The Groningen hypothermic liver perfusion system for improved preservation in organ transplantation
Plaats, Arjan van der

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2005

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 5

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

Published in Transplantation 2003; 76(1): 37-44.

5.1 Introduction

Reperfusion injury after cold ischemic storage prior to organ transplantation plays a critical role in the occurrence of primary non-function and delayed graft function\textsuperscript{1} which have remained major problems in liver transplantation\textsuperscript{2}. 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 also considered a major determinant of functional recovery after transplantation\textsuperscript{3}. 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
5. Hyperaggregation of HES and UW

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 influence 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 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
5.2. Materials and Methods

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 provide a plausible theory for the mechanisms involved, the red blood cell aggregation has been studied in a binary HES-HES mixture.

5.2 Materials and Methods

RBC aggregability and deformability were investigated in vitro with a Laser-assisted optical rotation cell analyzer (LORCA R&R Mechatronics, Hoorn, The Netherlands)\(^{18,19}\). This instrument 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° C, after in vitro admixture of UW/HES solutions with human fresh blood from healthy volunteers (n=8), drawn from the antecubital vein into 0.1mM ethylenediamine tetracetic acid.

Three commercially available HES solutions (6% in 9g/l sodium chloride) were selected based on their molecular weight and substitution ratio: HES 450/0.7 (Mw=450kDa, MS=0.7), HES 200/0.5 (Mw=200kDa, MS=0.5), HES 130/0.5 (Mw=130kDa, MS=0.5). Phosphate Buffered Saline (PBS, pH 7.4, 300mOsm/kg) was used as buffer fluid for the HES solutions. The final concentration of the HES solutions was 5% pH=7.4, isitone. The University of Wisconsin solution was used as commercially available (5% HES with a molecular weight cut off range of 100-1,000kDa and a mean of 250kDa). 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 450kDa and HES 130kDa solutions, using a mixing ratio of 5:1. After measuring the effect on the aggregation, HES 130kDa was added on the HES 450kDa-treated samples and vice-versa, to a final mixing ratio of 5:2. The experiment was performed at room temperature (22°C). For the evaluation of RBC aggregation we recorded several comparative parameters.
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5.2.1 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 120s 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 (\(T_2^{1/2}\) = 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 (5g/L) in PBS (50mM).

5.2.2 Viscosity measurements

For the measurements of the viscosity of blood and HES-treated blood we used an automated dynamic shear rheometer with cone-plane geometry (AR1000 Rheometer, TA Instruments). During measurements the temperature was set at 4°C and the shear rate of operation equaled the value of the corresponding shear rate which prevented aggregation for that specific sample.

5.2.3 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).
TappingMode 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).
5.3. Results

5.2.4 Statistical analysis

Differences between physiological and experimental aggregation parameters in different samples groups were evaluated using the paired two-tailed Student’s t-test. A p value of \(<0.05\) was considered statistically significant. The results are expressed as mean±SD.

5.3 Results

5.3.1 LORCA measurements

The molecular weight of HES had a highly significant influence on the kinetics of RBC aggregation (Figure 5.1). For a blood:HES ratio of 5:1 the aggregation index in the presence of HES 450kDa 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 130kDa treated samples was 13.64 ± 1.96. In addition, we determined the concentration-dependent effects of HES on RBC aggregation. Decreasing HES 450kDa and HES 200kDa concentrations resulted in a concomitant decrease of aggregation index, although 10% HES 450kDa still induced a significant increased aggregation (p<0.01).

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 UWsolution=2:1, the aggregation index decreased to 0.20 ± 0.42 (Figure 5.1). The kinetics of the aggregation process is also expressed by the half-time \((T \frac{1}{2})\) value. Since the \(T \frac{1}{2}\) is the time necessary to reach 50% of complete aggregation level, a lower \(T \frac{1}{2}\) reflects a faster aggregation process. The RBC aggregates formed three times faster when the cells came in contact with HES 450kDa \((T \frac{1}{2} = 6.67 ± 0.84)\) when compared to the control \((T \frac{1}{2} = 20.43 ± 4.59)\) (p<0.01). HES 130kDa inhibited the aggregation process, the half time necessary for RBC treated with HES 130kDa to reach complete aggregation \((T \frac{1}{2} = 29.17±6.68)\) 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 \((\tau)\)[mPa] required to dissociate the aggregates is calculated by multiplying the minimum shear rate \((\gamma)\)[s\(^{-1}\)] that prevents aggregation.
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The measured shear rates that prevented aggregation and the viscosity values measured for each sample at the corresponding shear rate are presented in Table 5.1. The values of the shear stress are calculated according to the presented formula and included also in Table 5.1. It was notable that the viscosity values of the control were higher than the viscosity measured for the HES 130kDa treated samples, the conditions of the measurement being the same ($\gamma = 80s^{-1}$, temperature 4°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 pretreated with HES 130kDa, large HES increased the aggre-
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Shear Rate \([s^{-1}]\) | Viscosity \([mPa \cdot s]\) | Shear Stress \([Pa]\)
---|---|---
UW-solution | 175 ± 29 | 14.9 ± 0.3 | 2.5 ± 0.2
HES 450kDa | 240 ± 70 | 12.4 ± 0.2 | 3.4 ± 0.2
HES 200kDa | 140 ± 12 | 15.1 ± 0.5 | 2.0 ± 0.1
HES 130kDa | 86 ± 15 | 18.2 ± 0.5 | 1.5 ± 0.05
Controls | 78 ± 3 | 22.7 ± 0.5 | 1.6 ± 0.1

Table 5.1: Shear Stress required to dissociate the aggregates (mean±SD)

Concomitant adding of HES 450kDa and HES 130kDa to the red blood cell suspension yielded values similar to those obtained by consecutive treatment with HES 450kDa and HES 130kDa (Figure 5.2).

5.3.2 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 5.2.

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</table>

Table 5.2: Statistics given by Image Pro-Plus Software after processing bright field microscopy images at a magnification of 200x.

The UW solution treatment of the RBC determined formation of branched rouleaux networks with a range of 23-56 cells per aggregate (Figure 5.3a). HES 450kDa induced formation of large size RBC aggregates with an irregular geometry: polymorph erythrocyte clusters were clearly visualized (Figure 5.3b). The morphology of the HES 130kDa-induced RBC aggregates consisted of various size linear rouleaux (Figure 5.3c). The image of the erythrocytes treated with HES-free UW solution confirmed the absence of RBC aggregation; at a magnification of 200x only 8 aggregates were counted, with a range of 2-3 cells per aggregate. A control was considered as well (Figure 5.3d). TappingMode atomic force microscopy technique revealed a three dimensional surface profile of RBC aggregates with micrometer resolution. This visualization ap-
5. Hyperaggregation of HES and UW

Figure 5.2: Aggregation of human red blood cells in binary HES-HES mixtures, a competitive assay. Sample 1: HES 450kDa, 2: Effect of HES 130kDa on HES 450kDa, 3: control, 4: HES 130 kDa, 5: Effect of HES 450 kDa on HES 130 kDa, 6: Simultaneous treatment with HES 450kDa/130 kDa. Box plots graph data represent statistical values. The boundary of the box closest to zero indicates the 25\textsuperscript{th} percentile, the line within the box marks the median of 6 measurements, and the boundary of the box farthest from zero indicates the 75\textsuperscript{th} percentile. Whiskers above and below the box indicate the 90\textsuperscript{th} and 10\textsuperscript{th} percentiles.
5.4. Discussion

The approach provided clear evidence of aggregation between intact red blood cells when treated with high molecular weight hydroxyethyl starch/UW solution (Figure 5.4 a, b respectively).

5.4 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 450kDa and HES 200kDa) 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 µm$^2$ in the HES 450kDa-treated samples and 4332 µm$^2$ 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$^{20}$ shows that in arterioles and venules, with a diameter of $24.7 \pm 9.1 \mu m$, the recorded shear rate has a mean value of $201 \pm 163 s^{-1}$. The minimal value of the shear rate that prevented UW-induced aggregation in our experiments was $175 \pm 29 s^{-1}$. 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$^{21}$.

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
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Figure 5.3: Bright field microscopy, magnification 500x. Bar scale represents 20µm.
a) UW-induced branched RBC rouleaux networks, b) HES 450kDa-induced RBC polymorph clusters, c) HES 130kDa-induced linear RBC rouleaux, d) Control - RBC suspended in autologus plasma.
5.4. Discussion

Figure 5.4: Tapping Mode Atomic Force Microscopy. Left: HES 450kDa treated red blood cells- cluster of 44.18µm/53.35µm with irregular geometry (scan size 60µm/60µm), Right: Branched RBC rouleaux network induced by UW solution - detailed topography (scan size 30µm/30µm).

and functional integrity of the organ. 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. 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.

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 immedi-
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ate 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 130kDa 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 130kDa 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 130kDa is proved to be an efficacious plasma volume expander in heart surgery\textsuperscript{26}. In addition, Zikria et al\textsuperscript{27} demonstrated that 100 to 300kDa 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 7\(\mu\text{m}\) to traverse capillaries with diameters no more than 3 – 5\(\mu\text{m}\). 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\textsuperscript{28,29}. 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 (\(p>0.05\)).

5.4.1 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\textsuperscript{30}. 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 pro-
tein 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\textsuperscript{31}. The second theory suggests that the aggregation is induced by macromolecular depletion from the membrane surface. In this theory the aggregation 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 concentration existing in the suspending medium between the neighboring surfaces\textsuperscript{32}.

5.4.2 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 450kDa and HES 200kDa 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 130kDa, 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 hyperaggregability induced by HES 450kDa and hypoaggregability induced by HES 130kDa are reversible phenomena’s, demonstrating in this way the nonspecific nature of HES adsorption on the surface of the cell.

5.5 Conclusion

In summary, our experiments conclusively showed that the physiological function of red blood cells to form aggregates is significantly affected in the presence of hydroxyethyl 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 130kDa-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.
5. Hyperaggregation of HES and UW

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 suggest, on the basis of our experimental data, that the use of low molecular weight HES (HES 130kDa) 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.

5.6 References


5. Hyperaggregation of HES and UW


5.6. References


