

THE EFFECTS OF LONG-TERM GRAFT PRESERVATION ON INTRAOPERATIVE HEMOSTATIC CHANGES IN LIVER TRANSPLANTATION

A Comparison Between Orthotopic and Heterotopic Transplantation in the Pig

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We compared hemostatic changes during OLT and HLT after various periods of graft storage, to investigate whether the host liver in HLT protects the recipient from hemostatic deterioration induced by severe graft storage damage. In particular, the mechanism of fibrinolytic deterioration was investigated. The effect of prostaglandin E₁ (PGE₁) on these parameters was also studied.

Material and Methods: 69 pigs underwent either OLT (N = 32) or HLT (N = 37) with a graft stored for 2 hr (N = 31), 24 hr (N = 16), 48 hr (N = 7), or 72 hr (N = 15). PGE₁ was given intravenously to both donor and recipient animals and was added to the preservation and flushing solutions. Fibrinolysis (euglobulin clot lysis time, t-PA activity and α_2 -antiplasmin) and coagulation parameters (activated partial thromboplastin time, prothrombin time, fibrinogen and platelet count) were measured at several intervals during transplantation.

Statistics: Univariate non-parametric tests were used for analysis of coagulation and fibrinolysis parameters. For the three main variables- i.e., the type of transplantation, the use of PGE₁, and the preservation time, multiple regression analysis was performed.

Results: Fibrinolytic activity increased during the anhepatic period of OLT. Graft reperfusion was followed by a rise in t-PA in both OLT and HLT. In HLT, t-PA quickly returned to normal, whereas a continuous increase was found in OLT. The coagulation parameters, in turn, remained unchanged during the anhepatic period and deteriorated in OLT compared to HLT. The duration of graft storage was directly related to the severity of the hemostatic changes, although this effect was more apparent in OLT than in HLT.

Conclusions: Changes in hemostasis are more pronounced in OLT than in HLT. This suggests that the host liver protects the recipient from the effects of graft storage damage, even after long preservation times. Early postreperfusion fibrinolytic activity was presumably due to t-PA release from the graft both in OLT and HLT. The further rise t-PA in OLT might be caused by the release of cytokines from the graft, that subsequently evoke endothelial t-PA release. In HLT, t-PA and cytokines may be cleared by the native liver. No positive or negative effect of PGE₁ on coagulation or fibrinolysis parameters was noticed.

KEY WORDS: Liver transplantation, fibrinolysis, DIC, PGE

INTRODUCTION

Orthotopic liver transplantation (OLT) has become an accepted method to treat patients with end-stage chronic liver disease. However, the procedure is frequently complicated by severe changes in hemostasis. Both increased fibrinolysis^{1,2} and disseminated intravascular coagulation^{3,4} have been implicated in playing a role. The most striking abnormalities occur late in the anhepatic period and become more marked after reperfusion of the graft^{5,6}. Earlier studies have demonstrated that the severity and duration of hemostasis abnormalities were mainly related to the quality of the donor liver^{7,8}. Release of activated hemostasis factors and/or humoral substances from the graft, that may interfere with hemostasis have been suggested to play a role.

Auxiliary heterotopic liver transplantation (HLT) has been proposed as an alternative to hepatic replacement. In HLT, the host liver is left *in situ* and the graft is transplanted in a heterotopic position. The anhepatic period is avoided and the function of the host liver retained. Hence, substances released from the allograft at reperfusion might be cleared and the deterioration of hemostasis less severe. Recently, we demonstrated that changes in fibrinolysis after reperfusion and more severe and substained in OLT than in HLT after 2 hours of simple cold graft storage⁹. This suggested that the native liver in HLT protects the recipient from the changes induced by preservation damage. We were now interested in whether the host liver in HLT also protects the recipient from the effects of long-term preserved grafts as well as the mechanism of hemostatic deterioration. We have thereby concentrated on fibrinolysis and included, additionally to previous studies, t-PA activity measurement.

Graft protection by addition of prostaglandin E₁ (PGE₁) to the flush solution has been demonstrated in human liver transplantation¹⁰. Furthermore, successful liver transplantations have been reported after long-term simple cold storage with University of Wisconsin (UW) solution without addition of prostaglandins: for 24 hours and more in man¹¹ and for 48 hours in dogs¹². It is not known if cytoprotective agents give additional protection. Therefore we also studied the effects on hemostasis of adding of PGE₁ to the preservation fluid.

METHODS

Sixty-nine female Yorkshire pigs were randomly allocated to OLT (N = 32) or HLT (N = 37). Thirty-one grafts were transplanted after 2 hr of cold storage, 16 after 24 hr, 7 after 48 hr and 15 after 72 hr. In the 2-hr experiments, Eurocollins' solution was used for graft storage because UW solution was not yet available. In the long-term preservation experiments (24-hr, 48-hr, and 72-hr), UW solution was used. Sixteen transplantations in the different preservation groups were performed using PGE₁ (Table 1). Body weights of the donor and recipient animals were similar, about 25 Kg. Histocompatibility was matched by the mixed lymphocyte culture-test¹³.

Surgical Technique

Donor and recipient operations were performed as described earlier¹⁴. In OLT, a venovenous heparin-coated iliacoportajugular bypass was introduced¹⁵.

Table 1 Number of animals in the various preservation groups

| | <i>Experimental Group</i> | | | | | | | | <i>Total</i> |
|----------------------|---------------------------|-----|-------|-----|-------|-----|-------|-----|--------------|
| | 2 hr ^a | | 24 hr | | 48 hr | | 72 hr | | |
| | OLT | HLT | OLT | HLT | OLT | HLT | OLT | HLT | |
| PGE ₁ , + | 0 | 0 | 4 | 4 | 1 | 2 | 1 | 4 | 16 |
| PGE ₁ , - | 16 | 15 | 4 | 4 | 1 | 3 | 5 | 5 | 53 |
| Total | 16 | 15 | 8 | 8 | 2 | 5 | 6 | 9 | 69 |

^aDuration of liver preservation.

Blood loss was estimated from the amount of liquid sucked away from the surgical field and collected in Buleaux bottles during the transplantation. Depending on the amount of blood loss, 500–1000 ml donor blood was given to the recipient. Usually, this was necessary around 30 minutes after graft reperfusion. Anesthetic techniques were the same for all animals.

Prostaglandin

When PGE₁ was given, this was started 60 min before donor hepatectomy by an intravenous infusion rate of 100 ng/kg/min¹⁶. In addition, 1 mg/L PGE₁ was added to the UW solution¹⁰. The recipient animal was treated with a similar infusion as the donor, starting about 60 min before the recirculation of the graft. Before implantation, the graft was rinsed via the portal vein with two liters of cold (4° C) 0.9% saline to which 1 mg/L PGE₁ was added. PGE₁ was supplied by The Upjohn Company, The Netherlands.

Blood Samples

For comparison, the moments of measurement were synchronized, relative to the moment of recirculation. Blood samples were drawn around every important operative step (Table 2) to assess hemoglobin levels, platelet counts, and various hemostasis parameters. Additionally, on reperfusion, the first 30 ml of perfusate were drawn from the infrahepatic vena cava of the graft. These first hepatic outflow samples (FR) after reperfusion were also subjected to hemostasis studies.

In OLT, R-5 is a measurement in the anhepatic phase. In HLT, there is no real anhepatic phase, but during the portal vein anastomosis there is a period of partial clamping of the portal vein. In the pig this often implies (almost) complete clamping.

Table 2 Intraoperative moments of hemostatic measurements

| | |
|--------|---|
| R-60 | Starting value: ± 1 hr before recirculation. |
| R-5 | Anhepatic phase: ± 5 min before recirculation. |
| FR | First hepatic outflow after reperfusion. |
| R + 5 | ± 5 min after recirculation. |
| R + 30 | ± 30 min after recirculation (during the construction of the arterial anastomosis). |
| R + 60 | ± 60 min after recirculation (5 min after the aorta anastomosis). |
| R + 90 | ± 90 min after recirculation (closure). |

Blood samples (20 ml) were taken from an arterial line. Eighteen ml of blood was divided equally into two polystyrene test tubes, containing 1 ml ice-cold trisodium citrate 0.11 mol/l and immediately placed on melting ice. Plasma was collected after centrifugation (2800 g, 4° C, 20 min), snap-frozen and stored in small aliquots at -70° C until used. Two ml blood was also collected into 0.045 ml 15% sol 6.75 mg EDTA.

A normal plasma pool was prepared from equal volume samples obtained from 10 animals immediately after induction of anaesthesia. This pool was defined as having 100% of normal coagulation and fibrinolysis proteins.

Hemostasis Studies

Tissue-type plasminogen activator (t-PA) was assayed according to Verheijen *et al.*¹⁷ using plasminogen, t-PA stimulator and S-2251 (from Kabi Diagnostica, Woerden, The Netherlands) and anti (porcine-heart) t-PA immunoglobulin (from Biopool AB, Umea, Sweden). To determine the euglobulin clot lysis time (ECLT), standard euglobulin fractions of plasma were prepared at pH 5.9 with a plasma dilution of 1:10¹⁸. Precipitates were redissolved in Tris/Tween buffer (0.1 M Tris/HCl, containing 0.1% Tween 80 [v/v] pH 7.5) and to 0.2 ml aliquots of the dissolved euglobulin fractions 0.1 ml portions of calcium-thrombin solution (CaCl₂ 25 mmol/l and thrombin 10 NIH U/l) were added to induce clot formation. The lysis time of the clot was recorded. The disappearance of air bubbles was regarded as the endpoint of lysis. α_2 -Antiplasmin (α_2 -AP) activity was measured according to Friberger *et al.*¹⁹ using Coatest* antiplasmin (Kabi Diagnostica, Woerden, The Netherlands). Fibrinogen was measured according to Clauss²⁰ using thrombin from DiaLab, Leusden, The Netherlands.

Routine clotting times, including the activated partial thromboplastin time (aPTT), reflecting the intrinsic coagulation pathway and the prothrombin time (PT), reflecting the extrinsic pathway, performed according to standard procedures. Reagents used were Actin* (Baxter Dade AG, Düringen, Switzerland) for the aPTT and Thromborel-S* (Behring Diagnostica, Amsterdam, The Netherlands) for the PT.

Statistics

Data were subjected to computerized statistical analysis (PATFILE statistical package). Continuous variables are given as median (mean, \pm standard error of the mean) and analyzed by nonparametric tests: in case of independent samples the Mann-Whitney test was used, and in case of paired samples the Wilcoxon rank-sum test. The coagulation changes were also analyzed in a multiple regression model (SPSS/PC+) using the three main variables in this study: the type of transplantation, the use of PGE₁ and the preservation time. The relative influence of these variables on a coagulation parameter is represented by the T-value (ratio between B and Standard Error of B) together with the significance of T.

RESULTS

Meaningful changes in hemostasis parameters during the operation are elaborated on in the following paragraph. Data are presented separately for each of the three

main variables in this study: the type of transplantation, the effect of graft preservation time, and the effect of PGE₁.

Type of Transplantation

Fibrinolysis parameters: The changes in t-PA activity were reciprocal to those in ECLT. During OLT, t-PA activity levels rose significantly during the anhepatic period ($p < 0.02$), and increased sharply after reperfusion ($p < 0.05$) till the end of the operation. In contrast, during HLT, t-PA activity levels remained low before reperfusion, increased temporarily after reperfusion ($p < 0.01$) and normalized again thereafter. The proportional increase in t-PA activity was similar in HLT and OLT immediately after reperfusion. At all sampling points, except R-60 and R + 5, the t-PA activity in OLT was significantly higher than in HLT (at R-5: $T = -2.20$, $p = 0.03$ and at R + 30 through R + 90: T between -2.79 and -3.12 , $p < 0.01$).

The t-PA activity levels were higher in the first venous outflow of the graft (FR) in HLT (1.22 IU/ml, median value) compared to OLT (0.36 IU/ml, $p < 0.02$), while this difference was not yet present in the portal vein blood entering the graft ($T = 1.06$, $p = 0.32$).

α_2 -AP levels declined significantly from R-60 through R + 5 ($p < 0.001$), and remained almost unchanged thereafter. α_2 -AP levels were lower in OLT than in HLT in the postreperfusion period from R + 30 through R + 90 (T between 2.08 and 3.37, $p < 0.05$).

Coagulation parameters: The PT and aPTT increased slightly in the period before reperfusion and increased significantly thereafter ($p < 0.001$). Before reperfusion and immediately thereafter, there was no significant difference between OLT and HLT for these tests, but in the postreperfusion period, at R + 30 and R + 60, the PT ($T = -3.48$ and $T = -3.55$, $p = 0.001$) and aPTT ($T = -3.83$ and $T = -4.11$, $p < 0.001$) were prolonged in OLT compared to HLT.

Fibrinogen levels practically halved before reperfusion ($p < 0.001$) without reaching a significant difference between both types of transplantation ($p = 0.98$). After reperfusion, fibrinogen levels remained stable and at R + 5 and R + 30 the fibrinogen level in OLT was significantly lower compared to HLT ($T = 2.05$ and $T = 2.09$, $p < 0.05$).

Graft Storage Time

Fibrinolysis parameters: The preservation time had an evident effect on the fibrinolysis parameters, although not yet apparent at R + 5 (for t-PA: $T = 0.45$, $p = 0.66$). Especially at R + 60 and R + 90, the t-PA activity (Figure 1), the ECLT and α_2 -AP deteriorated with increasing preservation times. The cold storage time had some effect on t-PA activity and ECLT before reperfusion, although these did not reach statistical significance in the multiple regression analysis ($T = 0.88$, $P = 0.39$ and $T = -0.86$, $p = 0.39$). The first venous outflow (FR) from the 2-hr and 24-hr preserved grafts contained high levels of t-PA activity. No increased t-PA levels could be demonstrated in the first venous outflow from the 48-hr and 72-hr preserved grafts.

Coagulation parameters: The multiple regression analysis for the effect of the graft storage time revealed a clear influence on the PT and aPTT levels (Figures 2 and 3, respectively). This was evident in the period after reperfusion, starting at R

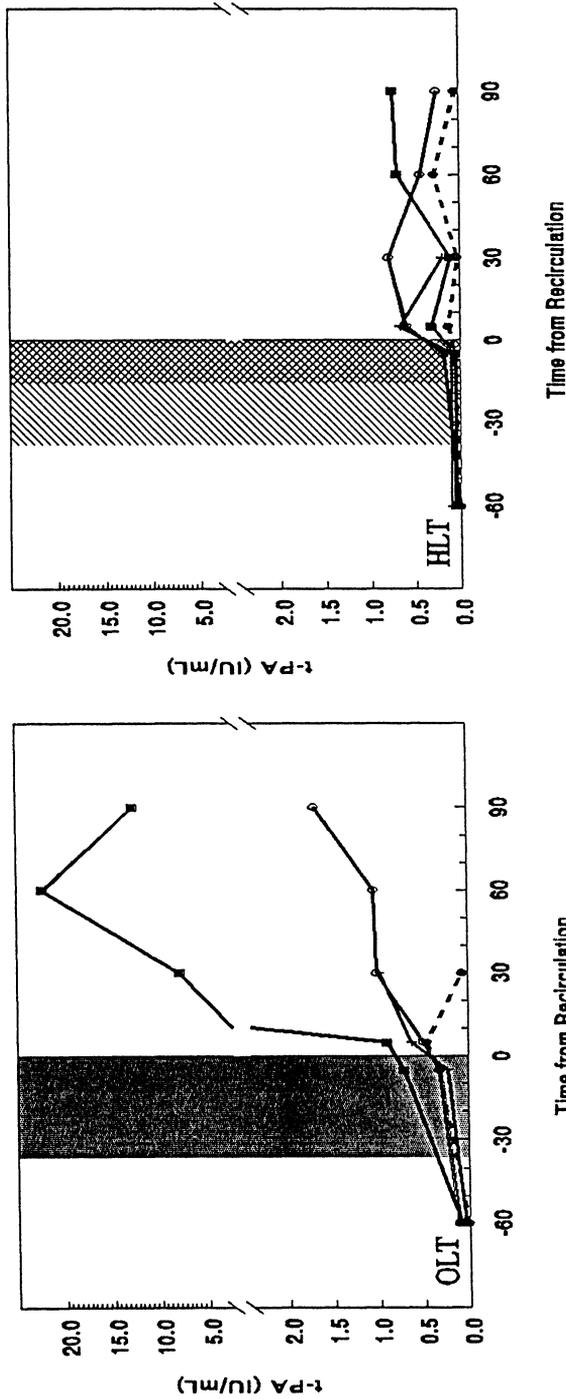


Figure 1 Tissue-type Plasminogen Activator (t-PA) activity levels during liver transplantation. Values are stated as medians. The horizontal axis gives the time in minutes from or to the moment of recirculation of the graft. Symbols: (OLT) Orthotopic Liver Transplantation is illustrated on the left and (HLT) Heterotopic Liver Transplantation on the right. Preservation time: + -- + : 2 Hr; ● -- ● : 24 Hr; ○ -- ○ : 48 Hr (line is interrupted as this group consists of only seven animals); ■ -- ■ : 72 Hr. Shaded areas: ■ is the anhepatic period in OLT; in HLT □ is the period in which the subhepatic inferior vena cava is partially clamped and □ in which also the portal vein is (partially) clamped.

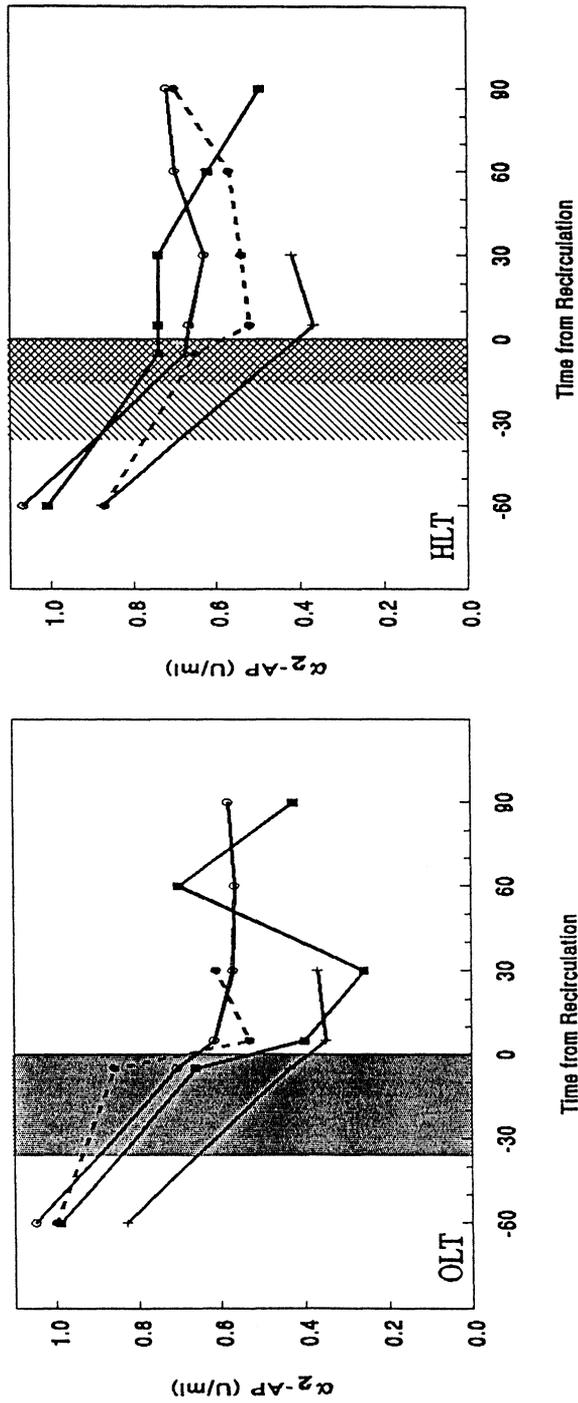


Figure 2 α_2 -Antiplasmin (α_2 -AP) levels during liver transplantation. Horizontal axis and symbols as in Figure 1.

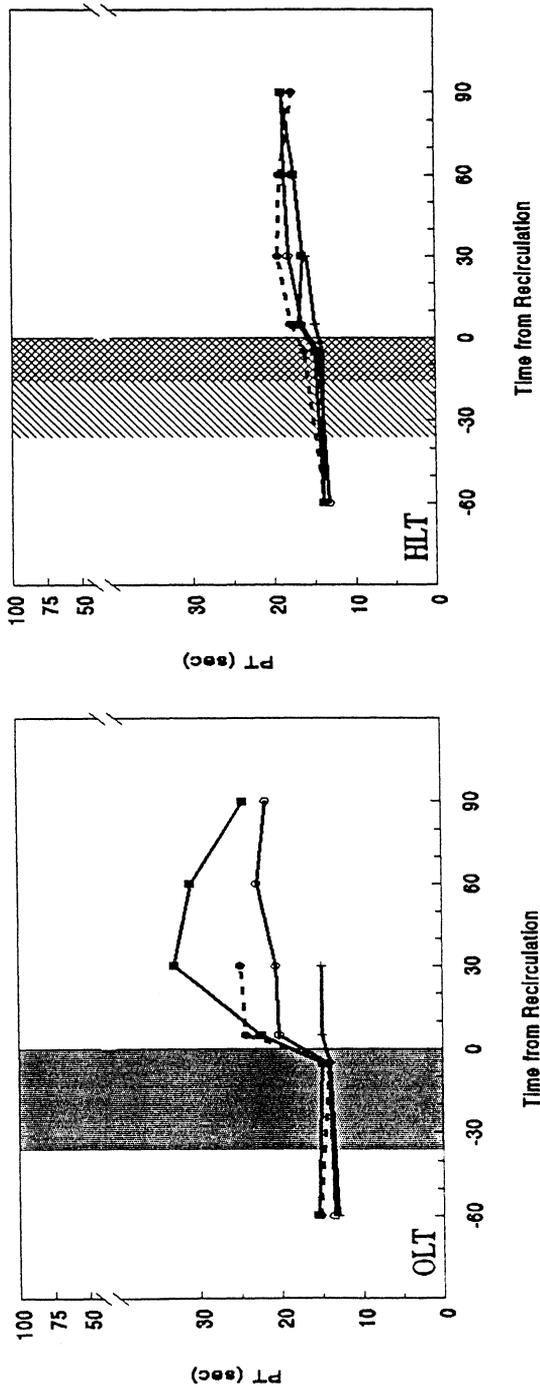


Figure 3 Prothrombin Times (PT) in during liver transplantation. Horizontal axis and symbols as in Figure 1.

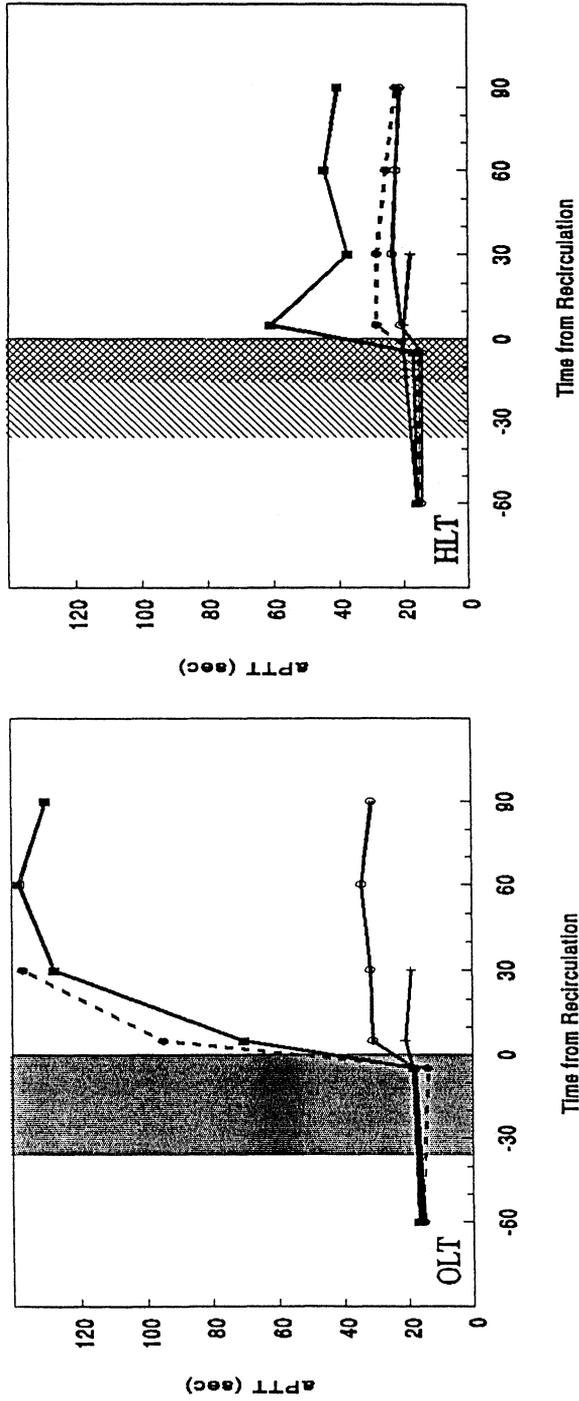


Figure 4 Activated Partial Thromboplastin Times (aPTT) during liver transplantation. Horizontal axis and symbols as in Figure 1.

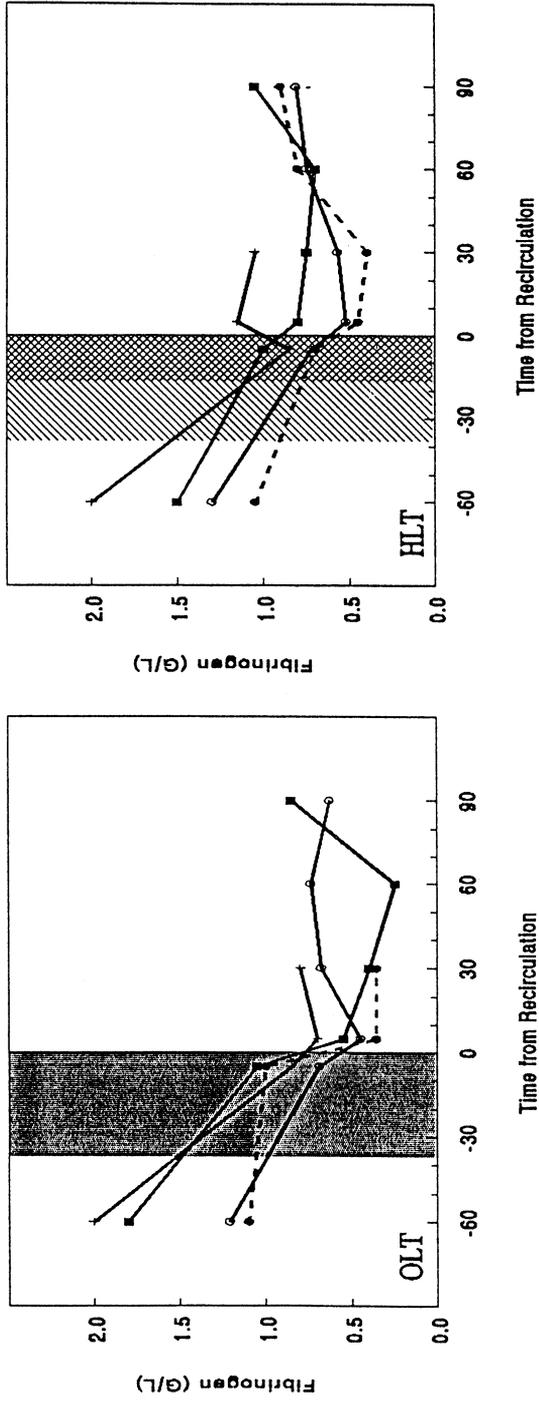


Figure 5 Fibrinogen levels during liver transplantation. Horizontal axis and symbols as in Figure 1.

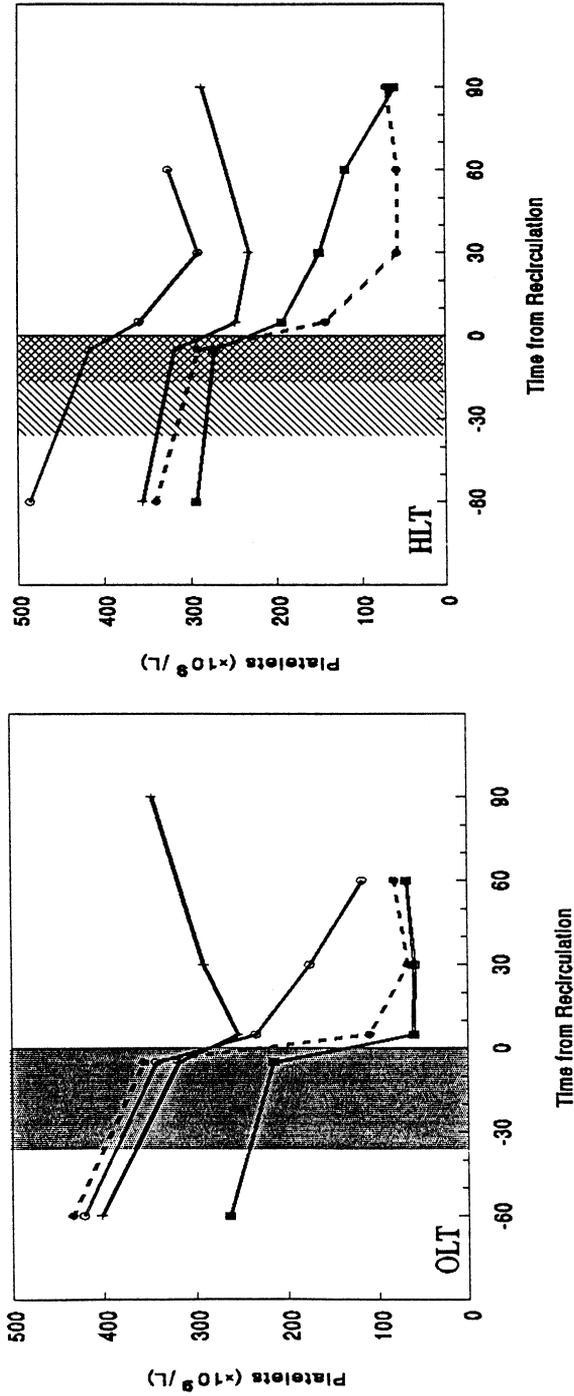


Figure 6 Platelets during liver transplantation. Horizontal axis and symbols as in Figure 1.

+ 5 ($T = 2.38$, $P = 0.02$ and $T = 3.88$, $p < 0.001$) and continuing till R + 30 for PT ($T = 3.17$, $p < 0.01$) and R + 60 for aPTT ($T = 3.93$, $p < 0.001$).

In contrast, fibrinogen levels were not significantly influenced by graft storage time. At R + 5 and R + 30 a longer ischemic period correlated with a decreased fibrinogen level, but the difference was not significant.

The preservation time had an evident effect on the platelet count (all T-values below -3.0 and $p < 0.005$).

Use of PGE

Neither coagulation nor fibrinolysis tests were affected by PGE₁ administration at any of the measured points (data not shown).

Blood Loss and Survival

The amount of blood loss and blood transfusion in the different groups are given in Table 3. In the multiple regression analysis blood loss was significantly controlled by the type of transplantation ($T = -2.33$, $p = 0.02$) and the preservation time ($T = 2.88$, $p < 0.01$). Because none of the animals survived OLT after 72 hr graft storage, many never received a second unit of blood. This explains why it appeared that HLT required more blood transfusion in the 72 hr experiment despite the higher blood loss in OLT. In the multiple regression the duration of preservation was a significant determinant of survival ($T = -3.59$, $p < 0.001$). Survival was not significantly different in the 2 hr preservation experiments. However, in the 24 hr and 48 hr experiments, survival was significantly longer after HLT compared to OLT ($p < 0.05$). Survival in the extended preservation experiments was dismal: In OLT all animals died the day of the operation after 48 hr and 72 hr graft preservation (three of which died within 30 minutes after reperfusion) and in HLT all animals after 72 hr graft preservation. Three animals survived more than 3 weeks after HLT of a 24 hr preserved graft. Two more animals survived after HLT for two days, one in the 24 hr group and one in the 48 hr group. In Table 4 the causes of mortality are categorized.

Table 3 Median blood loss and blood transfusion during the transplantation

| | 2 hr | | 24 hr | | 48 ^c hr | | 72 hr | |
|-----------------------------|------|------------------|-------|------------------|--------------------|------|-------|-------------------|
| | OLT | HLT | OLT | HLT | OLT | HLT | OLT | HLT |
| Blood loss ml | | | | | | | | |
| —median | 400 | 500 ^a | 850 | 425 ^b | 160 | 1125 | 1100 | 925 ^b |
| —lowest | 250 | 250 | 400 | 100 | | 700 | 600 | 200 |
| —highest | 1800 | 1200 | 2200 | 700 | | 1500 | 2000 | 1500 |
| Blood Transfusion ml | | | | | | | | |
| —median | 650 | 600 | 1000 | 600 ^b | 750 | 1100 | 600 | 1000 ^b |
| —lowest | 450 | 400 | 200 | 500 | | 900 | 450 | 375 |
| —highest | 2000 | 1100 | 1200 | 1200 | | 1250 | 1000 | 1500 |

^aThe blood loss in the 2 hr experiment is lower compared to the longer preservation groups ($p = 0.03$).

^bSignificantly different from OLT.

^cOnly one animal represented.

Table 4 Causes of death after liver transplantation

| | 2 hr | | 24 hr | | 48 hr | | 72 hr | | Total |
|--------------------------------|------|-----|-------|-----|-------|-----|-------|-----|-------|
| | OLT | HLT | OLT | HLT | OLT | HLT | OLT | HLT | |
| <i>Total</i> | 16 | 15 | 8 | 8 | 2 | 5 | 6 | 9 | 69 |
| Hemorrhage | 3 | 1 | 4 | 2 | 2 | 5 | 3 | 6 | 26 |
| Portal vein block ^a | | | | | | 3 | 1 | | 4 |
| Heart failure | | | 1 | 1 | | | | 1 | 3 |
| Cholangitis | 1 | | 1 | | | | | | 2 |
| Volvulus | 3 | 2 | | 1 | | | | | 6 |
| Rejection ^b | 2 | | | | | | | | 2 |
| Technical | | 4 | | | | | | 1 | 5 |
| Sacrificed | 3 | 5 | | | | | | | 8 |
| Others ^c | 2 | 2 | | 1 | | | | | 5 |
| Unknown | 2 | 1 | 3 | 2 | | | | | 8 |

^aTotal intrahepatic thrombosis of the portal venous system.

^bOnly if rejection was thought to be the direct cause of death.

^cPulmonary sepsis (n = 3), pneumothorax (n = 1), peritonitis (n = 1).

DISCUSSION

The results from this study clearly show that the host liver in HLT also protects the recipient from the deleterious effects of long-term preserved grafts on hemostasis. We found a dramatic and sustained increase in t-PA activity after reperfusion in OLT, while the increase in t-PA in HLT was limited and transient. In OLT, the deterioration of t-PA activity was directly related to the duration of graft storage. In HLT this effect was much less evident, which demonstrated that the protective effect of the host liver on the development of hyperfibrinolysis also holds true with longer graft storage times. There are several explanations for the increase of t-PA after reperfusion. First, endothelial cells, an important source of t-PA, are more susceptible to cold hypoxia and reperfusion than parenchymal cells^{21,22}. Free oxygen radicals have been implicated in this mechanism of graft injury^{23,24}. The damaged endothelium may release t-PA directly into the circulation. Another theory is that the tissue damage leads to the attraction of inflammatory cells, including macrophages^{25,26}. When activated, these cells are stimulated to produce cytokines (interleukin-1, tumor necrosis factor), which may actively interfere with coagulation and fibrinolysis²⁷. Recently, it has been shown that pretreatment with cyclosporine, a powerful inhibitor of the function of macrophages, ameliorated the hemostatic changes after reperfusion in pigs, as measured by prothrombin times, platelet counts, and fibrinogen levels²⁸. The initial increase in t-PA activity in HLT may be explained by a too abundant release of t-PA to be instantaneously cleared by the host liver. Comparison of t-PA activity levels in the portal vein inflow and the venous outflow revealed a gradient across the graft, which indicated that t-PA is also actively released by the graft. It was found that t-PA levels in the first hepatic outflow of the graft were higher in HLT than in OLT. A possible explanation is that the partial resection of the liver graft in HLT is responsible for this. This extra handling could make endothelial cells more liable to release t-PA²⁹. It was notable that the high t-PA activity levels were only found in the first hepatic outflow from

the 2 hr and 24 hr cold storage grafts and not in the 48 hr and 72 hr groups. Possibly, the endothelial cells in the latter group are damaged to such an extent that they no longer contain t-PA. Their contents, including t-PA, may have leaked into the preservation fluid, which is subsequently washed out during flushing before reperfusion. Despite this t-PA depletion, subsequent contact activation of the blood with the injured graft endothelium may evoke systemic t-PA release as suggested above. Probably, the same process occurred in HLT, but now the cytokines may be cleared by the host liver.

Fibrinogen levels, in turn, showed a serious decline in the *prereperfusion* period in both OLT and HLT. There was no difference between OLT and HLT, which suggests that the changes are related to the surgical procedure in general and not to the anhepatic period. Previous investigators have suggested that fibrinogenolysis during the use of an external bypass might also contribute to the decrease in fibrinogen levels^{15,30}. The latter is however likely to be of minor influence, as in HLT no signs of hyperfibrinolysis were present nor was a shunt used. Although part of the decline can be ascribed to hemodilution, there has to be another explanation as the hematocrit, a measure of hemodilution, did not equally decrease. Most likely a surgical induced activation of the coagulation system and extravasation of hemostasis proteins to the extravascular space are involved²⁹.

Similar to the fibrinolytic parameters, reperfusion induced a serious deterioration in coagulation parameters in OLT, whereas the changes in HLT remained limited. There was an apparent influence of the graft storage time. Presumably release of procoagulant factors from the preserved graft or local clotting activation on the damaged endothelium had initiated a consumption coagulopathy, as has been previously described. The present observations, however, show that HLT has also a protective effect on coagulation changes.

Remarkable effects of PGE₁ were found in preventing warm ischemic and reperfusion injury³¹ in both experimental³² and clinical¹⁰ studies. We therefore implemented a trial of PGE₁ in our experiment with long-term preservation. As determined by fibrinolytic and coagulation tests, our study provides no evidence of a protection against hemostatic disturbances. As opposed to the multitude of positive studies on PGE₁, it is difficult to explain why we found no protective effect at all. Besides having included not enough animals to detect small differences, there is probably only a narrow margin of therapeutic efficacy, if it exists, between trivial and irreversible damage. Apparently, the upper limit is surpassed with 24 hr preservation of the porcine liver. In addition, prostaglandins can be assumed to counteract a physiological protective mechanism that secludes marginally functioning areas in the graft. This may well prove useful in retrieving reversibly injured parenchyma³³ but in contrast, it could promote the entrance into the bloodstream of deleterious substances originating from injured cells.

In conclusion, it was demonstrated that in pigs changes in hemostasis after long-term graft storage are less dramatic in HLT compared to OLT. This suggests that HLT also protects the recipient from the effects of long-term preserved grafts. Addition of PGE₁ to the preservation fluid had no effect on the hemostatic changes in both OLT and HLT. Although these experiments are performed in the presence of a healthy host liver (although deprived from portal blood after the transplantation), while in the clinical situation the host liver is diseased, some remaining liver function is always present. The results are indicative of the beneficial role of the (remnant function of the) host liver on hemostasis.

Acknowledgements

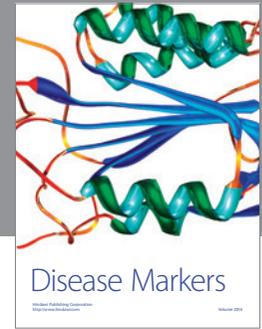
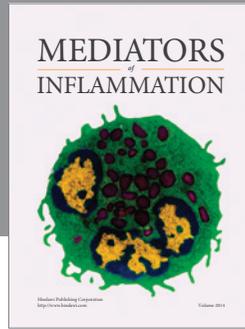
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