Chapter 7

Organ Perfusion During Organ Transplantation: Consequences and Importance of the Washing Out Procedure.

Hyperaggregating Effect of HES Components and University of Wisconsin Solution on Human Red Blood Cells: A Risk of Impaired Graft Perfusion in Organ Procurement?

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Consequences and Importance of Washing Out Procedure in Transplantation

Abstract

Study Objectives

To date, the standard preservation solution used during organ procurement and preservation of most organs is the University of Wisconsin (UW) solution. Despite its superiority over other cold storage solutions, the inclusion of hydroxyethyl starch (HES) as one of the components of the UW solution has been both advocated and denied. The aim of this study was to determine the effect of HES on red blood cell aggregability and to correlate aggregation parameters with HES molecular weight.

Methods

Human RBC aggregability and deformability were investigated in vitro, at 4°C, with a Laser-assisted optical rotation cell analyzer. The study of red blood cell aggregation in a binary HES-HES system gave an indication about the nature of HES-RBCs interactions. Bright field microscopy and atomic force microscopy were used to morphologically characterize the aggregates size and form.

Results

High molecular weight HES and UW solution had a potent hyper-aggregating effect; low molecular weight HES had a hypo-aggregating effect on RBC. RBC aggregates were of large size and their resistance to dissociation by flow induced shear stress was high.

Conclusions

Our in vitro experiments conclusively showed that the physiological function of red blood cells to form aggregates is significantly affected in the presence of hydroxyethyl starch. The use of high molecular weight HES in UW solution accounts for extended and accelerated aggregation of erythrocytes that may result in stasis of blood and incomplete wash-out of donor organs prior to transplantation.
7.1 Introduction

Reperfusion injury after cold ischemic storage prior to organ transplantation plays a critical role in the occurrence of primary nonfunction and delayed graft function which have remained major problems in liver transplantation. The viability of organ grafts depends on several factors such as cold ischemia time, the perfusion procedure, preservation methods and reperfusion quality. The efficacy of perfusion during the initial wash-out procedure, however, has to be considered a major determinant of functional recovery after transplantation.

Preservation solutions have been designed to ameliorate the adverse physiological and biochemical effects of ischemia under hypothermic conditions. Three principles are important in effective cold storage. First, the vascular wash-out during harvest should rapidly cool the organs, remove the blood and allow balance between the cold storage solution and the tissue. Second, the cold storage solution should prevent cell swelling and interstitial edema formation by including substances that are osmotically active and impermeable to the cell. Impermeants and saccharides achieve homeostasis of the intracellular water content. Homeostasis of the interstitial compartments is achieved by counteracting a hydrostatic force during the initial wash-out using colloids. The intravascular fluid compartment does not need an effective component in static cold-storage. Third, the cold storage solutions should prevent excessive cellular acidosis by containing sufficient concentration of hydrogen–ion buffer, histidine or citrate.

Since its introduction by Belzer et al. in the late eighties, the University of Wisconsin (UW) solution has become the standard solution for the preservation of most organs in transplantation. Despite the fact that UW solution made extended cold preservation feasible, some studies have demonstrated that prolonged cold ischemic time of hepatic allografts enhance bacterial infection, cause biliary and hepatic artery complications and increase the frequency of primary non function posttransplant.

The inclusion and importance of the colloid hydroxyethyl starch (HES) as one of the components of the UW solution has been both advocated and denied. HES prevents interstitial edema and has a beneficial effect on matrix metallo–proteinases but at the price of a higher solution viscosity. Due to the presence of HES, the viscosity of UW solution at 4°C increased by a factor of 2.5 when compared with the viscosity of the same solution at 37°C. Analyzing the effect of HES on the rheological properties of blood, Corry and collaborators have drawn the attention to the aggregating effect of HES on RBC.

The pathogenic potential of RBC aggregates prevails within the microcirculation, leading to altered flow dynamics and microvessel occlusive events. Furthermore, cell–cell interaction between platelets and erythrocytes can significantly enhance platelet reactivity with a prothrombotic effect. There is also strong evidence that RBC aggregation greatly enhances the tendencies of leukocytes to adhere to the postcapillary endothelium, a process recognized as essential in inflammation. Considering these aspects, HES induced–RBC aggregability could significantly influ-
ence the quality of organ preservation, increase damage due to ischemia/reperfusion and affect the outcome after transplantation.

This study concerns an extension to a previous observation made by our group\textsuperscript{18} that signalled a poor initial wash-out of rat liver when using UW solution. We concluded at that time that this effect is most likely the consequence of aggregate-formation induced by HES in combination with rat blood. The present study will reveal a detailed evaluation of the extent and kinetics of HES-induced human RBC aggregation, as well as a morphological characterization of these aggregates. In addition, to explain the mechanisms involved, red blood cell aggregation has been studied in a binary HES–HES mixture.

7.2 Methods

*RBC aggregability and deformability* were investigated in vitro with a Laser-assisted optical rotation cell analyzer (LORCA R&R Mechatronics, Hoorn, The Netherlands)\textsuperscript{19,20}. This instrument, based on the ektacytometric principle, is equipped with a video camera for detection of the laser diffraction pattern, a thermostation unit and an ellipse-fit computer program calculating the Elongation Index and Aggregation Index (AI). The experiments were performed at 4\degree C, after in vitro admixture of UW/HES solutions with human fresh blood from healthy volunteers (n=8), drawn from the antecubital vein into 0.1 mM ethylenediamine tetracetic acid.

Three commercially available HES solutions (6% in 9 g/l sodium chloride) were selected based on their molecular weight and substitution ratio: HES 450/0.7 (M\textsubscript{w}=450 kDa, MS=0.7), HES 200/0.5 (M\textsubscript{w}=200 kDa, MS=0.5), HES 130/0.5 (M\textsubscript{w}=130 kDa, MS=0.5). Phosphate Buffered Saline (PBS, pH 7.4, 300 mOsm/kg) was used as buffer fluid for the HES solutions. The final concentration of the HES solutions was 5%, pH=7.4, isotone. The University of Wisconsin solution was used as commercially available (5% HES with a molecular weight cut off range of 100–1000 kDa and a mean of 250 kDa). As a negative control, we tested the effect of a HES–free UW solution (prepared according to the UW–recipe without the addition of HES).

The samples were prepared not more than one hour before the measurements took place, the mixing ratios were: blood:HES = 5:1, 7:1, 10:1; blood:UW / HES–free UW = 5:1, 2:1. The hematocrit (Hct) was adjusted in all the samples to a constant value of 38%. A control (red blood cells suspended in autologous plasma, 38% Hct) was considered for every set of samples.

*Aggregation of human red blood cells in binary HES–HES mixtures*, a competitive assay: human erythrocytes were treated (as previous) with HES 450 kDa and HES 130 kDa solutions, using a mixing ratio of 5:1. After measuring the effect on the aggregation, HES 130 kDa was added on the HES 450 kDa–treated samples and vice-versa, to a final mixing ratio of 5:2. The experiment was performed at room temperature
For the evaluation of RBC aggregation we recorded several comparative parameters.

**LORCA Measurements**

For the determination of red cell aggregation, the blood is brought under a shear rate of $500 \text{s}^{-1}$, after which the shear is stopped at $t=0$. The backscattered intensity from the blood layer is measured during 120 s after shear stop. The intensity drops because of red blood cell aggregation. We considered the beginning point of the aggregation the extrapolated value of the decay curve towards $t = 0$. The kinetics of the aggregation was studied using two parameters: *aggregation index* and the *half time* ($T_{1/2} = \text{the time necessary to reach 50\% aggregation}$). *The minimal value of shear rate that prevents aggregation* gave an indication of the strength of the intercellular interaction by determining the aggregates’ resistance to dissociation by flow–induced shear stress. *The deformability* of the red cells has been determined by repeatedly measuring the diffraction pattern of red cells under various shear stresses in the range of 0.01–100 Pa, from which an Elongation Index has been calculated by LORCA software. The blood was diluted 200 times in PolyVinylPyrolidone (5 g/l) in PBS (50 mM).

**Viscosity Measurements**

For the measurements of the viscosity of blood and HES–treated blood we used an automated dynamic shear rheometer with cone–plate geometry (AR1000 Rheometer, TA Instruments). During measurements the temperature was set at $4^\circ \text{C}$ and the shear rate of operation equaled the value of the corresponding shear rate which prevented aggregation for that specific sample.

**Imaging Techniques**

Light Microscopy and Atomic Force Microscopy were used to morphologically characterize the aggregates’ size and form. Bright field microscopy provided a direct, large scale, two–dimensional visualization of the samples. Images were digitized and statistics of the aggregates’ area size were generated using the Image Pro–Plus software (version 3.0.1 Media Cybernetics). Tapping–Mode Atomic Force Microscopy provided three–dimensional imaging of unstained and uncoated RBC aggregates in air. Sample preparation consisted of a standard smear of 300 times diluted filtered blood on a glass surface. In this way, sample preparations and imaging environments known to generate artifacts are eliminated (e.g., dehydration, fixation, freezing, staining and coating).
Statistical Analysis

Differences between physiological and experimental aggregation parameters in different samples groups were evaluated using the paired two tailed Student T Test. A p value of < 0.05 was considered statistically significant. The results are expressed as mean±SD.

7.3 Results

LORCA Measurements

The molecular weight of HES had a highly significant influence on the kinetics of RBC aggregation (Fig. 7.1a). For a blood:HES ratio of 5:1 the aggregation index in the presence of HES 450 kDa was 39.76±5.99, an increase of more than 100% as compared to the control aggregation index, 18.16±3.43, (p<0.01). In contrast, the low molecular weight HES significantly reduced the RBC aggregability (p=0.019); the AI measured in the HES 130 kDa treated samples was 13.64±1.96. In addition, we determined the concentration–dependent effects of HES on RBC aggregation. Decreasing HES 450 kDa and HES 200 kDa concentrations resulted in a concomitant decrease of aggregation index, although 10% HES 450 kDa still induced a significant increased aggregation (p<0.01).

Figure 7.1: (a) Aggregation Index (AI) of human blood treated with different HES in various ratios (mean±SD). (b) Aggregation Index (AI) of human blood treated with UW solution/HES–free UW solution using different mixing ratios (mean±SD). Highly significant (** p<0.01) and significant (*) p<0.05) differences with whole blood are indicated.
Table 7.1: The shear stress required to dissociate RBC aggregates. Highly significant \((p<0.01)\) differences as compared with whole blood samples (controls) are indicated with **.

<table>
<thead>
<tr>
<th></th>
<th>Shear rate ((s^{-1})) mean±SD</th>
<th>Viscosity ((\text{mPa} \cdot \text{sec})) mean±SD</th>
<th>Shear stress ((\text{Pa})) mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>UW solution–treated samples</td>
<td>175±29</td>
<td>14.9±0.3</td>
<td>2.5±0.2 **</td>
</tr>
<tr>
<td>HES 450 kDa treated samples</td>
<td>240±70</td>
<td>12.4±0.2</td>
<td>3.4±0.2 **</td>
</tr>
<tr>
<td>HES 200 kDa treated samples</td>
<td>140±12</td>
<td>15.1±0.5</td>
<td>2.0±0.1 **</td>
</tr>
<tr>
<td>HES 130 kDa treated samples</td>
<td>86±15</td>
<td>18.2±0.5</td>
<td>1.5±0.05 **</td>
</tr>
<tr>
<td>Controls</td>
<td>78±3</td>
<td>22.7±0.5</td>
<td>1.6±0.1</td>
</tr>
</tbody>
</table>

The AI measured in the UW treated blood was 28.94±3.89 for the ratio 5:1 and 35.55±3.84 for the ratio 2:1; the control sample had an AI of 20.02±5.52. When treating the blood with colloid free–UW solution in a ratio of blood:HES–free UW solution = 2:1, the aggregation index decreased to 0.20±0.42 (Fig. 7.1b).

The kinetics of the aggregation process is also expressed by the half–time \((T_{1/2})\) value. Since \(T_{1/2}\) is the time necessary to reach 50% of complete aggregation level, a lower \(T_{1/2}\) reflects a faster aggregation process. The RBC aggregates formed three times faster when the cells came in contact with HES 450 kDa \((T_{1/2}=6.67±0.84\text{ s})\), as compared to the control \((T_{1/2}=20.43±4.59\text{ s})\) \((p<0.01)\). HES 130 kDa inhibited the aggregation process, the half time necessary for RBC treated with HES 130 kDa to reach complete aggregation \((T_{1/2}=29.17±6.68\text{ s})\) was significantly higher \((p=0.024)\) when compared to control.

Resistance to dissociation by flow induced shear stress expresses the strength of the aggregates. The shear stress required to dissociate the aggregates is calculated by multiplying the minimum shear rate that prevents aggregation with the viscosity of the blood at that shear rate:

\[
\text{Shear Stress (mPa)} = \text{Shear Rate (s}^{-1}) \times \text{Viscosity (mPa.s)}
\]

The measured shear rates that prevented aggregation, the viscosity values measured for each sample at the corresponding shear rate, and the calculated shear stresses are presented in Table 7.1. It was notable that the viscosity values of the control were higher than the viscosity measured for the HES 130 kDa treated samples, the conditions of the measurement being the same (shear rate = 80\text{ s}^{-1}, \text{ temperature } 4^\circ\text{C}).

Erythrocyte deformability measured by means of Elongation Index parameter with LORCA, showed no significant differences between HES treated samples and control samples.

Aggregation of human red blood cells in binary HES–HES mixtures, a competitive assay: for red blood cells pretreated with HES 450kDa, aggregation index decreased
when adding small starch (from 65.8±4.7 to 55.4±2.6). For red blood cells pre-treated with HES 130 kDa, large HES increased the aggregation index from 37.6±4.1 to 57.5±5.2. Concomitant adding of HES 450 kDa and HES 130 kDa to the red blood cell suspension yielded values similar to those obtained by consecutive treatment with HES 450 kDa and HES 130 kDa (Fig. 7.2).

**Imaging Techniques**

The large-scale light microscopic images showed clear differences between the extent of aggregation in the HES–treated samples and the control samples. The statistics on these images, given by Image Pro–Plus software are shown in Table 7.2. The UW solution treatment of the RBC determined formation of branched rouleaux networks with a range of 23–56 cells per aggregate (Fig. 7.3a). HES 450 kDa induced formation of large size RBC aggregates with an irregular geometry: polymorph ery-
thromocyte clusters were clearly visualized (Fig. 7.3b). The morphology of the HES 130 kDa induced RBC aggregates consisted of various size linear rouleaux (Fig. 7.3c). The image of the erythrocytes treated with HES–free UW solution confirmed the absence of RBC aggregation; at a magnification of 200× only 8 aggregates were counted, with a range of 2–3 cells per aggregate. A control was considered as well (Fig. 7.3d). Tapping–Mode atomic force microscopy technique revealed a three dimensional surface profile of RBC aggregates with micrometer resolution. This visualization approach provided clear evidence of aggregation between intact red blood cells when treated with high molecular weight hydroxyethyl starch/UW solution (Fig. 7.4a,b, respectively).

Table 7.2: Statistics given by Image Pro–Plus Software after processing bright field microscopy images taken at a magnification of 200×.

<table>
<thead>
<tr>
<th></th>
<th>HES 450 kDa</th>
<th>HES 130 kDa</th>
<th>UW solution</th>
<th>HES – free UW</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count</td>
<td>1881</td>
<td>1289</td>
<td>1545</td>
<td>349</td>
<td>164</td>
</tr>
<tr>
<td>Total aggregate count</td>
<td>154</td>
<td>216</td>
<td>64</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Single cells (%)</td>
<td>4.9</td>
<td>35</td>
<td>2.9</td>
<td>95.1</td>
<td>94.5</td>
</tr>
<tr>
<td>Cells in aggregate (%)</td>
<td>95.1</td>
<td>65</td>
<td>97.1</td>
<td>4.9</td>
<td>5.5</td>
</tr>
<tr>
<td>Cells / aggregate (range)</td>
<td>10–28</td>
<td>4–7</td>
<td>23–56</td>
<td>2–3</td>
<td>2–3</td>
</tr>
<tr>
<td>Area Max. (µm²)</td>
<td>6740</td>
<td>1398</td>
<td>4332</td>
<td>72</td>
<td>137</td>
</tr>
</tbody>
</table>

7.4 Discussion

In the present study, we conducted a comparative analysis of various parameters expressing the aggregation status of RBC in samples treated with University of Wisconsin solution and different molecular weight HES solutions. Our findings indicate that high molecular weight hydroxyethyl starch solutions (HES 450 kDa and HES 200 kDa) as well as UW solution have a potent hyperaggregating effect on human RBC. RBC aggregates formed in the presence of this colloid are of large size; the maximum size aggregate area was 6740 µm² in the HES 450 kDa treated samples and 4332 µm² in the UW–treated samples. In addition, their resistance to dissociation by flow induced shear stress is increased by 50–100% compared to control samples. These data suggest that gravity–induced hydrostatic perfusion pressures presently used in procurement can not easily dissociate the abnormal RBC aggregates. Some authors have advocated a more physiologic method in which the UW solution is flushed under pressure (100 mmHg) similar to the mean arterial blood pressure with the advantage of perfusing the small intrahepatic vessels. Measurement of the microvascular blood flow patterns in physiologic conditions using intravital microscopy shows that in ar-
Figure 7.3: Bright field microscopy, magnification 500×. Bar scale represents 20 µm. (a) UW–induced branched RBC rouleaux networks, (b) HES 450 kDa induced RBC polymorph clusters, (c) HES 130 kDa induced linear RBC rouleaux, (d) Control–RBC suspended in autologus plasma.
	erioles and venules, with a diameter of 24.7±9.1 µm, the recorded shear rate has a mean value of 201±163 s⁻¹. The minimal value of the shear rate that prevented UW–induced aggregation in our experiments was 175±29 s⁻¹. These data indicate that even with a high–pressure perfusion, the low shear rates generated in certain areas and the small vessel diameter compared to the aggregates size make this vessel category prone to mechanical obstruction. In addition, increasing the perfusion pressures could represent and additional stress factor for sinusoidal endothelial cells.
These cells are already particularly vulnerable to cold ischemia/reperfusion injury and thus are believed to be the primary target of this injury.\textsuperscript{22} The presence of remaining host erythrocyte aggregates after the initial wash–out of the donor organ could contribute to an inadequate microvascular perfusion with preservation solution and therefore to a poor maintenance of graft viability during ischemic storage. The areas of the respective organs that are only marginally equilibrated with University of Wisconsin solution are less protected during the subsequent ischemic storage period, thus contributing to an overall reduced structural and functional integrity of the organ.\textsuperscript{23} Preservation injuries are considered to be major contributors to primary allograft failure after liver transplantation. Inadequate preservation with UW solution for 16 hours becomes histologically evident 24 hours after reperfusion: submassive confluent necrosis of hepatocytes associated with loss of intercellular borders mainly in the midzonal region, with selective sparing of periportal and centrilobular zones.\textsuperscript{22} In this respect, Busquets et al. reported the presence of preservation injuries in 17\% of the liver grafts preserved in UW solution and associated the presence of these lesions with an increase of posttransplant biliary complications.\textsuperscript{24} In addition, mobilization of resting host red blood cells during reperfusion time, the presence of lysed erythrocytes and endothelial cells due to cold ischemia and inadequate microvascular perfusion with preservation solution may lead to a local hypercoagulable state. Local activation of the coagulation system on graft reperfusion may
cause intravascular and/or intracardiac thrombus formation and pulmonary thromboembolism. Suriani et al. suggested that subclinical thromboembolism on graft reperfusion is common. He reported echodense masses in the right atrium within one min after reperfusion in 70% of the patients undergoing liver transplantation. Thus, it could be possible that by identifying the RBC hyperaggregating effect of UW solution as an etiology–related factor for these complications immediate function, patient and graft survival would improve.

In our study, low molecular weight HES treatment of blood yielded a decline of blood viscosity values. Furthermore it significantly decreased the red blood cell aggregability and slowed the process in time. The aggregate’s resistance to dissociation by flow induced shear stress was significantly lower in the HES 130 kDa treated samples when compared to control. The visualization revealed various sized linear rouleaux morphology with a range of 4–7 red blood cells per aggregate.

Questions might arise regarding the efficacy of HES 130 kDa in maintaining the colloid osmotic pressure during the wash–out procedure and preservation period. Hydroxyethyl starches have been used for many years in order to prevent and treat hypovolemia during major surgery: they decrease the transvascular fluid flux and edema formation via maintenance of the colloid osmotic pressure and preservation of the microvascular barrier. In that respect, HES 130 kDa is proved to be an efficacious plasma volume expander in heart surgery. In addition, Zikria et al. demonstrated that 100 to 300 kDa fraction of HES significantly minimized tissue edema in an ischemia–reperfusion model of increased vascular permeability, independent of the colloid osmotic pressure effect. They hypothesized that this finding was related to a biophysical effect of starch effectively sealing the separated endothelial junctions.

Under normal conditions erythrocytes deformability allows individual red blood cell with a mean resting diameter of 7m to traverse capillaries with diameters no more than 3–5 μm. Rigid cells in the postoperative blood flow could present a block in the microcirculatory passageway. Any decrease in the deformability would result in impaired perfusion of organs and peripheral tissues. Therefore the present study was designed to investigate the influence of HES on RBC deformability as well. We found no significant effect of HES on RBC deformability (p>0.05).

**Theoretical models of erythrocyte aggregation**

Membrane adhesion processes, including erythrocyte aggregation, can be classified into two categories: specific binding and nonspecific binding. Specific binding occurs via interaction of macromolecules with their specific receptors on the erythrocyte membrane. For nonspecific binding mechanism, two major theoretical models have been proposed. The first theory is based on the surface adsorption of macromolecules to form bridging configuration between adjacent erythrocytes. The adsorption is believed to be favored by Van der Waals forces, hydrogen bounds or electrostatic attractions. According to this theory, polymers and plasma protein with
a large molecular mass insert between adjacent erythrocytes, increase the intercellular
distance and induce erythrocyte aggregation by decreasing the electrostatic repulsive
forces of erythrocytes. The second theory suggests that the aggregation is induced
by macromolecular depletion from the membrane surface. In this theory the aggre-
gation is independent of both the molecular mass and the surface adsorption. The
attraction of colloid particles producing the aggregation is induced by variations in
the surface energy and differences in osmotic pressure due to a profile of polymer con-
centration existing in the suspending medium between the neighboring surfaces.

**Hypotheses on the mechanism of hydroxyethyl starch induced RBC aggregation**

Our study documented that the extent of HES induced RBC aggregation varied with
the molecular weight. Colloids with high molecular weights such as HES 450 kDa
and HES 200 kDa induced a significantly higher aggregation when compared to the
physiological aggregation. Concentration of the colloid was shown to be pivotal in
the aggregation process. The observed strong correlation of erythrocyte aggregation
with the molecular weight and concentration of HES can be explained by the theory of
macromolecular bridging. In contrast, the colloid with a small molecular weight, HES
130 kDa, had an inhibiting effect on the extent and kinetics of the aggregation. These
findings are consistent with the assumption that inhibition of aggregation occurs
because of increase of small molecules in the depletion region.

The study of red blood cell aggregation in a binary HES–HES system showed that
both hyper-aggregability induced by HES 450 kDa and hypo-aggregability induced by
HES 130 kDa are reversible phenomena, demonstrating in this way the nonspecific
nature of HES adsorption on the surface of the cell.

**In summary,** our experiments conclusively showed that the physiological function
of red blood cells to form aggregates is significantly affected in the presence of hy-
droxyethyl starch. The aggregation of erythrocytes was extended and accelerated
with increasing the molecular weight of HES and its concentration. As a new and
unexpected finding, a significantly lower aggregation was observed in HES 130 kDa–
treated erythrocytes compared to the aggregation in controls. In addition, the use of
a colloid–free UW solution resulted in a complete abolition of RBC aggregability.

The causes of hepatic dysfunction or allograft failure after liver transplantation are
multifactorial and identifying risk factors predictive of both patient and graft survival
is crucial to improve outcome after transplantation. To date, several risk factors have
been shown to negatively affect the graft survival, such as donor/recipient age, size
of body/weight index, prolonged donor stay in the intensive care unit and long cold
ischemic time, perfusion during initial wash–out and preservation methods. Most
of these factors are static, but some of them are subject to manipulation, for example
the use of high molecular weight HES in the formulation of UW solution. We sug-
gest, on the basis of our experimental data, that the use of low molecular weight HES (HES 130 kDa) will improve the quality of the University of Wisconsin solution, have a beneficial effect on organ preservation and possibly reduce the chance of postreperfusion primary nonfunction and posttransplant biliary lesions with delayed recovery in organ transplantation.

**Acknowledgements:** We gratefully acknowledge M.D. Morariu, Ph.D., for technical contribution and for sharing expertise in blood rheology and imaging techniques.

**References**


Recent improvements in the clinical care of patients have their roots in two distinct fields of modern medicine: biomedical research and clinical ethics. In order to improve the ability of clinical medicine to apply successfully and ethically the new developments in medical science, research must be undertaken to understand the full effects of medical treatments and the proper threshold for medical intervention. Moreover, it is of critical importance how physicians understand the risks and benefits of treatment and how to guide the decision making for individual patients. The ethical aspects of treatment decisions are of equal importance, with emphasis on patients expectations when they consent to manipulation involving risk factors and on their participation in a treatment decision. Only by investing time and effort in both medical education and research, the ethical ideals that underlie the physician–patient relationship can be fulfilled.

The present thesis describes a generally recognized pathophysiologic mechanism: impairment of organ perfusion with its diagnostic and therapeutic challenges. Out of the multitude of possible etiologic factors for organ perfusion impairment, we chose to investigate two extreme situations of acute organ support: (1) organ perfusion during cardiopulmonary bypass with cardiac arrest and (2) organ perfusion during
organ donation and procurement prior to transplantation. Even if these two clinical fields seemed to be segregated at a first approach, our results conclusively showed a parallelism in etiology, pathologic mechanisms, and therapeutic approaches.

Using this original approach, we investigated several issues of concern for both cardiac surgery and organ transplantation: use of artificial colloids, use of prophylactic corticosteroids, diagnostic value of organ injury markers, consequences of hemodilution, hypothermia induced injury, and vascular endothelial activation.

Effects of hydroxyethyl starches (HES) on red blood cell aggregation

The use of HES solutions as priming and plasma substitution fluids in patient undergoing cardiopulmonary bypass results in altered red blood cell aggregation. In parallel with the decrease in red blood cell aggregation, blood viscosity declines also. The subsequent variations in blood rheology activate the vascular endothelium with pro-inflammatory and pro-thrombotic effects. A distinct effect of different molecular weight starches was evident post-bypass. While the markers of endothelial activation went down to baseline levels in HES 200/0.5 group, HES 130/0.4 was associated on the first postoperative day with sustained endothelial activation.

In organ transplantation, the use of high molecular hydroxyethyl starches (HES 450/0.7 and higher) as components of the University of Wisconsin preservation solution accounts for accelerated and augmented red blood cell aggregation. The aggregates are of large size and their resistance to dissociation by flow induced shear stress is high. These data suggest that gravity-induced hydrostatic perfusion pressures presently used in procurement can not easily dissociate the abnormal red blood cell aggregates. In addition, the small vessel diameter in marginal areas compared to the aggregates size make these vessel category prone to mechanical obstruction during organ procurement. The presence of remaining host erythrocyte aggregates, trapped in the microvasculature after the initial wash-out of the donor organ could contribute to an inadequate microvascular perfusion with preservation solution and therefore to a poor maintenance of graft viability during ischemic storage. The areas of the respective organs that are only marginally equilibrated with University of Wisconsin solution are expected to be less protected during the subsequent ischemic storage period, thus contributing to an overall reduced structural and functional integrity of the organ. Low molecular weight HES treatment of blood yields a decline of blood viscosity values, decreased the red blood cell aggregability and slows the process in time.

Our results documenting the effect of hydroxyethyl starches on red blood cell aggregation suggest the necessity of a more careful selection of HES solutions when
considering a therapeutic strategy. In cardiac surgery, hypertensive and atherosclerotic patients who have already a high basal levels of circulating von Willebrand factor might benefit from HES 200/0.5. HES 130/0.4 could represent a first choice for patients with bleeding tendencies and patients with acquired von Willebrand syndrome after aortic stenosis. In organ preservation prior to transplantation, the exclusive use of low molecular weight HES will improve the quality of the University of Wisconsin solution by preventing intravascular red blood cell aggregation. By preventing mechanical obstruction during wash-out, use of HES 130/0.4 might have a beneficial effect on organ preservation and possibly reduce the chance of postreperfusion primary nonfunction and posttransplant biliary lesions with delayed recovery in organ transplantation.

**Prophylactic corticosteroid treatment**

The assumption that prophylactic corticosteroid therapy, by its virtue to inhibit the inflammatory response, would also transfer a protective effect of organ injury associated with cardiopulmonary bypass, was rejected by the results presented in this thesis. Dexamethasone treatment offered no protection against transient, perioperative renal, intestinal and hepatic injury in patients undergoing on-pump coronary artery bypass grafting. In fact, dexamethasone treatment seemed to be detrimental, resulting in a pronounced postoperative pulmonary dysfunction, prolonged time to tracheal extubation and by initiating postoperative hyperglycaemia. An important observation was the strong positive correlation found between high blood glucose level, as side effect of dexamethasone, and end organ injury. The necessity of a stricter management of serum glucose emerged, suggesting insulin therapy at serum glucose lower than 10 mmol.L$^{-1}$ (as standard) in order to avoid kidney and intestinal injury. This message is also important for the clinicians responsible for the management of the brain dead organ donors, confronted as well with the use of corticosteroid therapy and glucose management. An early institution of insulin therapy might decrease brain death–related donor organ damage.

**Diagnostic value of organ injury markers**

With the goal of diagnosing impairment in organ perfusion and subsequent organ injury, the present work investigated in a multitude of clinical settings the use of both standard and newly available organ injury biomarkers. Most of the standard laboratory investigations proved to require long assay times, to lack sufficient specificity and/or sensitivity. In contrary, using newly available, sensitive and specific organ injury biomarkers we were able to document transient, subclinical cardiac, renal, intestinal and hepatic tissue injury even in low risk patients undergoing cardiopulmonary bypass. Similar, these new markers proved to be useful when investigating
early brain death–related donor organ damage.

Fatty acid binding proteins (FABP) are cytosolic proteins with various tissue specific isotypes, released in circulation and subsequently in urine in case of cellular damage. We investigated the use of heart (H), intestinal (I), and liver (L) type fatty acid binding proteins. In cardiac patients undergoing cardiopulmonary bypass, plasma H–FABP correlated with other cardiac injury markers (cardiac Troponin I and creatine kinase MB). The advantage of including H–FABP in the diagnosis of myocardial injury is the early peak arising already one and a half hour after reperfusion, which was significantly earlier than the peaks of cardiac Troponin I (fourteen hours) and creatine kinase MB (sixteen hours). Urinary concentration of H–FABP proved to be a better indication of kidney damage than of myocardial damage, explained possibly by a primary release of H–FABP in urine from the damaged distal renal tubules. In our study on the patients undergoing cardiac surgery, the urinary peak of H–FABP did not correlate with the others cardiac markers but correlated strongly and significantly with the urinary peak of N-acetyl-glucosaminidase (NAG, proximal tubules injury) and peak microalbuminuria (glomerular injury). Similar in brain dead rats donors, H–FABP and NAG urine concentration reached significantly higher values as early as half hour and one hour, respectively, after brain death induction, as compared with sham operated animals. A highly positive correlation was documented between the two renal tubules markers, consolidating the diagnose of renal tubular damage during brain death.

I/L–FABP are cytosolic proteins readily released into the circulation following enteroocytes damage, with a 40-fold higher content of L–FABP, reported as useful urine markers for the detection of intestinal injury. Both urinary I–FABP and L–FABP increased significantly during CPB, reaching peak values in the urine collected during the first two hours and six hours postoperative, respectively. Urine I–FABP correlated significantly with urine L–FABP. The increased values of I–FABP and L–FABP during CPB reported in this study verify the indirect evidence of mucosal integrity loss during CPB reported previously as perioperative reduction in intramucosal pH, increase in gut permeability and endogenous endotoxemia. Using the test of I–FABP concentration in the urine of brain dead rats, we were able to detect intestinal injury as early as two hours after brain death (data not shown).

Additional to fatty acid binding proteins, we would like to emphasize the utility of N–acetyl–glucosaminidase in diagnosing proximal tubules injury, and of α-Glutathione S–transferase in diagnosing hepatic injury.

Consequences of hemodilution

Using a complex operative strategy in patients undergoing on-pump coronary artery bypass grafting, we showed an important attenuation of the transient renal and intesti-
nal postoperative injury achieved by means of limiting intra–operative hemodilution and blood transfusion requirements. Variation in hematocrit explained more than a third of the variation of both postoperative NAG and I–FABP. A decrease with one unit (1%) in hematocrit predicted significantly an increase with a quarter of the peak postoperative NAG values. The same decrease with one unit (1%) in hematocrit predicted significantly an increase with a tenth of the peak postoperative I–FABP values in patients undergoing on–pump cardiac surgery. Hemodilution, besides lowering the oxygen carrying capacity of blood, alters as well blood rheology with possible pathological consequences.

With the aim set on investigating variation in blood rheology during isovolemic hemodilution and subsequent effects on vascular endothelial activation, we designed an animal study to answer previously formulated hypothesis in clinical studies. To bring relevance, the study addressed two different hydroxyethyl starch (HES) solutions commonly used in the clinical practice as priming solutions for the heart–lung machine and as plasma expanders. An important observation was that hemodilution up to 50% resulted in negligible hypoxia/reperfusion injury, as quantified by the reactive oxygen species production measured in the vital organs. Low red blood cell aggregation, as documented in this model of acute isovolemic hemodilution, was associated with activation of vascular endothelium, especially in lungs and small intestine. Translation of these data in clinical terms suggests that acute hemodilution may lead to inflammatory stress of pulmonary capillaries. Subsequent diffusion limitation may be expected. Similar, an increased inflammatory response in the small intestine associated with acute hemodilution, might contribute to a loss in barrier function of the intestinal mucosa with subsequent translocation of endotoxins and/or bacteria. Additionally, the data presented in this study suggest a new pathway for the erythrocyte involvement in clot formation: due to their function to aggregate, erythrocytes can modulate endothelial activation with von Willebrand factor release, with a subsequent pro–thrombotic effect. The investigations on acute isovolemic hemodilution might be clinically relevant for the patients undergoing on–pump cardiopulmonary bypass, the patients in traumatic–hemorrhagic shock with sustained fluid resuscitation, or the brain dead organ donors with large volume of fluid infusion to correct for hypotension. Based on the results demonstrating increased endothelial activation, we hypothesize that lower incidence of thrombotic events and decreased inflammatory reactions could be achieved by avoiding excessive hemodilution.

_Hypothermia–related injury_

Contrary to conventional thinking about the benefits of corporeal hypothermia on systemic protection against global ischemic injury during extracorporeal circulation, an increasing number of clinical studies support corporeal normothermia. The re-
sults of our clinical investigation comparing normothermia with hypothermia showed a negative correlation between body temperature during cardiopulmonary bypass and postoperative I–FABP urine concentrations. In other words, lower body temperatures during CPB were associated with higher intestinal damage. These findings confirm at a different level, the studies performed in organ transplantation that document a higher organ damage when cold ischemic preservation time is extended. In the clinical setting of organ transplantation, cooling down the organ followed by rewarming is a generally recognized trigger of injury.

Vascular endothelial activation

Pro–inflammatory and pro–coagulatory vascular endothelial activation was demonstrated to be a central pathological finding in both cardiac surgery and brain death organ donation. In cardiac surgery with cardiopulmonary bypass, endothelial activation was demonstrated to arise in the first hours after myocardial reperfusion, as documented by elevated plasma concentrations of von Willebrand factor, thrombomodulin, tissue Plasminogen Activator and E–Selectin. At gene regulation level, endothelial activation is shown in our pig experimental study to arise already three hours after induction of isovolemic hemodilution. In brain dead donor rats, endothelial activation was documented as early as half hour after brain death induction.

The etiology of endothelial activation is multifactorial: systemic inflammatory response, surgical stress, and systemic mobilization of wound–release factors. As original contribution, the present thesis introduces a new etiologic factor: decreased red blood cell aggregation as a trigger of impaired blood rheology and thus mechanical endothelial activation. We hypothesize that the drop in RBC aggregation added to plasma viscosity reduction during hemodilution alone, or even more during extracorporal circulation, are important factors contributing to variation in shear stress at the vascular endothelial wall. The variation in shear is known to lead to a complex signaling response eventuating in pro-inflammatory and pro–coagulatory vascular endothelial activation.

In conclusion, the work described here aims to add a new foundation stone on the scientific basis for diagnosing and treatment, by contributing to current clinical debates and suggesting new directions for clinical and fundamental research. Additionally, the results included in this thesis emphasize the need of collaborative decision making between physicians with different expertise, and between physicians and researchers.
Perspectives

Development and validation of new extracorporeal assist devices are highly desirable when performing artificial organ support. Pulsatile perfusion remains a challenging therapeutic choice. Either used as bridge to transplantation, bridge to recovery, or during coronary artery bypass grafting with cardiac arrest, an effective pulsatile perfusion might improve clinical outcomes.

In the same line of research, hypothermic machine perfusion providing a pulsatile blood flow is known to offer better protection against cold ischemic injury when compared with cold storage in marginal donor organs. Special effort has to be invested in testing in both experimental and clinical settings the benefits on graft viability when perfused with this newly available hypothermic, pulsatile machine preservation systems.

Last but not least, special scientific attention has to address the pathophysiology of disease and placing it in a clinical relevant context. In this respect, our efforts in documenting new mechanisms of endothelial activation related to variation in blood rheology parameters, the potential consequences of red blood cell aggregation on (micro)circulation might prove to be valuable in managing complications in both cardiac and transplant patients.