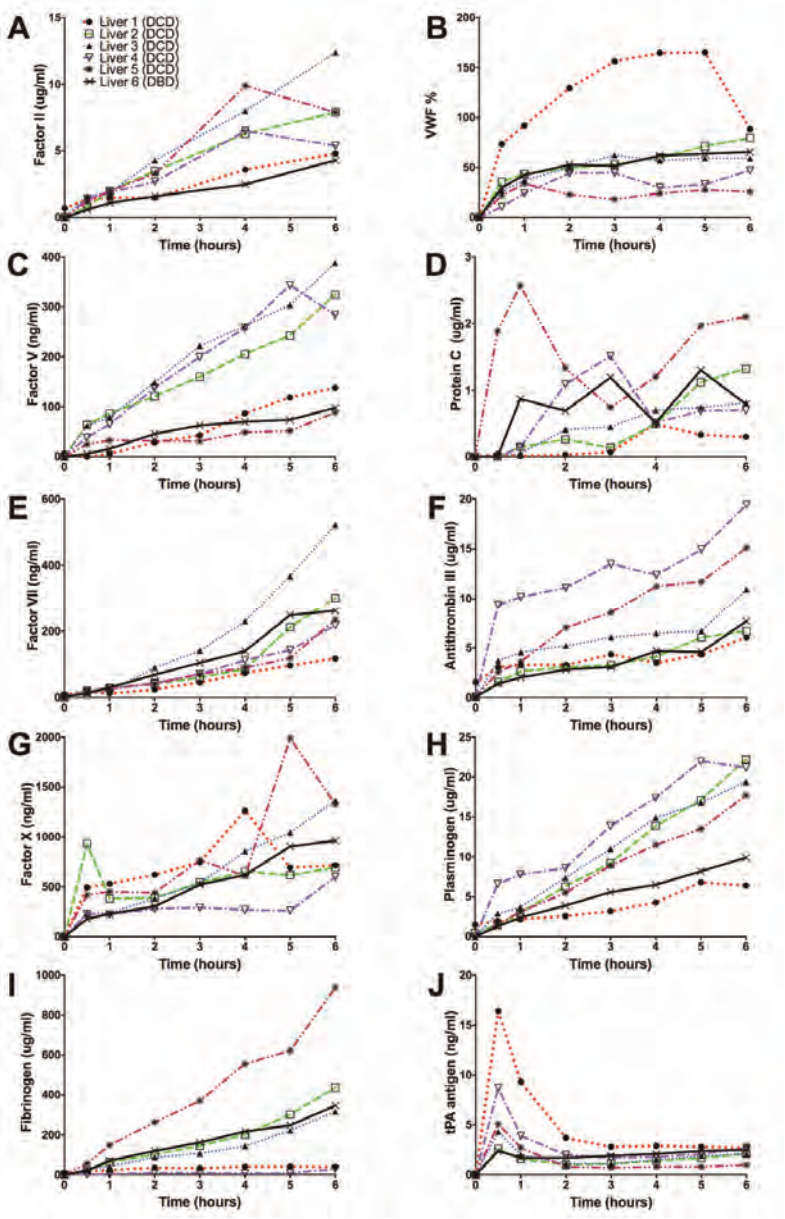

Figure 1. Increases in concentrations of pro-coagulant, anti-coagulant and fibrinolytic proteins in 6 human donor livers during 6 hours of normothermic machine perfusion (NMP) using a plasma-free solution. *Panel A; Factor II. Panel B; Factor V. Panel C; Factor VII. Panel D; Factor X. Panel E; Fibrinogen. Panel F; Von Willebrand factor (VWF). Panel G; Protein C. Panel H; Antithrombin III. Panel I; Plasminogen. Panel J; Tissue-type plasminogen activator (tPA)*

Table 2. Hemostatic protein production during 6 hours NMP of human donor livers using a plasma-free perfusion solution, compared to a plasma-based reference solution.

Protein	Median concentration after 6 hours NMP	Percentage of concentrations measured in reference solution*
Factor II (µg/ml)	6.65 [4.30 – 12.40]	**10%** [9 – 19]
Factor V (ng/ml)	211 [89 – 388]	**9%** [6 – 12]
Factor VII (ng/ml)	248 [118 –522]	**59%** [36 – 100]
Factor X (ng/ml)	839 [599–1363]	**9%** [9.6 – 10]
Fibrinogen (µg/ml)	332 [29 – 938]	**31%** [6.5 – 58]
VWF (%)	62.4 [26 – 88]	**76%** [49 – 55]
Protein C (µg/ml)	0.80 [0.30 – 2.10]	**71%** [39 – 171]
Antithrombin III (µg/ml)	9.31 [6.04 – 19.44]	**41%** [33 – 66]
Plasminogen (µg/ml)	18.6 [6.4 – 22.2]	**18%** [8 – 19]
tPA antigen (ng/ml)	2.3 [1.0 – 2.7]	**116%** [56 – 108]

Values are presented as median and range.

* *A plasma-based reference solution with a similar composition to the plasma-free NMP solution served as a baseline reference for comparison with the concentrations measured in the plasma-free NMP solution.*

DISCUSSION

The results of this study demonstrate that after 6 hours of NMP, isolated donor livers perfused with a plasma-free solution are able to generate substantial amounts of hemostatic proteins. The production of pro-coagulant proteins ranged between 9-57% of the levels measured in the plasma-based reference solution. Anti-coagulant and fibrinolytic protein levels amounted to 41-71% and 18-116% of the levels measured in the plasma-based reference solution, respectively. In addition, appreciable amounts of the endothelial-derived proteins, VWF and tPA, released into the perfusate indicate that besides adequate

hepatocyte synthetic activity, the secretory function of sinusoidal endothelium is also maintained during NMP.

To prevent coagulation activation and subsequent clot formation within the perfusion circuit and more importantly, in the liver itself, an anticoagulant agent is typically added to the perfusion solution during NMP. To date, anticoagulation management by most groups involves the use of heparin. Current NMP anticoagulation protocols in literature describe the addition of bolus doses of; 10,000IU[15], 20,000IU[39] or 25,000 IU[14] added to the perfusion fluid during priming of the device before the start of NMP. In some cases, additional continuous doses such as 830 IU per hour[46] or 1000 IU per hour[41], are given throughout NMP. Even though heparin is present within plasma-free perfusates with the aim of preventing coagulation activation during NMP, heparin will only function as an anticoagulant in the presence of antithrombin. In this study, the concentrations of the procoagulant proteins (II, V and X) are generally lower, relative to the concentration in the plasma-based reference solution than the anticoagulant and fibrinolytic proteins, which together with the profound production of tPA may indicate that a favourable anticoagulant and pro-fibrinolytic environment is present, at least in the first 6 hours of NMP. Additionally, antithrombin is generated in appreciable quantities and as the production of pro- and anticoagulants favours the anticoagulants, it is likely that heparin anticoagulation during plasma-free perfusate NMP is effective. Nevertheless, with production of all procoagulant proteins starting immediately after initiation of NMP, more direct anticoagulants such as direct thrombin inhibitors (for example, Bivalirudin, which in contrast to inhibitors such as Dabigatran, Argatroban, does not require metabolic activation by the liver) may be more effective in the initial phase of NMP as such anticoagulants are not dependent on antithrombin.

To our knowledge, we are the first to quantify the levels of hemostatic proteins in the perfusate of plasma-free NMP. This study shows that NMP results in the restoration of normal metabolic and synthetic function of donor livers culminating in significant production of hemostatic proteins. The study by Banan et al.[41], in which INR was used as a marker for

hepatic synthetic function, observed decreasing INR levels in all livers in their cohort during 8 hours of NMP. They attributed these decreases to production of coagulation factors, which is in line with the findings of this study. Given these findings, future research is required to determine whether measurement of perfusate levels of hemostatic factors can be used to assess functional capacity and as viability markers during NMP.

This study demonstrates the capability of donor livers perfused with a plasma-free perfusion fluid to produce substantial amounts of hemostatic proteins during a relatively short period of NMP. Despite the liver being responsible for the synthesis of these proteins, Lisman et al[51]., previously elucidated that plasma levels of hemostatic proteins are not controlled by the liver. Hormonal systems, extra-hepatic sensors and clearance of these proteins were proposed as possible mechanisms responsible for the regulation of the plasma levels of hemostatic proteins. Given the absence of these influences during NMP of an isolated liver, it is likely that the generation of these proteins will proceed throughout NMP. Particularly for studies where donor livers are perfused for extended periods of time (10+ hours), one can speculate that this could potentially lead to generation of a perfusion solution with plasma-like or even supra-physiological concentrations of these proteins towards the end of NMP. Therefore, future research is required to further investigate whether current anticoagulant protocols using heparin are sufficient to prevent coagulation both in the very early and late phases of NMP.

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