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

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*DOI:*
10.33612/diss.161905515


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CHAPTER 5

# Anti-coagulant management and synthesis of hemostatic proteins during machine preservation of livers for transplantation

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*Semin Thromb Hemost 2020 Sep;46(6):743-750*

**ABSTRACT**

Liver transplantation remains the only curative treatment for patients with end-stage liver disease. Despite a steadily increasing demand for suitable donor livers, the current pool of donor organs fails to meet this demand. In order to resolve this discrepancy, livers traditionally considered to be of sub-optimal quality and function are increasingly utilized. These marginal livers, however, are less tolerant to the current standard cold preservation of donor organs. Therefore, alternative preservation methods have been sought and are progressively applied into clinical practice. *Ex situ* machine perfusion is a promising alternative preservation modality particularly for sub-optimal donor livers as it provides the ability to resuscitate, recondition, and test the viability of an organ prior to transplantation. This review addresses the modalities of machine perfusion currently being applied, and particularly focuses on the hemostatic management employed during machine perfusion. We discuss the anticoagulant agents used, the variation in dosage and administration, as well as the implications of perfusion for extended periods of time in terms of coagulation activation associated with production of coagulation factors during perfusion. Furthermore, in regard to viability testing of an organ prior to transplantation, we discuss the possibilities and limitations of utilizing the synthesis of liver-derived coagulation factors as potential viability markers.

**Liver transplantation and the current preservation modalities**

Orthotopic liver transplantation (OLT) remains the most effective and only curative treatment for patients with end-stage liver disease. Despite this, OLT remains a complex procedure that is associated with several potentially life-threatening complications[1,2]. In fact, less than two decades ago, OLT was frequently associated with high rates of intraoperative blood loss, often resulting in a need for substantial amounts of red blood cell (RBC), fresh frozen plasma (FFP), and platelet concentrate transfusions, which significantly influence post-operative patient outcomes and the viability of the transplanted organ[3,4]. Fortunately, with the improvement of both surgical and anesthesiological techniques, the rates of both intraoperative blood loss and transfusion requirements during OLT have dramatically decreased. In fact, several transplant centers have even reported transfusion-free OLT procedures in recent years[5,6].



Liver transplantation is currently limited by the persistent shortage of donor organs available for transplantation to meet the ever-growing demand. This discrepancy has resulted in increasing mortality rates on waiting lists worldwide, prompting the use of extended criteria donor (ECD) livers in order to expand the donor organ pool[7,8]. Such livers include; livers donated after circulatory death (DCD), livers from older donors, and livers that exceed the traditionally accepted degree of steatosis. However, studies have shown that these ECD livers are more likely to develop primary non-function, early allograft dysfunction, or biliary complications due to their increased vulnerability to ischemia-reperfusion injury incurred during the OLT procedure[9].

*Current preservation modalities of donor livers for transplantation*

Static cold storage (SCS) is the current standard of care for the preservation of donor livers. This method is capable of adequately slowing down the metabolic processes in the organ and minimizing ischemic injury. However, it is limited by the duration in which donor organs can be preserved. Moreover, unlike low-risk livers that are capable of tolerating moderate ischemia, ECD donor livers tolerate ischemia poorly[10]. Furthermore, DCD livers inevitably undergo an additional hypotensive warm ischemic period during circulatory arrest of the donor, which all together put these livers at risk of developing severe graft injury[11]. In order to circumvent injury in these increasingly utilized ECD livers, other preservation methods and techniques have been explored and are being applied into current clinical practice. The most notable alternative preservation method is *ex-situ* machine perfusion (MP). Using MP, donor livers are preserved under conditions simulating *in vivo* physiology by providing a continuous circulation of oxygen, nutrients, and other (metabolic) substrates for a given period of time. There are different ways to perform MP, each with a distinct methodology and different risks and benefits. Therefore, depending on the specific objective of the preservation technique required to achieve, a certain type of MP is applied[12]. Such objectives may include organ resuscitation and reconditioning, viability testing, and extended preservation.

*Hypothermic machine perfusion (HMP)*

Hypothermic machine perfusion is performed at 4-12 °C and can be carried out with or without active oxygenation of the perfusion fluid. Those performing HMP without active oxygenation have demonstrated that given the direct contact of the perfusion fluid with atmospheric air, the perfusate contains sufficient oxygen to perfuse and oxygenate the liver graft[13]. HMP of the liver can be performed by perfusing the liver either through the portal vein alone[14,15] or via both portal vein and hepatic artery[16] (Figure 1). Consensus on which methodology results in superior outcomes is yet to be reached and larger RCTs investigating

the clinical efficacy of these techniques are currently ongoing (NCT01317342 and NCT02584283).

In current clinical practice, HMP is typically performed in ECD donor liver grafts that have undergone circulatory arrest and thus a warm ischemic phase (DCD livers), in livers derived from older donors, livers that have been stored on ice for a prolonged period of time (extended cold ischemia), or livers that are significantly steatotic. The period of warm ischemia during the (DCD) procurement process and the subsequent cold ischemia during transportation lead to depletion of key intracellular energy sources, such as adenosine 5′-triphosphate (ATP). Moreover, as a result of ischemia, a number of structural and metabolic changes occur in the cells that trigger a cascade of reactions leading to injury and damage upon reperfusion, also commonly known as ischemia-reperfusion (IR) injury. This can manifest clinically during transplantation as systemic (hemodynamic, hemostatic, or metabolic) dysregulation in the recipient which frequently increases the risk of post-transplant complications and possible graft failure[17,18].



The low temperature applied during HMP slows down metabolism and the need for oxygen, allowing the restoration of cellular energy stores and replenishing of ATP[16,19,20]. Furthermore, HMP results in a decreased release of injurious mitochondria-derived reactive oxygen species in the recipient after implantation and reperfusion. These reactive oxygen species are responsible for the induction of downstream inflammatory reactions which further perpetuate graft injury[21-23].

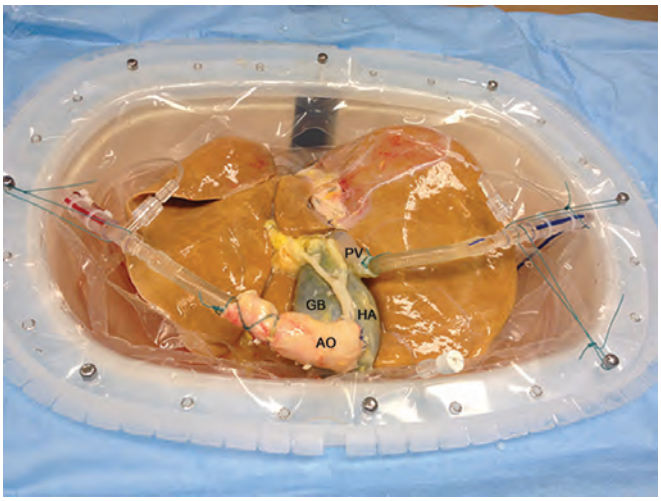


***Figure 1: Human donor liver undergoing (dual) hypothermic machine perfusion (HMP) with University of Winsconsin fluid at 10 °C.*** *AO: supratruncal aorta segment used for hepatic artery cannulation; HA: Common hepatic artery (cannulated via aorta); PV: Portal vein (cannulated with portal vein cannula); GB: Gall bladder*

*Normothermic machine perfusion (NMP)*

On the other hand, donor livers can also be perfused at normal core body temperature (normothermic machine perfusion (NMP)). NMP provides the opportunity to recondition the liver grafts by circulating nutrients and oxygen at 37°C enabling aerobic metabolism to continue during the preservation phase whilst limiting ischemic injury. The set-up for NMP closely resembles *in vivo* circulation with dual perfusion (through both portal vein and hepatic artery), a perfusate closely resembling the composition of whole blood, and active oxygenation (Figure 2).

NMP is currently applied in an effort to resuscitate marginal liver grafts which would traditionally be declared as unfit for transplantation, or to extend the preservation period[24-26]. NMP can be performed immediately after procurement until transplantation, thus significantly minimizing SCS as the preservation modality for these grafts[27]. This procedure reduces IR injury. NMP cannot always be performed immediately after procurement, for example when there is no mobile NMP device available in the procurement center, or when the

procurement is at a different hospital than the transplantation. In those cases, NMP can also be performed end-ischemically i.e., following a period of static cold storage during transportation. An important feature of NMP is the opportunity to assess the viability of the (metabolically active) organ prior to transplantation. This is carried out by measuring markers released during activation of key metabolic processes that are activated upon reperfusion and by measuring bile production and composition.

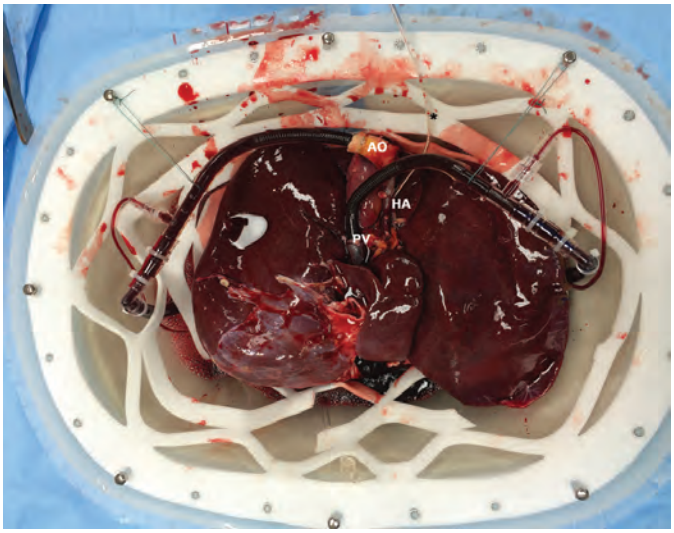


***Figure 2: Human donor liver undergoing normothermic machine perfusion (NMP) with a bovine-derived hemoglobin based oxygen carrier (Hemopure®) and plasma-free perfusion solution at 37 °C.*** *AO: supratruncal aorta segment used for hepatic artery cannulation; HA: Common hepatic artery (cannulated via aorta); PV: Portal vein (cannulated with portal vein cannula); *bile cannula for collection of bile via extra-hepatic bile duct.*

*Perfusion fluids utilized in machine perfusion of donor liver grafts*

Depending on the MP modality used, different perfusion solutions are used. Current clinical application of HMP involves the use of University of Wisconsin/ Belzer's solution or modifications thereof. This colloid solution is consistent with an extra-cellular fluid in order to attenuate cell swelling and lysis and some have argued that utilization of these solutions during HMP require no oxygen carrier given the low levels of metabolism at hypothermic temperatures. On the other hand, conditions maintained during NMP, which mimic those under physiological *in vivo* conditions, necessitate the need for a perfusion fluid that mimics the composition of whole blood. To date, perfusion fluids used in NMP studies have consisted of a combination of packed red blood cells or more recently, an alternative oxygen carrier such as HBOC-201® (a bovine-derived free hemoglobin oxygen carrier solution) together with either plasma-based solutions or plasma-free solutions consisting of colloids such as Gelofusine or albumin, or crystalloids such as sterofundin[28-32]. 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.

**Anti-coagulation during machine perfusion: current clinical practice**

During HMP, plasma is never used as a perfusion solution. Moreover, the hypothermic temperatures largely prevent synthesis of coagulation factors by the liver. Therefore, addition of an anticoagulant agent to the circuit during the HMP is not necessary. However, during NMP, potential activation of coagulation and subsequent clot formation due to the presence of FFP in the perfusion fluid and/or de novo synthesis of coagulation factors is a concern[17,33]. The absence of an endothelial layer as the perfusate circulates through silicon tubing of the NMP circuit, for example, could potentially activate coagulation. Hence, in order to prevent the occurrence of thrombotic events, the current practice has been to add an anticoagulant to the perfusion solution during NMP. Thus far, anticoagulant management by all groups

performing machine perfusion consists of the use of unfractionated heparin. However, the manner in which the heparin is administered to the perfusion circuit varies amongst the different groups. Some studies have reported administering a single bolus dose of unfractionated heparin at the start of perfusion (dosage range reported 20,000 – 30,000 IE)[31,34,35], others have reported bolus doses at regular intervals throughout the duration of perfusion or alternatively, administering a single bolus dose at the start of perfusion, followed by continuous infusion throughout perfusion or simply continuous infusion throughout the entire perfusion[36].

Despite the different anticoagulation protocols being applied during NMP, no major or minor thrombotic complications have been described. Detailed studies assessing the efficacy of the various anticoagulation protocols during NMP remain scarce. To our knowledge, our group is the only group so far that has specifically assessed whether coagulation activation occurs during NMP. We studied ECD livers during 6 hours of NMP perfused with a plasma-based solution to which a bolus dose of 20,000 IE of heparin was administered at the start of NMP. Our results showed no significant increase in markers of coagulation activation in the perfusate nor did we see any evidence of (micro-)thrombus formation on histology[37]. In contrast, we observed a significant increase in plasmin-antiplasmin complexes and D-dimers over time. Thus, our findings suggested that end-ischemic *ex situ* NMP of ECD livers results in activation of fibrinolysis, which may aid in the dissolution of any preexisting clots that could have been formed as a result of coagulation activation in the donor during the agonal and circulatory arrest phase[33]. The activation of fibrinolysis during NMP is likely explained by release of tissue-type plasminogen activator from injured endothelium during reperfusion.

**Hemostasis and the prevention of coagulation activation during prolonged normothermic machine perfusion**

Given the physiological conditions maintained during NMP, donor livers typically resume normal metabolic and synthetic functions, such as lactate clearance, bile production and synthesis of proteins soon after initiation of NMP[24,38]. It is widely known that the liver is principally responsible for the synthesis of pro- and anticoagulant proteins along with components of the fibrinolytic system. However, the extent and rate of the synthesis of hemostatic proteins during *ex situ* NMP is yet to be thoroughly investigated. Nevertheless, our group recently demonstrated substantial hemostatic protein production during NMP, with release of hemostatic proteins in the perfusate already within minutes after reperfusion. After only six hours of NMP with a plasma-free perfusion solution, we showed that all studied DCD livers (n=6) were capable of synthesizing substantial amounts of pro-coagulant, anti-coagulant and fibrinolytic proteins, with some of these proteins reaching concentrations exceeding physiological plasma levels[39] (Figure 3). Our proof-of-concept findings indicated that restoration of the synthetic function of the hepatocytes culminates in the production of substantial amounts of hemostatic proteins in a relatively short period of time. These results are especially relevant as some transplant centers performing NMP are increasingly exploring the potential to perfuse livers for extended periods of time (15 to 24+ hours)[32,36,40]. In fact, in most of the centers where NMP is clinically applied, the majority of the livers undergo a minimum of eight hours of NMP prior to transplantation. Therefore, the composition of the perfusate following an extended period of NMP could potentially resemble plasma at the end of perfusion. These findings indicate that adequate anticoagulation, even for non-plasma-based perfusion solutions, is necessary.

Although the liver is responsible for the synthesis of these proteins, our group previously elucidated that maintenance of plasma levels of hemostatic proteins is not controlled by the liver. Alternatively, hormonal systems, extra-hepatic sensors and rate of clearance of these proteins were proposed as the factors mainly responsible for maintaining plasma levels of

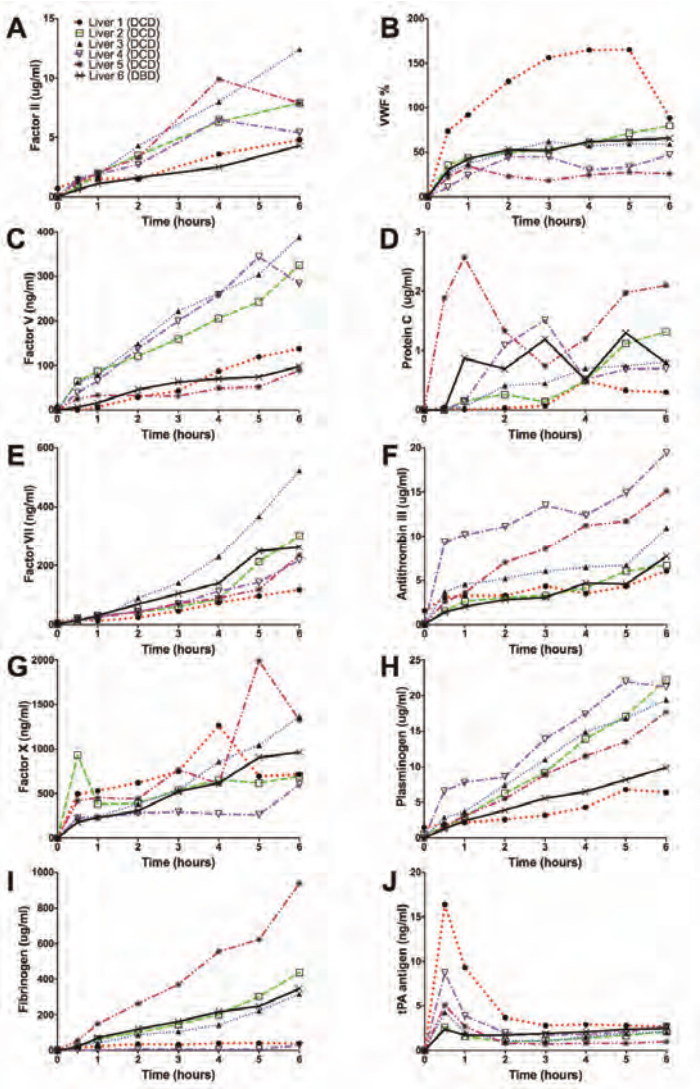


***Figure 3: Production of pro-coagulant, anti-coagulant and fibrinolytic proteins in 6 human donor livers during 6 hours of normothermic machine perfusion (NMP) using a plasma-free solution.*** *Panel A; Factor II. Panel B; Factor V. Panel C; Factor VII. Panel D; Factor X. Panel E; Fibrinogen. Panel F; Von Willebrand factor (VWF). Panel G; Protein C. Panel H; Antithrombin III. Panel I; Plasminogen. Panel J; Tissue-type plasminogen activator (tPA)*

** This figure was obtained with permission from Karangwa et al[39]. Production of physiologically relevant quantities of hemostatic proteins during ex situ normothermic machine perfusion of human livers. Liver Transpl. 2018;24(9):1298-1302.*

*Link to formal publication: https://doi.org/10.1002/lt.25290*

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of these proteins[41]. Given the absence of the majority of these influences during NMP of an isolated liver, the production of hemostatic proteins potentially proceeds uninterruptedly throughout NMP, resulting in a perfusate with protein levels exceeding normal physiological levels. Hence, adequate and perhaps, standardized anticoagulation protocols are crucial in preventing the occurrence of thromboembolic events during NMP, as we suggest in our previous study[39].

As previously discussed, the dosing and frequency of heparin administration widely varies amongst the several groups performing NMP. In view of the fact that donor livers are capable of producing substantial amounts of hemostatic proteins, and because the liver may clear heparin from the perfusate, anticoagulation protocols that involve continuous administration of heparin or administration at regular intervals as opposed to single bolus doses are likely to be more favorable and protective for livers undergoing *ex situ* perfusion for extended periods of time.

Heparin is currently the only anticoagulation agent being used during *ex situ* NMP of donor livers. Thus far, it has proven to be highly efficient in preventing thrombin formation as no thromboembolic events have been reported so far by any of the groups performing NMP. This is regardless of whether the perfusate used is plasma- or non-plasma based. Even though heparin is added to plasma-free perfusates with the aim of preventing coagulation activation during NMP, heparin will only function as an anticoagulant in the presence of antithrombin. At baseline, plasma-free perfusates are devoid of hemostatic proteins. Results from our study assessing hemostatic protein production during NMP with a plasma-free perfusate showed that in the first six hours of NMP, the concentrations of the procoagulant proteins are generally lower, relative to normal levels in plasma as compared to anticoagulant and fibrinolytic proteins. Together with the profound tPA release by the endothelium, our findings suggested that a favorable anticoagulant and pro-fibrinolytic environment is present in the initial phases of NMP. Moreover, during the six hours, considerable amounts of antithrombin were generated. Therefore, the administered of

heparin is likely to effectively maintain an anticoagulated environment during NMP. Nonetheless, as hemostatic protein production occurred almost immediately after reperfusion, the use of more direct anticoagulant agents such direct thrombin inhibitors, specifically those that do not require metabolic activation by the liver, may be effective in ensuring that thrombin formation in the initial phase of NMP is avoided. However, the use of these agents may be limited by the higher cost[42] as well as the shorter half-life compared to heparins[43,44]. This deems their use less suitable for extended perfusion durations. The use of direct thrombin inhibitors during NMP is a topic that requires further research.



**Hemostatic protein production during machine perfusion as a potential functional viability marker**

With the increasing utilization of sub-optimal donor livers, NMP is crucial as it allows for the pre-transplant assessment of the organ. Due to the factors that contribute to injury in higher risk livers, such as donor characteristics (e.g. older donors, steatosis, etc.), mechanism of death (circulatory vs. brain death), and procurement processes (agonal phase after withdrawal of life-support, prolonged extraction time of the donor organ, inadequate flush-out of the organ etc.), these higher-risk liver grafts are frequently predicted to be of suboptimal function upon reperfusion. Assessing such an organ's viability prior to transplantation is thus essential to minimize the risk of primary non-function, early allograft dysfunction or the development of (severe) vascular and biliary complications.

In current practice, functional assessment of donor livers during NMP typically uses the same biochemical parameters clinically employed (i.e., pH, lactate clearance, glucose and electrolyte concentration, and bile production). These parameters provide insight into injury and function of both hepatocellular and biliary components[24,26,45]. Nonetheless, universally validated viability criteria are yet to be established. Besides the widely used clinical biochemical markers, coagulation factors have also been used as markers to demonstrate

liver synthetic function during NMP. Studies have shown that the production of Factor V during NMP correlated to organ viability which indicates that coagulation factor production could potentially function as reliable viability marker[46-48].

Ideally, NMP viability markers should be measurable real-time. However, in the majority of the studies on the production of hemostatic proteins during NMP, enzyme-linked immunosorbent assays (ELISA's) were used to perform these analyses. Unfortunately, ELISAs are limited by the duration needed to perform the assay. Functional assays would be preferable, although the presence of heparin in the perfusate would affect some assays. Other groups have therefore opted for the measurement of INR as a measure of coagulation factor synthesis[35,49]. Similarly, these studies demonstrated a gradual decrease in INR back to normal levels in viable liver grafts during NMP which supported the notion that viable livers were capable of restoring their synthetic function. In comparison to ELISA assays for coagulation factors, INR is faster (results generated within minutes) and allows for (near) real-time assessment of liver function prior to transplantation. Moreover, in regard to logistics and costs, INR is significantly more favorable than ELISA kits. Nevertheless, the INR can only be reliably measured if the perfusate also contains sufficient amounts of fibrinogen, and we have demonstrated that fibrinogen levels remain relatively low at least in the first 6 hours of NMP. Therefore, development of more rapid and sensitive assays to measure the concentration of coagulation factors in the perfusate is necessary for real-time viability assessment of donor livers. Moreover, more studies with bigger sample sizes are required to determine whether factors such as donation after circulatory death vs. after brain death, donor age, cold and warm ischemia times, degree of steatosis etc. affect the synthetic function of donor livers. In so doing, the reliability and validity of using synthesis of coagulation factors as a viability marker can be further evaluated.

**Conclusion**

The burden of the shortage of suitable organs for transplantation has resulted in the increased use of higher risk donor livers. In order to minimize the morbidity associated with transplantation of these organs, alternative preservation methods such as machine perfusion with pre-transplantation organ assessment are being explored and this technology has gradually made an appearance into current clinical practice. To-date, anticoagulant prophylaxis during machine perfusion is carried out with the use of heparin. Heparin has proven to be an effective anticoagulant, as no thrombo-embolic events during liver machine perfusion have been described so far. Nevertheless, during NMP, donor livers have been shown to produce substantial amounts of hemostatic proteins within a relatively short amount of time. This prompts the call for awareness to the need for adequate, continuous or regularly-administered anticoagulant agents during (extended) NMP and perhaps the development of a standardized protocol for anticoagulant administration, especially for extended perfusion. Furthermore, coagulation factors have the potential to be suitable markers for viability (as markers for hepatocellular synthetic function). However, a need for rapid, real-time assays to measure these proteins during NMP is required in order for this to be clinically implementable.

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PART II

# Reperfusion Injury