Improving liver preservation
't Hart, Nils
Chapter V

Effect of University of Wisconsin Organ Preservation Solution on Haemorheology

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During the past decades liver transplantation has become a routine therapeutic method for patients suffering from end stage liver diseases. Significant improvements in transplantation techniques, immunosuppression and treatment of complications have been gained in the last ten years and transplantation results have improved ever since. The number of transplants depends on predominantly deceased donors, the amount of donors becoming available, however, is not sufficient to cope with the increasing demand for liver transplantation. Consequently, the waiting lists are steadily increasing. To prevent the discard of valuable donor livers, increasing the donor pool and decreasing the waiting lists to a more acceptable level, the viability of the graft ought to be better maintained during organ procurement, allocation, transportation and finally transplantation. It is a fact that during preservation liver function and its integrity gradually decreases. This downward slope in viability depends on several factors such as cold ischemia time, perfusion procedure, preservation solution and preservation method. Improvement of these factors can result in a significantly better preservation and possibly an expansion of the donor pool. To reach our goal to include more donor categories in the donor pool we have now focused on the importance of wash-out of the donor organ as an essential part of liver preservation. Many times during the procurement operation little attention is paid to a thorough wash-out of donor blood which is important for liver viability. Preservation, thus, remains a critical issue and improvements in preservation, and the procurement technique in particular, are required to further optimise transplantation results.

To date, the initial wash-out procedure is performed with the University of Wisconsin-Cold Storage Solution (UW-CSS) in the majority of the donor procedures\(^1,2\). UW-CSS was developed in the late 1960s by the group of Belzer at the University of Wisconsin. They aimed at developing one solution suitable for both static cold-storage and dynamic machine perfusion techniques. Until the introduction of UW, static CS solutions did not contain any colloid. In contrast, during continuous machine perfusion, a high colloidal substance is always included to counteract extravasation of fluids and prevent formation of tissue edema due to hydrostatic pressure\(^3,4,5\). Based on their experiences in kidney preservation, Belzer included diafiltrated hydroxyethyl starch (HES) in UW-CSS as they intended to diminish edema formation during organwash-out\(^6-9\). Another reason to include a colloid was that it was known that viability of abdominal and thoracic organs improved when preserved in colloid containing solutions\(^10\). Besides beneficial effects of these colloids, its disadvantages have been reported as well, in particular concerning HES as it tends to increase the viscosity of the preservation solution\(^11-13\). Until now, a number of authors have stated that the high viscosity of UW-CSS hampers effective wash-out. Studies focusing on the analysis of the behaviour of UW-CSS in combination with blood are, however, not published. The effect of UW-CSS on whole blood could, nevertheless, be more important than viscosity alone especially during initial wash-out at time of organ procurement.
Cold-storage with UW-CSS allows liver preservation up to approximately 12-15 hours\textsuperscript{8,14}. Another method to store organs is continuous hypothermic machine perfusion (HMP). Belzer \textit{et al.} introduced this technique and showed that it is beneficial in kidney preservation\textsuperscript{1,2}. Due to the successful results in kidney preservation, several groups became interested in applying HMP for the liver\textsuperscript{11}. It is known now that the HMP technique is able to extend the duration of preservation and that it potentially allows transplantation of livers retrieved from older, marginal or Non-heart-beating donors. One of the advantages of HMP over cold-storage is wash-out of waste products and the continuous supply of nutrients. On the other hand, it remains unknown what exactly improves organ viability when it is preserved with HMP instead of cold-storage. Irrespective of the method used, preservation starts with an appropriate and effective wash-out of the donor organ. Since an incomplete wash-out is detrimental for preservation and exposes the procured liver to increased ischemia-reperfusion injury with a decreased functional recovery after transplantation, a complete wash-out is mandatory using better procurement strategies or continuous machine perfusion\textsuperscript{15-17}. For an optimal preservation result we postulate that both during initial wash-out and HMP preservation, physiologic perfusion pressures should be used to minimise the change of endothelial cell damage due to shear stress. In our rat liver procurement experiments, portal perfusion was performed at a physiological pressure of 12 mmHg. This technique resulted in an incomplete wash-out of donor blood from the (micro)vasculation of the rat liver. Repeating the procurement experiment with a modified UW-CSS without hydroxyethyl starch, a better wash-out of blood and a complete and even distribution of the solution throughout the entire liver was found.

In this study, we analysed the viscosity of cold UW-CSS and the aggregation behaviour of blood in the presence of HES containing solutions. The original UW-CSS was studied and compared to modified UW-CSS (UWmod), both in combination with whole blood.
Materials and Methods

Albino Oxford rats (250-350 g) were used as blood donors. In all experiments, blood (6 ml) was obtained by cardiac puncture from anaesthetised rats using Halothane/O₂/N₂O. Five minutes prior to blood harvesting, one ml saline containing 500 iE/ml heparin was administered intravenously.

**Sample preparation.** Blood samples were collected in heparin-coated test-tubes at 4 °C and used immediately. Whole rat blood was standardised to a haematocrit of 45% by adjusting the amount of blood plasma; mixtures were prepared immediately prior to the experiments. All experiments were performed in six fold, using four experimental groups containing: A) UW-CSS (ViaSpan, DuPont, Wilmington, USA), B) UWmod, that was prepared according to the original recipe, without addition of HES, C) a mixture of UW-CSS and whole rat blood (1:1) and D) a mixture of UWmod with whole rat blood (1:1). Whole rat blood at 37 °C and 4 °C served as controls.

**Viscosity.** Viscosity of the preservation solution was analysed by answering the following questions:

- **Magnitude:** Does HES influence the viscosity of UW-CSS under hypothermic conditions, and what is the effect on viscosity when UW-CSS and whole blood are combined?
- **Non-Newtonian behaviour:** Whole blood is known for its non-Newtonian behaviour, which implies that the viscosity is significantly higher at low shear-rates than it is at high shear-rates\(^{18}\). UW-CSS could show this behaviour as well. Does UW-CSS show non-Newtonian characteristics, and if it does, is this effect still present and prominent when blood is combined with UW-CSS?
- **Bingham-effect:** Some fluids show the Bingham-effect, which means that there is a threshold stress (\(\tau_{\text{thres}}\)) that has to be overcome to allow fluids to flow. It is known that blood shows the Bingham-effect\(^{18}\). Does a threshold stress need to be overcome before UW-CSS starts to flow?

These qualities of viscosity were analysed with a cone-plate rheometer (AR 1000, TA Instruments, New Castle, USA), measuring the shear-stress of a 0.5 ml sample at increasing shear-rates. This method allows the determination of viscosity (Pa·s) of solutions, which is defined as shear-stress (Pa) divided by shear-rate (s\(^{-1}\)). In addition, the cone-plate rheometer allows analysis of the behaviour of solutions at low shear-rates. Shear-rate to shear-stress and shear-rate to viscosity curves were obtained by applying shear-rates up to 100 s\(^{-1}\). The experiments were performed at 4 °C. The controls, whole rat blood without additives, were analysed at 4 °C and 37 °C.

**Red blood cell (RBC)-aggregation.** Corry *et al.* have shown that HES increases red blood cell aggregation when it is combined with blood\(^{19}\). To study the influence of HES on the aggregation behaviour of rat blood, measurements were performed using a Laser-assisted Optical Rotational Cell Analyser (LORCA, R&R Mechatronics, Hoorn,
The LORCA analyzer consists of a temperature controlled couette system with a sample volume of 1.5 ml. Blood cell aggregation is measured using the intensity of the laser light that is projected on the sample and back-scattered by blood (Figure 1). The amount of aggregation is presented by means of the aggregation amplitude, which is defined as the difference between the intensity of back-scattered laser light between total dis-aggregation and complete aggregation.

A gradual dilution of donor blood occurs during human organ retrieval at time of organ perfusion with UW-CSS. To represent the gradual dilution of whole blood with UW-CSS, occurring during the initial organ wash-out, different dilution ratios of UW-CSS to blood were studied. The chosen dilution ratio's of blood with UW-CSS and UWmod were 4:1, 3:2 and 1:1. Also, erythrocyte aggregation was measured in whole blood at 4°C.

Figure 1: Blood cell aggregation: lightscatter before and after suddenly stopping disaggregation. Erythrocyte morphology corresponding to various parts of the curve is indicated. From left to right: elongated flow orientated and disaggregated blood cells, undeformed randomly orientated and disaggregated blood cells, rouleaux aggregation.

**Microscopic examination.** RBC-aggregation in solutions combining UW-CSS with whole blood (1:1) and UWmod with whole blood (1:1) at 4 °C were studied with light microscopy. The solutions were processed on a glass plate as a standard smear and stained with May-Grunwald-Giemsa. Microscopic examination (Leica Microsystems, Wetzlar, Germany) in combination with an image-processing program (Leica Qwin 2.8, Cambridge, UK) was used to quantify the erythrocyte aggregates.
**Statistical analyses.** Comparison between the results (mean +/- SEM) was performed using the unpaired two-tailed Student’s t-test. Differences were considered to be statistically significant with a P-value of < 0.05.
Results

Viscosity. Whole blood demonstrated a characteristic non-linear viscous behaviour in the shear rate to viscosity graphs, UW-CSS showed this non-Newtonian property as well. This effect was also observed in the solutions containing whole blood and UW-CSS. The viscosity of whole blood in combination with UW-CSS was found to be 1.3 times higher than that of whole blood at 37 °C. In contrast, UWmod showed a constant viscosity for different shear-rates. For UWmod in combination with whole blood a 1.8 times higher viscosity was found in a similar linear pattern. The whole blood controls showed a higher non-Newtonian viscosity curve at 4 °C compared to 37 °C.

The shear-rate to shear-stress curves demonstrated a linear relationship. The shear-stress of the solutions at zero shear-rate (extrapolated from the mean) were slightly higher than zero, but no considerable increase in $\tau_{\text{thres}}$ was observed (Table 1). The measured shear-rate to viscosity and shear-rate to shear-stress curves of UW-CSS and UWmod, as well as the mixtures of these solutions with whole blood (1:1) at 4 °C are shown in Figure 2a and 2b, respectively.

Table 1: Viscosity and threshold stress values of the studied solutions (mean +/- SEM). * = p ≤ 0.05 versus 37 °C whole rat blood.

<table>
<thead>
<tr>
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<th>Viscosity [Pa·s]</th>
<th>$\tau_{\text{thres}}$ [Pa]</th>
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<tbody>
<tr>
<td>WB 37°C</td>
<td>$9 \cdot 10^{-3}$ +/- $1.7 \cdot 10^{-3}$</td>
<td>0.03</td>
</tr>
<tr>
<td>WB 4°C</td>
<td>$18 \cdot 10^{-3}$ +/- $3.6 \cdot 10^{-3}$ *</td>
<td>0.07</td>
</tr>
<tr>
<td>UW-CSS 4°C</td>
<td>$11 \cdot 10^{-3}$ +/- $0.4 \cdot 10^{-3}$ *</td>
<td>0.001</td>
</tr>
<tr>
<td>UWmod 4°C</td>
<td>$5 \cdot 10^{-3}$ +/- $0.4 \cdot 10^{-3}$ *</td>
<td>0.009</td>
</tr>
<tr>
<td>UW-CSS + WB 4°C</td>
<td>$12 \cdot 10^{-3}$ +/- $1.4 \cdot 10^{-3}$ *</td>
<td>0.07</td>
</tr>
<tr>
<td>UWmod + WB 4°C</td>
<td>$9 \cdot 10^{-3}$ +/- $1.2 \cdot 10^{-3}$</td>
<td>0.01</td>
</tr>
</tbody>
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Figure 2: Shear-rate to viscosity (a) and shear-rate to shear-stress curves (b) of UW-CSS, blood and blood in combination with UW-CSS.
**RBC-aggregation.** The combination of blood with UW-CSS at ratios of 3:2 and 1:1 showed a significant increase in aggregation when compared to whole rat blood (P < 0.05). Although results did not reach statistically significant levels, UWmod combined with blood in similar ratios showed a decreasing trend in aggregation. The results of the aggregation measurements with the LORCA are presented as percentages of whole blood aggregation (Figure 3).

![Figure 3: Aggregation amplitudes of blood in combination with different concentrations UW-CSS and UWmod, relative to blood. * = p < 0.05 versus whole rat blood.](image)

**Microscopic examination.** The randomly distributed structure of the solutions containing blood and UWmod is clearly different from the structure of blood in combination with the original UW-CSS. The aggregates that were formed in the solutions containing UW-CSS and whole blood had a mean ( +/- SEM) length of 30 +/- 8 µm and a mean width of 18 +/- 6 µm (Figure 4).
Figure 4: Erythrocyte smears, stained with May-Grunwald-Giemsa. 4a: Erythrocytes in combination with UWmod (1:1). 4b: Erythrocytes in combination with UW-CSS (1:1).
Discussion

The University of Wisconsin organ preservation solution is widely used to wash-out and preserve donor organs. One of its components is hydroxyethyl starch which was originally added to the solution to prevent edema formation during the initial wash-out procedure. In this study we analysed the viscosity and aggregation qualities of UW-CSS, with and without HES, and both solutions were further studied in combination with whole blood. Our interest in HES is based on previous publications by several groups who stated that a reduction in viscosity is required for effective machine preservation of the liver. We found, however, that the viscosity of UW-CSS is just slightly higher in comparison to UW without HES. We do not consider this increase in viscosity to be an important factor in poor organ wash-out during the retrieval procedure or the following machine perfusion preservation. In contrast to viscosity, aggregation of blood in the presence of HES is found to be a significant factor, generating aggregates that could exceed the diameter of liver microvasculature.

In the past, several authors have used a HES-free wash-out solution prior to organ preservation with the original UW-CSS\textsuperscript{23-27}. In 1990 Pienaar et al. described successful machine preservation experiments of the liver, after a so-called preflush of lactated Ringers solution to wash-out blood from the donor liver\textsuperscript{28}. Judged by the fact that several authors have left HES out of their initial wash-out and preservation solutions, it is likely that they must have encountered difficulties with the initial wash-out of the liver at low perfusion pressures\textsuperscript{11-13}. These same authors have suggested that these difficulties were due to the high viscosity of UW-CSS. However, viscosity measurements showed for UW-CSS a value of $11 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$ Pa·s which is only a factor 1.3 times higher than viscosity of blood at 37 °C. The viscosity of blood at a temperature of 4 °C is 2 times higher when compared to measurements at 37 °C. The combination of UW-CSS and whole blood ($11 \cdot 10^{-3}$ Pa·s) resulted in a viscosity in between the viscosity levels of UW-CSS at 4 °C and whole rat blood at 4 °C. Significantly lowering the viscosity is possible by excluding hydroxyethyl starch from UW-CSS. Despite the fact that UWmod showed lower viscosity values, it is not justified to state that the high viscosity of UW-CSS is the causative factor resulting in a difficult or even poor wash-out of the donor liver. As UW-CSS viscosity at 4 °C is only a factor 1.3 higher this would result in a 1.3 times decrease in flow during wash-out when compared to physiologic conditions with whole blood at 37 °C. It is unlikely that this relatively slight increase in viscosity could explain poor organ wash-out. Also, we were able to demonstrate a considerable non-linearity in viscosity of UW-CSS as illustrated in figure 2a. Since this phenomenon is absent in the modified version of UW, we concluded that this effect is the result of omitting HES from UW-CSS. In contrast to UW-CSS and whole blood, UWmod combined with blood showed a low viscosity and a linear pattern in the viscosity-shear rate curve.
The non-linear in the solution containing UW-CSS and whole blood is, thus, due to both whole blood and HES as it is included in UW-CSS but not in UWmod. An increase in the threshold pressure (Bingham effect) is not present, as shown in figure 2b, and does, therefore, not cause a poor organ wash-out in our rat liver wash-out experiments.

The effect of HES-chains on whole rat blood dominates the results of the LORCA measurements (Figure 3). RBC aggregates are formed when UW-CSS is combined with blood. The amplitude of aggregation demonstrates an increasing pattern with an increasing concentration of UW-CSS in combination with blood. The addition of 20% UW-CSS does not have a significant effect, but the addition of 40% and 50% UW-CSS significantly increased the aggregation amplitude. In contrast, blood combined with UWmod did not show this effect. The effect of increased RBC aggregation is clearly visualized in the microscopic images (Figure 4). These smears consisted of whole blood combined with UW-CSS and showed large erythrocyte aggregates. Without HES, single blood cells diffuse in a uniform pattern throughout slide. The shape and size of the aggregates induced by HES exceeds the diameter of hepatic sinusoids. The mean diameter of a sinusoid is approximately 10 µm, the mean length and width of the aggregates are 30 µm and 18 µm respectively. This might cause a blockade of sinusoids resulting in a poor initial wash-out during liver procurement, as it has been observed in wash-out experiments using non-heart beating donor livers\textsuperscript{15}. Taking into account that the average diameter of capillaries are approximately 10 µm, the erythrocyte-HES aggregates might block microvasculature and may have an important role during procurement and the initial wash-out procedure.

The combination of UW-CSS and blood produces large aggregates which may play an important role in human donor organs during initial blood wash-out at physiological perfusion pressures. We observed aggregation in rat blood and found the same effect in whole human blood\textsuperscript{29}. Furthermore, additional experiments showed that a decrease in rouleaux formation can be obtained using the 130 kDa form of HES which is considerably smaller than the currently used form of 250 kDa in UW-CSS. Our findings with human blood confirmed that solutions containing high molecular weight hydroxyethyl starch (HES 450 kDa and HES 200 kDa), have a potent hyperaggregating effect on human RBC. RBC aggregates formed in the presence of this colloid are large, with a maximum aggregate size of 6740 µm\textsuperscript{2} in the HES 450 kDa-treated samples and 4332 µm\textsuperscript{2} in the UW-treated samples\textsuperscript{29}. In addition, it has been shown that the shear-rates that will occur during the wash-out procedure do not allow an easy dissociation of aggregates during perfusion\textsuperscript{29}. Dissociation of these aggregates by flow induced shear-stress is 50-100% easier when 130 kDa is used compared to samples treated with the original UW-CSS. These data suggest that currently used gravity-induced hydrostatic perfusion pressures, can not easily disrupt the RBC aggregates formed during clinical organ procurement. It is, therefore, important to improve organ procurement and especially the
initial wash-out procedure to improve graft quality and subsequently viability. We now propose to ameliorate wash-out and use a preflush of donor livers without the HES component.

In conclusion, UW-CSS induces aggregate-formation due to HES in combination with whole blood. Both, aggregate formation and a slightly elevated viscosity of UW-CSS, prevent effective donor blood wash-out. Since aggregates could hamper perfusate flow, the initial donor liver wash-out ought to be improved before potentially beneficial effects of continuous HMP can be expected. We propose to preflush the donor liver in-situ with a flushing solution without HES, or to find an alternative for HES to optimise initial wash-out and subsequent organ preservation.
Reference List


