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ORIGINAL ARTICLE

Improved microcirculation by low-viscosity histidine–tryptophan–ketoglutarate graft flush and subsequent cold storage in University of Wisconsin solution: results of an orthotopic rat liver transplantation model

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Summary

As previously shown in a model of isolated rat liver perfusion, the combined use of an initial graft flush with low-viscosity histidine–tryptophan–ketoglutarate (HTK) solution followed by cold storage in University of Wisconsin (UW) solution markedly improved the preservation during an extended cold storage period. In this study, we aimed to transfer our results into an *in vivo* model of orthotopic rat liver transplantation, and to elucidate the potential mechanism of the improved preservation by focusing on the hepatic microcirculation. Livers were harvested from male Wistar rats. Aortic perfusion with a pressure of 100 cm H_2O was performed with either UW (group UW) or HTK (groups UW and HTK_UW), followed by additional back-table perfusion with UW (group HTK_UW). After 20-h cold storage at 4 °C, livers were orthotopically transplanted with reconstructing the hepatic artery. As measured by bile flow and liver enzymes, HTK flush followed by UW storage was superior compared to single use of either UW or HTK solution. The hepatic microcirculation was significantly improved, as shown by the increased percentage of reperfused sinusoids and reduced sinusoidal leucostasis. HTK and UW effectively reduce ischaemia-reperfusion injury after liver transplantation. By combining the comparative advantages of both solutions, a cumulative effect resulting in an improved preservation was shown. Thus, this mechanism improves microcirculatory reperfusion.

Introduction

There is clear evidence that microcirculatory disturbances due to endothelial damage contribute towards early graft dysfunction or failure. Furthermore, the biliary tree is exclusively supplied by the hepatic artery, resulting in a high susceptibility of perfusion deficits, such as ischaemic type bile lesions (ITBL) or ischaemic bile lesions. There are some reports on a higher incidence of biliary complications in livers preserved in University of Wisconsin (UW) solution compared to histidine–tryptophan–ketoglutarate (HTK) solution. This can be explained in part not only by the high viscosity of UW but also by the high potassium concentration which may cause an initial vasoconstriction and the hyperaggregating effect of HES, as the key antiedematous ingredient in UW is responsible for the poor perfusion of potential grafts [1].

University of Wisconsin solution has a three times higher viscosity at 4 °C compared to HTK [2]. We assumed that initially rinsing the liver *in situ* with HTK allows a rapid and complete clearance of red blood cells from the microvasculature, and enhances the preservation in UW solution. Initially rinsing the organ with low-viscosity HTK solution followed by storage in UW

solution may exert the cumulative protection by high-volume and high-flow cooling. Basically, our aim was to wash out the whole graft with the best available solution for short-term preservation, followed by back-table fluid exchange and storage in UW solution, as already suggested by Wilson *et al.* [3]. We were able to demonstrate a clear benefit in an *in vitro* model of isolated rat liver perfusion by using the combined preservation concept compared to each preservation solution alone. Parameters observed were enzyme leakage, bile production and portal venous resistance on reperfusion. Moreover, intrahepatic glycerol concentrations showed an improved parenchymal protection already during the cold ischaemic period [4].

To elucidate the mechanism of our previously shown superior preservation by initially rinsing the organ with HTK followed by storage in UW, we observed the initial microcirculation during the early critical phase of reperfusion of transplanted rats.

Materials and methods

All experiments with regard to the protection of animals were performed according to the regulations of the federal law. The principles of laboratory animal care (NIH Publication No. 85-23, revised 1985) were followed.

Experimental protocol

Male Wistar rats weighing between 250 and 280 g were housed in temperature and light controlled cages with access to tap water and food *ad libitum*. After 1 week of acclimatization, the orthotopic rat liver transplantation was performed with hepatic artery reconstruction. Donor livers were freed from all attaching ligaments, and after cannulation with a 14-G catheter, the infrarenal aorta was flushed according to our protocol with 15 ml UW (Group UW) or 40 ml HTK (Group HTK). Aortic perfusion was performed by gravity with a pressure of 120 cm H_2O. After explanting the organ, a back-table portal perfusion with a pressure of 20 cm H_2O was performed in the UW group with 5 ml of UW, and with 20 ml HTK in the HTK group. A third group of livers, which were initially rinsed *in situ* with 40 ml HTK, was perfused with 5 ml UW (Group HTK_UW). The common bile duct was cannulated with a 27-G polyethylene tube, allowing for collection of total bile outflow during reperfusion. Then livers were stored for 20 h in the respective preservation solution at 4 °C. The sample size for each group was $n = 5$.

For the recipient operation, rats were anaesthetized by isoflurane inhalation. Polyethylene catheters placed in the carotid artery and the jugular vein allowed for assessment of systemic haemodynamics and injection of fluorescent dyes. Transplantation was performed with all venous anastomoses made by hand sutures using Prolene 7-0 (Ethicon, Norderstedt, Germany) monofile suture material. The hepatic artery was reconstructed by stent implantation. Portal clamping time was the same in all three groups (UW 19 ± 2 min; HTK 18 ± 1 min; HTK_UW 19 ± 1 min; NS).

The bile duct was not anastomosed, but cannulated with 27 g tubing for collection of bile fluid during reperfusion. After completing all anastomoses, anaesthesia was maintained and intravital fluorescence microscopy was performed.

Intravital fluorescence microscopy

A fluorescence microscope equipped with a 100 W mercury lamp (Axiotech vario; Zeiss, Jena, Germany) and different filter sets for blue (excitation/emission wavelength: 450–490/>520 nm) and green (530–560/>580 nm) light epi-illumination was used. Microscopic images were taken with a water immersion objective (x20/0.50W; Zeiss) recorded by a CCD video camera (FK 6990A-IQ; Pieper, Berlin, Germany) and transferred to a video system (S-VHS Panasonic AG 7350-E; Osaka, Japan). Sodium fluorescein (2 μmol/kg i.v.; Merck, Darmstadt, Germany) served as tissue contrast enhancement with assessment of sinusoidal perfusion [5,6]. Leucocyte–endothelial cell interaction was assessed after *in vivo* white blood cell staining by rhodamine-6G (1 μmol/kg i.v.; Merck) using the green filter system [7].

For analysis of apoptotic cell death, *in vivo* staining of hepatocellular nuclei was achieved by intravenous injection of bisbenzimide (Hoechst 33342; 10 μmol/kg; Sigma, Taufkirchen, Germany) and ultraviolet epi-illumination (330–380 > 415 nm) [8,9].

Quantitative video analysis

Assessment of hepatic microcirculation parameters were performed off-line by frame-to-frame analysis of the videotaped images using a computer assisted image analysis system (CapImage; Zeintl, Heidelberg, Germany). Ten different lobules per animal were evaluated to determine sinusoidal perfusion by counting the number of perfused sinusoids, given in percentage of all sinusoids visible and crossing a 200 μm raster line [10]. Leucocyte–endothelial cell interaction was also analysed in 10 hepatic lobules per animal including the number of stagnant leucocytes located in sinusoids, which were not moved during an observation period of 20 s. Furthermore, in each liver, 10 postsinusoidal venules were observed for 20 s, and not moving or detaching leucocytes were counted (given as cells/mm^2 endothelial surface, calculated from the diameter and length of the

vessel segment studied, assuming cylindrical geometry) [5,7].

Apoptotic cell death was analysed in 10 lobules per animal by counting the number of cells that showed apoptosis-associated condensation, fragmentation and crescent shaped formation of chromatin (given as cells/mm^2) [8].

Bile flow

Functional integrity and homogenous flow distribution in the livers were estimated by collecting the bile produced in µl/g liver weight/60 min over the whole reperfusion period.

Liver enzymes

Sixty minutes after perfusion started liver enzyme release of alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) was determined in arterial blood samples in U/ml.

Cleaved caspase-3 activity

To study active caspase-3 using immunohistochemistry, 5-µm sections of paraffin-embedded liver specimens were incubated overnight at room temperature with a rabbit polyclonal cleaved caspase-3 antibody (1:50; Cell Signalling technology, Frankfurt, Germany). This antibody detects endogenous levels of large fragments (17/19 kDa) of activated caspase-3, however not full length caspase-3. Biotinylated anti-mouse/rabbit immunoglobulin antibody was used as a secondary antibody for streptavidine–biotin complex peroxidase staining (Link, LSAB-HRP; Dako-Cytomotion, Hamburg, Germany) and 3,3′diaminobenzidine was used as the chromogen. The sections were counterstained with hemalaun and analysed for cleaved caspase-3 positive hepatocytes [given in '*n*' per high power field (HPF)].

Statistical analysis

All data are expressed as mean ± SD. Once normality and equal variance were proven in all groups, the differences were assessed with ANOVA followed by the appropriate *post hoc* comparison test. Statistical significance was assumed at $P < 0.05$. Statistics were performed using the SigmaStat software package (Jandel, San Rafael, CA, USA).

Results

Bile flow

Cumulative bile within the single HTK preservation was 11.9 ± 4.5 µl/60 min/g liver weight, which was comparable to the single UW preservation (group 2: 14.3 ± 8.4 µl/60 min/g liver weight). In contrast, low-viscosity HTK flush/UW storage revealed significantly higher values of bile flow (33.7 ± 10.2 µl/60 min/g liver weight) (Fig. 1).

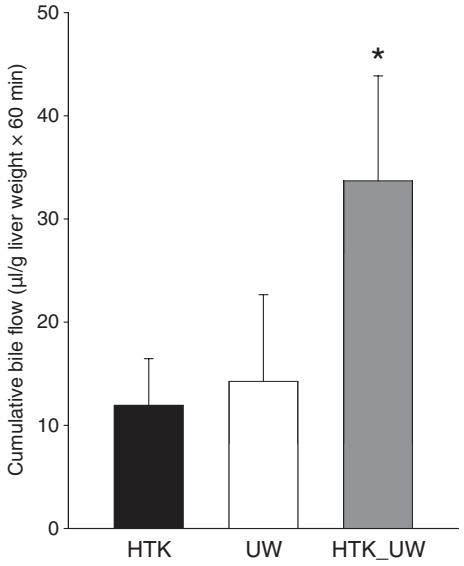

Figure 1 Cumulative bile flow over the first 60 min of liver reperfusion at 20-h cold preservation. Livers were harvested by aortic perfusion with either UW (group UW), or HTK (groups HTK and HTK_UW), followed by additional back-table perfusion with UW (group HTK_UW). Data are given as mean ± SD. *$P < 0.05$ versus HTK and UW each; $n = 5$.

Liver enzymes

Released liver enzymes were measured after 1 h of reperfusion and taken as a general parameter of parenchymal injury of the liver during reperfusion. Both UW groups, either with (group HTK_UW) or without (group UW) HTK preflush had a significantly lower enzyme release of ALT and AST (Fig. 2a and b) compared to single storage in HTK (group HTK).

Intravital fluorescence microscopy

Sinusoidal perfusion rate was significantly improved in the HTK_UW group (96.9 ± 2.3%) compared to the HTK (86.2 ± 7.7%) and UW group (84.5 ± 7.2%) (Fig. 3a). Accordingly, the number of stagnant leucocytes counted in 10 liver lobules during a time-frame of 20 s, was significantly lower in the HTK_UW group (37.3 ± 12.5 group HTK_UW vs. 67.3 ± 23.5 group HTK and 64.2 ± 19 group UW) (Fig. 3b).

There were no statistical differences observed in the groups with regard to adherent leucocytes in hepatic

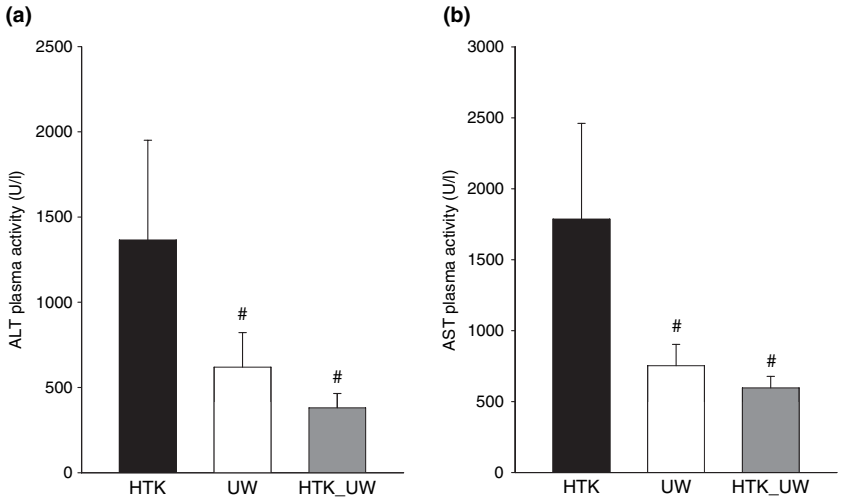

Figure 2 Plasma activities of ALT (a) and AST (b) after 60 min of hepatic reperfusion at 20-h cold preservation. Livers were harvested by aortic perfusion with either UW (group UW) or HTK (groups HTK and HTK_UW), followed by additional back-table perfusion with UW (group HTK_UW). Data are given as mean ± SD. $^{\#}P < 0.05$ versus HTK; $n = 5$.

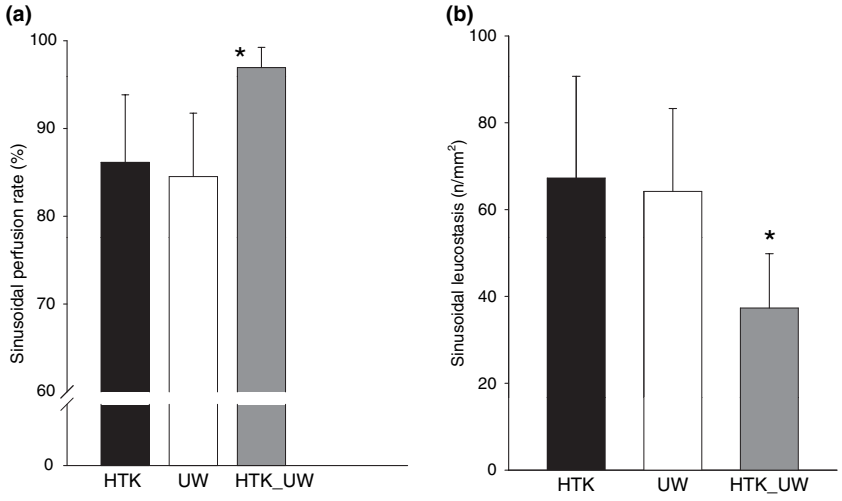

Figure 3 Sinusoidal perfusion rate (a) and sinusoidal leucostasis (b), as assessed by intravital fluorescence microscopy, after 60 min of reperfusion in livers at 20-h cold preservation. Livers were harvested by aortic perfusion with either UW (group UW) or HTK (groups HTK and HTK_UW), followed by additional back-table perfusion with UW (group HTK_UW). Data are given as mean ± SD. $^{*}P < 0.05$ versus HTK and UW each; $n = 5$.

venules, a parameter of the immunological activity and antigenecity of the endothelium (Fig. 4).

Hepatocellular apoptosis

Apoptosis was determined by intravital staining using bisbenzimide, and verified by immunohistochemistry for cleaved caspase-3, a recognized parameter of apoptotic cell death. For both parameters in all groups, only a small number of apoptotic cells were observed for both parameters without any statistically significant differences (Fig. 5a and b).

Discussion

This study was aimed to elucidate the mechanism of the beneficial effect of a low-viscosity donor liver preflush using HTK with subsequent storage in UW. This concept was evaluated against the single use of either HTK or UW as the respective storage solution.

As already well described, the severity of the ischaemia-reperfusion injury is directly related to the extent of microcirculatory reperfusion failure [6,11]. As an improved microcirculation is considered the main advantage of a low-viscosity preflush, we focused on the initial status of sinusoidal perfusion after 20 h of cold ischaemia and transplantation.

There was a significant increase in the percentage of perfused sinusoids in the HTK_UW group 1 h after transplantation. A lower number of trapped leucocytes was also observed in the HTK_UW group (Fig. 3). No statistical meaningful difference among the groups preserved either with HTK or UW alone was observed. Especially, the biliary tree is very susceptible to microcirculatory deficits, e.g. provoked by thrombosis of the hepatic artery. There are conflicting reports on the

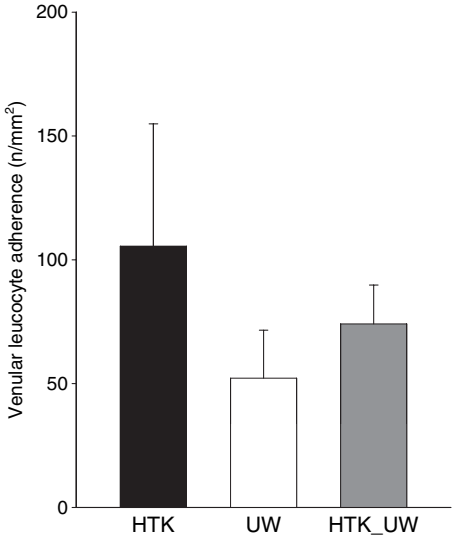

Figure 4 Venular leucocyte adherence, as assessed by intravital fluorescence microscopy, after 60 min of reperfusion in livers at 20-h cold preservation. Livers were harvested by aortic perfusion with either UW (group UW) or HTK (groups HTK and HTK_UW), followed by additional back-table perfusion with UW (group HTK_UW). Data are given as mean ± SD; $n = 5$.

effectiveness of preserving the biliary tree with HTK or UW. Moench and Otto [12] were not able to prove a difference, whereas Canelo *et al.* [13] showed a significant benefit of livers stored in HTK with regard to the incidence of ischaemic type biliary lesions. Our data reveal a significant higher bile production after 60 min of reperfusion for the HTK_UW group (Fig. 1). Bile formation at the canalicular level results from an oxygen-dependent active transport of bile acids and other solutes [14]. Therefore, we conclude that improved microcirculation early after transplantation enables an accelerated cellular resuscitation along with secretion of a balanced bile salt composition.

With regard to the antigenecity of the organ, we observed the interaction of leucocytes and endothelium in postsinusoidal venules. As shown in Fig. 4, there were no significant differences between the groups, although livers preserved with UW showed a reduced leucocyte–endothelium interaction compared to HTK preservation alone.

As repeatedly reported, UW is the more effective preservation solution for storage times longer than 12 h than HTK [15,16]. A recent study from Mangus *et al.* [17] reported a significantly higher enzyme loss of AST and ALT already after 7 h of cold ischaemia for livers stored in HTK compared to UW-preserved livers. Interestingly, all livers were flushed before harvesting with a low-viscosity sodium chloride solution. In this study, all livers preserved in UW also had a significantly lower enzyme release (Fig. 2) compared to storage in HTK alone. Livers from group 3 (HTK_UW) revealed the lowest enzyme deliberation.

To evaluate the impact of apoptosis 1 h after reperfusion, intravital staining with bisbenzimide revealed in all groups a low number of apoptotic cells, without any significant differences among the groups (Fig. 5). This finding could be confirmed by determination of cleaved caspase-3 staining, an established parameter of apoptosis (Fig. 5). The low number of apoptotic cells is probably related to the short reperfusion time, although potential differences may occur at a later time point after transplantation. Furthermore, the influence of the endoplasmic reticulum stress induced cell death as recently discussed [18] might not be visible 1 h after reperfusion.

In conclusion, the combined use of an initial rinse with HTK and subsequent storage in UW allows a safe and,

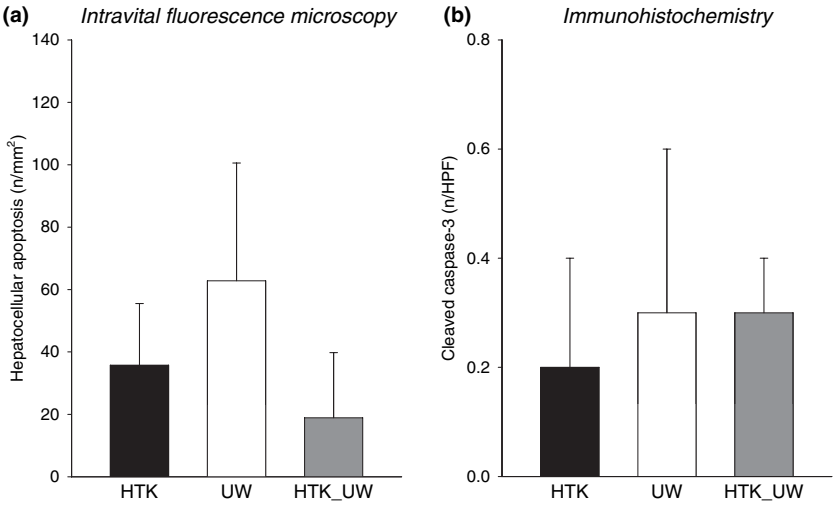

Figure 5 Hepatocellular apoptosis after 60 min of reperfusion in livers at 20-h cold preservation. Livers were harvested by aortic perfusion with either UW (group UW) or HTK (groups HTK and HTK_UW), followed by additional back-table perfusion with UW (group HTK_UW). Hepatocellular apoptosis was studied by (a) *in vivo* bisbenzimide staining and intravital fluorescence microscopy and by (b) immunohistochemistry for cleaved caspase-3. Data are given as mean ± SD; $n = 5$.

compared to the single use of each solution, improved preservation. As shown in this study, a significantly better microcirculation results in a higher metabolic activity and in reduced membrane damage. The potential beneficial effects on the biliary tree have to be evaluated by further long-term survival studies.

Authorship

PO: designed research, performed research, analysed data and wrote paper. HG: performed research. EC: performed research and collected data. NU and NP: designed research. SW and SV: analysed data. VB and PG: designed research, analysed data and wrote paper.

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