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Hyaluronic acid uptake in the assessment of sinusoidal endothelial cell damage after cold storage and normothermic reperfusion of rat livers

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Abstract The uptake of hyaluronic acid (HA) was used to assess preservation damage to sinusoidal endothelial cells (SEC) during cold storage and subsequent normothermic reperfusion of rat livers. After 8, 16, 24, and 48 h storage in University of Wisconsin (UW) solution, livers were gravity-flushed via the portal vein with a standard volume of cold UW solution containing 50 μg/l HA. The effluent was collected for analysis of HA, aspartate aminotransferase (AST), and lactate dehydrogenase (LDH). The mean uptake of HA at 0 h was 59.1 % ± 4.6 % (mean ± SEM). After 8 h of storage, HA uptake was similar (55.5 % ± 7.3 %), whereas after 16 h of storage it was reduced to 34.7 % ± 5.8 %. At 24 and 48 h of storage, no uptake of HA was found. In a second series of experiments, livers were stored in UW solution and subsequently reperfused for 90 min with a Krebs-Henseleit solution (37 °C) in a recirculating system containing 150 μg/l HA. Following 8 h of storage, 34.6 % ± 8.0 % of the initial HA concentration was taken up from the perfusate. After 16 and 24 h of storage, no uptake of HA was found. The results of this study indicate that damage to SEC occurs progressively during storage, leading to zero uptake of HA by the rat livers at 24 h of cold ischemia time. Additional reperfusion injury to the SEC was demonstrated by the reduced ability of the SEC to take up HA following normothermic reperfusion. The uptake of exogenous HA in preserved livers, used as a tool to assess SEC injury, enables the detection of early preservation damage.

Key words Liver preservation, hyaluronic acid · Hyaluronic acid, liver preservation · Sinusoidal endothelial cells, liver preservation

Introduction

Preservation injury in liver grafts is the result of events taking place in the liver during cold storage and subsequent normothermic reperfusion. Several studies have shown that the endothelial cells lining the liver sinusoids are an important site at which damage in cold-preserved livers occurs [3, 18, 21]. Damage to these sinusoidal endothelial cells (SEC) gives rise to microcirculatory disturbances and adherence of leukocytes and platelets, ultimately leading to microvascular perfusion failure and decreased graft function [6, 20]. Parameters that indicate damage to SEC may, therefore, be helpful in assessing preservation injury in liver grafts and in estimating the resulting viability after transplantation.

Purine nucleoside phosphorylase (PNP), an enzyme primarily localized in the SEC, has been proposed as a marker of SEC injury [14, 19, 24, 26]. In a previous study, we showed a gradual release of PNP into the vascular system of the liver with progressive cold ischemia

time (CIT). There was a simultaneous release of parameters of liver parenchymal cell damage such as aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) [27].

This study deals with the ability of SEC to take up exogenous hyaluronic acid (HA) conferred through the vascular system of the liver during cold storage and subsequent reperfusion. HA is a high molecular weight glycosaminoglycan produced by connective tissue and synovial membranes throughout the body. It enters the circulation from the lymphatic system and is specifically taken up and degraded by endothelial cells of the liver sinusoids [7, 9, 11, 12, 28]. Several studies have reported clinical significance of elevated serum HA concentrations in liver disease [5, 8] and in monitoring function of a liver graft following transplantation [2, 23, 25]. We investigated the use of the uptake of exogenous HA as a parameter reflecting function of SEC during cold storage and subsequent reperfusion in preserved rat livers.

In this study, we used two different models. In a first series of experiments, rat livers were cold-flushed with University of Wisconsin (UW) solution and stored for up to 48 h at 4 °C. Uptake of HA by the SEC was assessed by measuring the HA in the effluent after repeated cold flushes of the liver with a standard volume of UW solution containing HA. In a second series of experiments, the livers were stored for 8, 16, and 24 h at 4 °C in UW solution and subsequently reperfused with a recirculating Krebs-Henseleit solution containing HA under normothermic and oxygenated conditions. SEC function was assessed by determining the clearance of HA from the perfusate. In addition, AST and LDH were measured in the perfusate as parameters of parenchymal cell injury, and cell death was assessed using the trypan blue exclusion test [3]. Tissue damage in the livers was further evaluated via electron microscopy and enzyme histochemical methods for the demonstration of 5'-nucleotidase (5'-NT) and LDH [13]. The activity and localization of 5'-NT has been shown to be a sensitive marker for cell injury in rat livers [15, 16].

Materials and methods

Animals and preparation of livers

Inbred female Wistar rats, weighing 250–280 g, were anesthesized with pentobarbital sodium (50 mg/kg) intraperitoneally. Before cannulating the portal vein, the rats were heparinized (10 units/100 g) via a tail vein. The livers were flushed in situ via the portal vein with 40–50 ml cold (2 °–4 °C) UW solution (DuPont Pharmaceuticals, The Netherlands). Before excision of the liver, the suprahepatic caval vein was cannulated with a plastic catheter (8 Fr; Baxter, Trieste, Italy) in order to collect the effluent. The cut end of the infrahepatic caval vein was ligated.

Hyaluronic acid (HA) uptake during cold storage

After in situ wash-out and excision, the livers were immediately gravity-flushed (0 h) with a standard volume of cold UW/HA solution (4 °C, 15 cm H_2O) via an 8 Fr plastic catheter fixed into the portal vein. The standard flush volume of the UW/HA solution consisted of 7 ml UW solution and 50 μg/ml HA (range 47–55 μg/ml). HA was obtained from rooster comb (M 1.3 10E6, Sigma, St. Louis, Mo., USA). The effluent was collected and analyzed for HA content, AST, and LDH. HA was measured using a radioactivity binding assay (detection limit ~ 5 μg; Pharmacia Diagnostics, Uppsala, Sweden). The test is based on the use of specific HA binding proteins (HAPB) isolated from bovine cartilage. HA reacts with ^{125}I-labelled HAPB in solution. The unbound ^{125}I – HAPB is then quantitated by incubation with HA covalently coupled to sepharose particles of small size and low density. Separation is performed by centrifugation followed by decanting. The radioactivity bound to the particles is measured in a gamma counter and the response is inversely proportional to the concentration in the sample. The total time needed is about 2 h. The uptake of HA by the liver was expressed as the percentage of the initial HA concentration remaining in the effluent.

Gravity flushes were repeated at 8, 24, and 48 h of cold storage using the same standard flush volume containing HA. In an additional series of experiments ($n = 3$), rat livers were stored for up to 16 h and gravity-flushed.

In control experiments, standard flushes of livers were performed without adding HA to the flush solution at 24 and 48 h of cold storage. The effluent was collected and examined for passive release of HA.

Since heparin is a known competitive ligand for the HA receptor on SEC, and since the rats were heparinized prior to excison of the liver, the heparin content in the effluent was examined. Heparin was measured with the anti-Xa test (Coatest, KabiVitrum, Amsterdam, The Netherlands) [4].

In additional experiments ($n = 4$), livers were gravity-flushed at 0 and 24 h, without intermittent flushes at 8 or 16 h, to evaluate a possible effect of saturation for uptake of HA by SEC.

HA uptake during reperfusion following cold storage

Following in situ wash-out and hepatectomy, livers were stored for 8 ($n = 6$), 16 ($n = 3$) or 24 h ($n = 3$) in UW solution. Subsequently, the livers were placed in a perfusion cabinet [22] at 37 °C and reperfused with 200 ml recirculating Krebs-Henseleit solution (144 mM Na^+, 6.0 mM K^+, 127.4 mM $H_2PO_4^-$, 1.2 mM $MgSO_4$, 24.0 mM HCO_3^-, 1.3 mM $CaCl_2$), pH 7.4, containing 0.5 % bovine serum albumin (Boseral, Organon Teknika, Boxtel, The Netherlands). HA was added to the perfusate, resulting in an average HA concentration of 150 μg/l (120–163 μg/l). The mean perfusate flow was 20 ml/min. The perfusion pressure did not exceed 15 cm H_2O. The perfusate was saturated with O_2:CO_2 (95 %:5 %). The first 10 ml of the effluent containing the UW solution remaining in the vascular system was drained before the liver was perfused via recirculation. Perfusate samples of 3 ml were collected at 0, 15, 30, 60, and 90 min of reperfusion, for determination of HA, AST, and LDH. Uptake of HA by the liver was expressed as the decrease in percentage of the initial HA concentration in the perfusate.

Histology and enzyme histochemistry

At the end of each experiment, the livers were perfused with 50 ml UW or Krebs-Henseleit solution (50 ml) containing 200 μM trypan blue (Serva, Heidelberg, Germany). Histological examination was performed in biopsies obtained from the left lateral lobe of livers at 48 h ($n = 6$) of cold storage and after 90 min of reperfusion following 8 ($n = 3$), 16 ($n = 3$), and 24 h ($n = 3$) CIT. For enzyme histochemistry, biopsies of 5 mm^3 were taken, chilled in liquid nitrogen, and stored at −70 °C. The morphological appearance was studied by examining architectural pattern, size, and shape of hepatic cells and liver sinusoids. 5'-Nucleotidase activity was detected in unfixed cryostat sections of the tissue samples using the lead salt method [15]. The activity of LDH was localized by incubating sections according to the tetrazolium salt procedure, as described previously [13]. At the end of separate experiments, livers were infused with McDowell fixative (4 % formaldehyde/1 % glutaraldehyde in 100 mM cacodylate buffer, pH 7.4) via the portal vein (perfusion fixation technique). Then the livers were minced into small pieces and processed for electron microscopy by rinsing in the same buffer, postfixating in 1 % OsO_4, dehydrating, and embedding in epoxy resin. Ultrathin sections (30–70 nm thick) were contrasted with lead citrate and uranyl citrate and studied with a Zeiss EM −10c transmission electron microscope.

Statistical analysis

Results are expressed as mean values ± SEM. The data were analyzed with Student's t-test; P values below 0.05 were considered statistically significant.

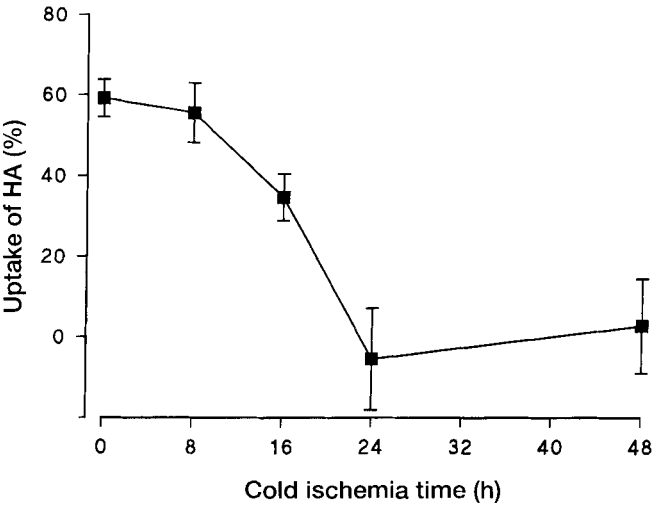


Fig. 1 Ability of SEC to take up HA from the influent in relation to cold ischemia time. Rat livers ($n = 6$) were flushed with 7 ml UW solution (4 °C) containing HA (50 μg/l). Uptake of HA was calculated as the difference in HA concentration between influent and effluent, and expressed as the percentage of the HA concentration in the influent

Results

At the end of the initial wash-out using 40–50 ml UW solution, the effluent was completely clear, indicating that the liver was free of blood. Heparin, a known competitive ligand for the HA receptor on endothelial cells, could not be demonstrated in the effluent (results not shown).

In control experiments ($n = 3$), in which no HA was added to the standard flush volume of UW solution, the concentration of HA in the effluent of livers stored for 24 h was below the detection level (~ 5 μg/l) of the radiometric assay for HA. After 48 h of storage, the HA concentration in the effluent had increased to 7.8 μg/l (range 5.0–10.0 μg/l).

HA uptake during cold storage

At 0 h (Fig. 1), the mean HA concentration in the effluent was 40.9 % ± 4.6 %, indicating that 59.1 % of the HA contained in the standard flush volume was taken up by the liver. After 8 h of storage, the mean percentage of HA uptake was 55.5 % ± 7.3 %, which is statistically not significant from the uptake at 0 h, indicating an unaltered capacity of the SEC to take up HA. After 24 h of cold storage, however, no uptake of HA could be demonstrated [the concentration of HA in the effluent was 105.7 % ± 12.6 %, which is significantly higher ($P = 0.0018$) than the concentration at 8 h of storage]. In fact, a higher concentration of HA was found in the effluent than was present in the standard flush volume, presumably due to passive release of HA from injured SEC.

Because the SEC apparently lost their ability to take up HA between 8 and 24 h of storage, additional experiments were performed with livers that were stored for 16 h. At 16 h ($n = 3$), the remaining concentration of HA was 65.3 % ± 5.8 %, which means an uptake of 34.7 %, suggesting a gradual loss of SEC function between 8 and 24 h of storage (Fig. 1).

When livers were exclusively flushed at 0 h and 24 h of CIT (without intermittent flushing of the liver), no uptake of HA was found in livers stored for 24 h, indicating that the inability of SEC to take up HA was not the result of saturation of SEC with HA.

Figure 2 shows a gradual increase in AST and LDH activity in the effluent, indicating progressive parenchymal cell damage. After 48 h of storage, the amount of AST (in units) in the effluent showed an 11-fold increase (from 0.07 ± 0.002 U at 0 h, to 0.77 ± 0.2 U after 48 h of CIT). The amount of LDH (in units) concomitantly showed a sevenfold increase (from 0.50 ± 0.1 U at 0 h, to 4.1 ± 0.4 U at 48 h of CIT).

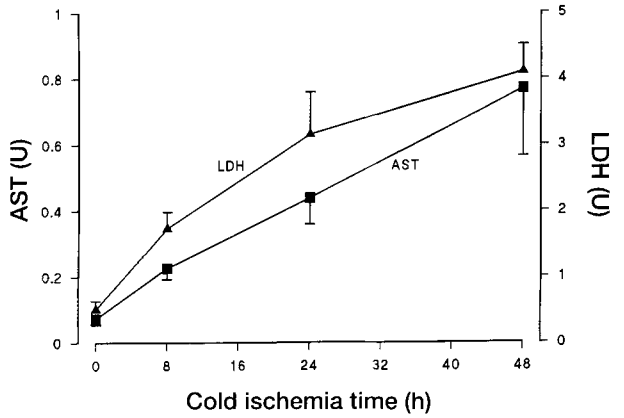


Fig. 2 The amount (units) of parenchymal enzymes, AST and LDH, released into the liver effluent. Rat livers ($n = 6$) were stored for up to 48 h at 4 °C and flushed at intervals with cold UW solution (7 ml). Enzyme levels (mean ± SEM, $n = 6$) increased gradually up to 48 h of cold ischemia time

HA uptake during reperfusion following cold storage

Figure 3a shows the decrease in HA concentration in the effluent during 90 min of reperfusion of livers using normothermic Krebs-Henseleit solution containing HA (150 µg/l). After 8 h of storage ($n = 6$), 34.6 % ± 8.0 % of the initial concentration was taken up by the liver at the end of reperfusion. After 16 h ($n = 3$) and 24 h ($n = 3$) of storage, no uptake of HA could be demonstrated during the 90-min reperfusion period.

Figure 3b shows the release of AST (in units) in the perfusate. During 90-min reperfusion following 8 h of storage, AST in the perfusate increased from 0 U to 2.56 ± 0.3 U. Following 16 h and 24 h of storage, AST increased from 0.26 U to 9.20 ± 0.8 U and from 0.14 U to 5.94 ± 2.5 U, respectively. These values are significantly higher than the amounts measured immediately after cold storage. In none of the three groups, i.e., after 8, 16, and 24 h of storage, did the concentration of LDH in the perfusate increase significantly during reperfusion (results not shown).

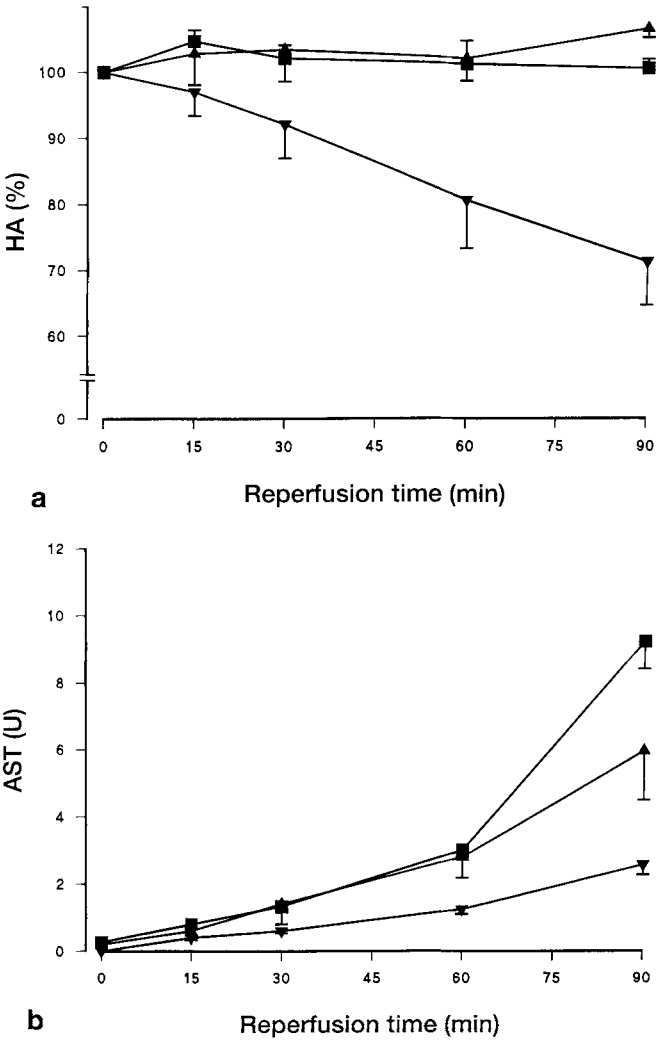


Fig. 3 **a** Uptake of HA by rat livers following 8 (▼-▼), 16 (■-■), and 24 (▲-▲) h of cold storage. The livers were reperfused for 90 min at 37 °C with 200 ml Krebs-Henseleit solution containing 150 µg/l of HA. HA uptake was expressed as the percentage of the initial concentration of HA in the perfusate at 0 h. **b** Release of AST (in units) during 90 min of reperfusion at 37 °C with 200 ml Krebs-Henseleit solution following 8 (▼-▼), 16 (■-■), and 24 (▲-▲) h of cold storage

Morphology and enzyme histochemistry

Table 1 summarizes the morphological and enzyme histochemical findings of rat livers during cold storage and subsequent reperfusion as assessed semiquantitatively. Histological examination revealed a normal architecture of the liver lobule and hepatocytes with normal size and shape for up to 16 h of storage. After 24 h of storage, liver tissue was swollen and hepatocytes appeared circular. After 48 h of storage, liver parenchyma were severely damaged.

After 8 h of storage, the localization of 5'-NT at bile canalicular and sinusoidal plasma membranes had already changed into a diffuse form and the activity in periportal areas was decreased (Fig. 4a). Hardly any 5'-NT activity could be detected in livers after 24 h of storage. After 90 min of reperfusion following all periods of storage investigated, 5'-NT activity in livers was completely lost. In contrast, localization of LDH activity remained essentially unaltered in livers stored for up to 16 h. Only slight changes were observed after 24 h of storage (Fig. 4b). During reperfusion, remarkable decreases in enzyme activity were observed in pericentral

Table 1 Semiquantitative scoring of histological and enzyme histochemical features of rat liver tissue during cold ischemia and after 90 min of subsequent reperfusion (+++ high, ++ moderate, + low, 0 absent)

	Storage time				After 90 min of reperfusion		
	8 h	16 h	24 h	48 h	8 h	16 h	24 h
Morphological evidence of tissue damage	0	+	++	+++	+++	+++	+++
5′-NT activity	++	+	0	0	0	0	0
LDH-activity	+++	+++	++	+	+	+	+
Trypan blue uptake	0	0	0	+	++	+++	+++

areas after periods of storage longer than 16 h (Fig. 4c). No trypan blue-positive nuclei were found in any biopsies up to 24 h of storage. At 48 h of storage, some trypan blue-positive nuclei in SEC and hepatocytes were observed. However, after reperfusion following 8 h of storage, trypan blue-positive nuclei in SEC and hepatocytes were observed, and this phenomenon became even more prominent after 24 and 48 h of storage and subsequent reperfusion.

Figure 5 shows an electron micrograph of a rat liver sinusoid and surrounding liver parenchymal cells after 24 h of cold storage. Endothelial cells were generally present, but the endothelial lining was frequently interrupted and blebs were observed in the sinusoidal lumen. Intracellularly, organelles seemed to be intact. In liver parenchymal cells, no signs of severe damage, such as presence of flocculent densities in mitochondria, were found.

Discussion

Rat livers flushed with Euro-Collins solution and stored for more than 8 h are not viable when transplanted, although no marked hepatic cell damage is evident morphologically [21]. This observation led to the notion that damage to other liver cells is responsible for the loss of graft viability during preservation. Ultrastructural examinations have shown that SEC are the predominant site at which critical preservation injury in livers occurs [3, 21]. SEC have been suggested to be the specific target for cold ischemic injury, whereas during warm ischemia the damage is mainly located in hepatocytes [17]. SEC may be damaged by the combination of ischemic injury during storage and the injury sustained during reperfusion. The efficacy of UW solution for long-term preservation of liver grafts is based on suppression of hypothermia-induced cell swelling and protection of the microvasculature of the liver [20, 30]. In view of the wide-spread application of UW solution in clinical liver transplantation, this solution was used in the present study to assess SEC injury in preserved livers.

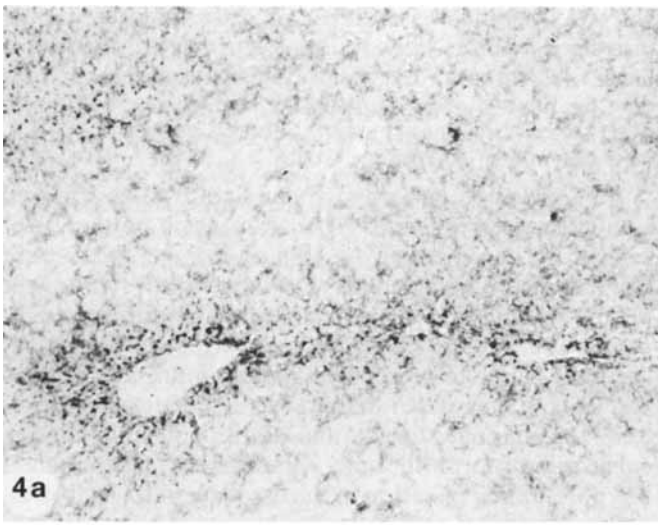
A unique feature of HA is that it is specifically taken up and metabolized by SEC in the liver. Therefore, the ability of the liver to take up HA reflects the functioning of SEC, and this characteristic was used in our study to assess preservation injury in livers during cold storage and subsequent reperfusion. The uptake of HA is receptor-mediated with high affinity ($K_d = 6 \times 10^{-11}$) combined with fluid-phase endocytosis [29]. The clearance capacity is estimated to be about 10–20 mg/day per kg, with a high reserve capacity (up to 30 mg/day per kg [10]. Studies with radioactive-labeled HA have shown that after binding to SEC, it is rapidly endocytosed and degraded into lysosomes to D-glucuronic acid and N-acetylglucosamine [28, 29].

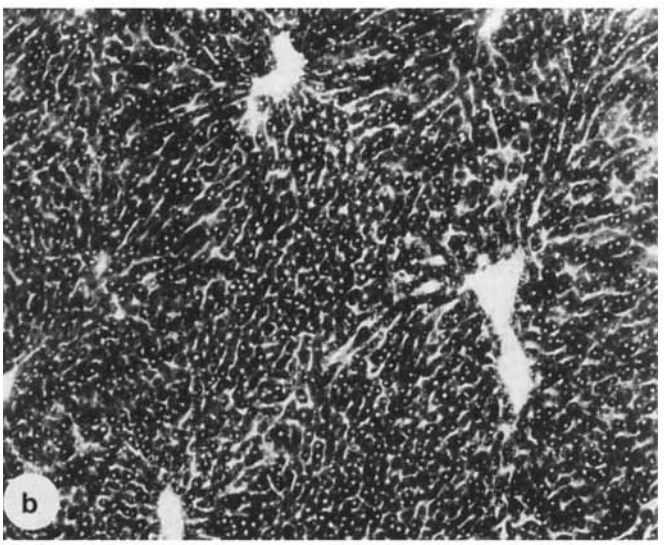
Based on these data, we added HA to the preservation solution in a concentration of 50 μg/l, which is approximately 1.5 times the physiological concentration in serum. In the reperfusion experiments, HA was added to the recirculating medium in a concentration of 150 μg/l. A possible saturation effect, as a result of repeated flushes, was not responsible for the lack of uptake of HA after 24 h of storage because no uptake of HA was found after 24 h of storage when the preceding standard flushes were omitted.

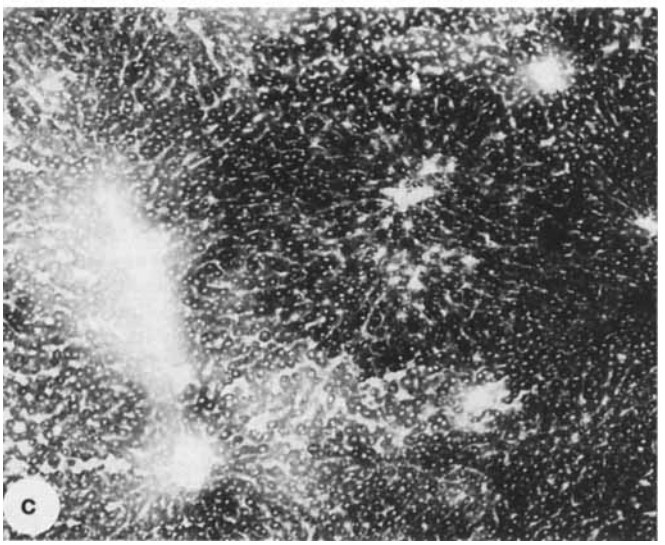
When flushing livers with a preservation solution and measuring HA in the effluent, different processes contributing to the amount of HA in the effluent should be taken into account, namely, passive leakage of HA out of SEC as a result of cell damage and production of HA by hepatic fibroblasts or Ito cells. The latter is of no relevance for the present experiments since the suggested production process only starts after a lag phase of several days [1]. The passive release of HA into the effluent was considered to be insignificant because the levels of endogenous HA in the effluent after 48 h of storage were just above the detection level of the radiometric HA assay used.

In the cold storage model, the results demonstrate that after 8 h of ischemia, the function of SEC was unaffected; i.e., uptake of HA was similar to that at 0 h. After 24 h and 48 h of storage, no uptake of HA could be detected, suggesting abolished SEC function. In an additional series of experiments, it appeared that after 16 h of storage, the ability of the livers to take up HA was still present, although decreased, indicating that ischemic injury to the SEC is a gradual process. Parenchymal cell damage during storage was marked by a continuous increase in hepatocellular enzymes (AST and LDH) released into the effluent with increasing periods of storage.

In the reperfusion model in which livers were recirculated with normothermic Krebs-Henseleit solution for 90 min, uptake of HA was only detectable when the







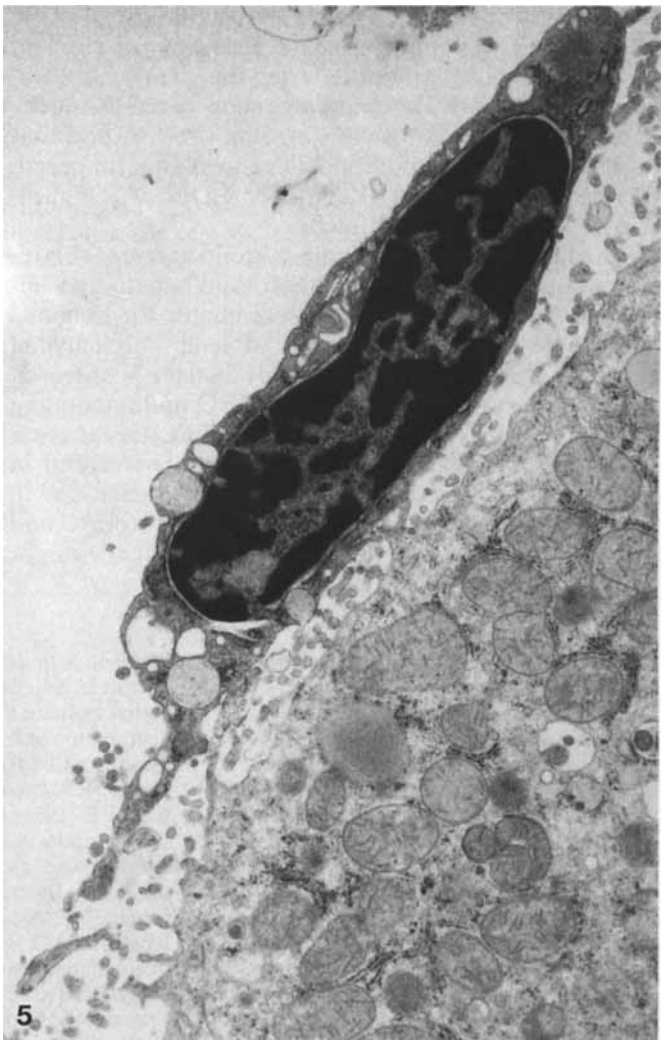


Fig. 4 **a** Localization of 5'-nucleotidase activity in rat liver after 8 h of cold ischemia time (CIT). Activity was diffusely localized and had disappeared in periportal areas. **b** Localization of LDH activity in rat liver after 24 h of CIT. Rather normal activity was found in both periportal and pericentral areas. **c** Localization of LDH activity in rat liver after 90 min of reperfusion and after 16 h of CIT. Enzyme activity was decreased in pericentral areas

Fig. 5 Electron micrograph of a sinusoid in the rat liver after 24 h of cold storage without reperfusion. Frequent interruptions of the endothelial lining were seen. Hepatocytes did not appear to be damaged and cell organelles such as mitochondria and endoplasmic reticulum remained intact ($\times$ 9,000)

preceding storage time was not longer than 8 h. Hence, whereas SEC function persisted after 16 h of storage, no uptake of HA was found during the 90 min of subsequent reperfusion. These findings show that SEC injury during storage occurs as an ongoing process, and that during reperfusion, additional damage is superimposed. During reperfusion, AST, but not LDH, was significantly enhanced in the effluent.

The light microscopic findings are in agreement with the parameters for damage to SEC and hepatocytes. 5'-NT can be used as a sensitive parameter for ischemic damage of plasma membranes of liver parenchymal cells. The storage time at which this damage is observed is similar to the period at which SEC malfunctioning starts (i.e., after 8 h of storage). Therefore, this enzyme histochemical parameter may also be a useful tool in predicting the function of the SEC. The decrease in LDH activity in hepatocytes after 24 h of storage and the sustained decrease during reperfusion after storage correlate with the appearance of enzymes in the perfusate. Electron microscopic findings have shown that after 24 h of storage, the endothelial lining was frequently interrupted, suggesting a relation between SEC damage and decreased HA uptake.

In conclusion, progressive damage to SEC occurred in livers stored for up to 24 h, after which no uptake of HA could be detected. At 16 h of storage, livers were capable of taking up HA; however, after subsequent normothermic reperfusion, zero uptake was found, indicating additional SEC injury due to reperfusion. Measuring the uptake of exogenous HA in preserved livers provides a useful parameter for assessing damage to SEC during cold storage and additional injury during subsequent normothermic reperfusion. This method has potential application in the assessment of early preservation injury.

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