

A histopathological study of anoxic-resuscitated liver allografts

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Summary. The possibility of resuscitating livers after warm ischaemia has been recently suggested. The aim of the present investigation was to analyse the effects of anoxia on the morphology of hepatic cells, to determine whether these effects are reversible after providing a resuscitation period between warm ischaemia (WI) and cooling, and to study the behaviour of the resuscitated liver in the recipient organism. Ten female, Large-White pigs acted as donors for 10 recipient animals of the same kind who received an orthotopic liver graft. Recipients were divided into two groups depending on whether the livers they received had undergone a resuscitation period (Group I (n=5) where animal livers were subjected to 5 min warm ischaemia (WI) without resuscitation, and Group II (n=5) where the livers were subjected to 5 min WI followed by 5 min resuscitation). Morphological and ultrastructural studies of liver cells were performed using light and electron microscopy. ATP, ADP and AMP levels were determined in liver biopsies by high performance liquid chromatography (HPLC). Plasma AST and bilirubin levels in the two groups were compared 24 h after transplantation. After 5 min of anoxia, hepatocytes showed two morphological patterns in response to WI. Some were appreciably condensed with dark mitochondria, peroxisomes and some cytoplasmic vacuoles. Others showed electronlucent organelles, inflamed mitochondria with broken cristae and disorganized endoplasmic reticulum. Hepatocytes showed globular microvilli and bleb formation with migration towards the sinusoids. One hour after the revascularisation of the resuscitated livers, the hepatocytes showed nearly normal morphological characteristics. However, the hepatocytes of non-resuscitated organs continued to show alterations. Kupffer cells were activated in the livers of both experimental groups. Ultrastructural changes and total tissue adenine nucleotide (TAN) levels recovered

completely in resuscitated livers soon after transplant. These results suggest that when short WI periods are followed by equivalent periods of resuscitation, the hepatocytes of transplanted livers recover from the effects of anoxia.

Key words: Liver transplant, Resuscitation, Anoxia, Warm ischaemia, Cardiac arrest

Introduction

New surgical techniques and the use of immunosuppressant drugs (Kim et al., 1991a,b, 1994), and in particular the development of solutions for the preservation of donated organs (Belzer and Shouhard, 1988; Kalayoglu et al., 1989; Southard and Belzer, 1993), have resulted in an increase in the number of liver transplants performed. However, the lack of donors and the conditions under which organs are donated continue to limit this figure. Currently only brain-dead patients, i.e., cadavers with beating hearts, may be considered for organ donation. This situation is further complicated in the fatty livers (from obese or alcoholic donors) show a high incidence of primary dysfunction following implantation (Todo et al., 1989).

Recently, Schön et al. (1993) have suggested the possibility of resuscitating livers after warm ischaemia (WI), so that organs from persons who have suffered cardiac arrest may be transplanted successfully. Damage to the tissues caused by anoxia is sometimes irreversible and may be the cause of functional failure of the transplanted organ. The noxious effects of WI in the liver are irreversible if the liver is immediately cooled and transplanted. The resistance of hepatocytes and sinusoidal cell walls to ischemia determines whether reversibility of damage is possible (Myagkaya et al., 1984; Holloway et al., 1989). Microcirculatory cells are more susceptible to damage by hypoxia than are parenchymal cells. Sinusoidal cell injury occurs prior to hepatocyte damage (Vasilescu and Tasca, 1991).

The purpose of this investigation was to study the

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ultrastructural alterations caused by anoxia that occur in hepatocytes and to determine whether cell damage can be repaired by providing a resuscitation period between WI and cooling. The behaviour of resuscitated livers in recipients was also analysed.

Materials and methods

Experimental animals

Twenty female, Large-White pigs weighing 15-20 kg were used as liver donors and recipients. Experimental design and animal care were in accordance with the guidelines issued by The Animal Care Committee of the European Committee of the European Economic Community (EEC-28871-22A9).

Experimental design and biopsy times

Two initial groups were formed consisting of 10 donor and 10 recipient pigs. Recipient animals were divided into 2 further groups: Group I (n=5) (animal livers subjected to 5 min WI without resuscitation) and Group II (n=5) (animal livers subjected to 5 min WI followed by 5 min resuscitation). These groups differed only with respect to the resuscitation period. The duration of cold ischaemia, total duration of ischaemia and time to vascular suture were the same in both.

Liver biopsies were taken from each graft at the following times: at baseline (B), at the end of warm ischemia (WI), at the end of resuscitation (R), at the end of the cooling process (C), at the end of preservation (P), just before graft reperfusion, and, in the transplanted liver, at 1 hour after graft reperfusion (T).

Surgical technique

The pigs were anaesthetised using Ketamine and was maintained by isofurane inhalation. The abdomen was opened by the midline incision and all donors livers were subjected to a period of hepatic WI by clamping the hepatic portal vein. Group II livers were then allowed a period of resuscitation (by releasing the clamp). The donor livers were then flushed with Euro-Collins solution, stored for 2 h in the same solution (4 °C) and transplanted.

Spontaneous porto-jugular bypass was established before the anhepatic phase. No heparin was used. Arterial reconstruction was performed by endo-to-end anastomosis between the donor celiac trunk and the recipient hepatic artery using surgical loops (2x1). No biliary reconstruction was performed. A silicon tube was introduced into the donor bile duct through the abdominal wall for measurement of the 24-hour biliary output following transplant.

Morphological and ultrastructural studies

Morphological and ultrastructural studies were

performed using light and transmission and scanning electron microscopy.

Liver samples were cut into small sections. Those used in light microscopy were fixed by immersion in a 10% formol solution, embedded in paraffin and cut with a microtome to obtain 5 μ m-thick transverse and longitudinal sections. Cells were stained with hematoxylin and eosin and Masson's trichrome. Tissue for use in electron microscopy was fixed in a 3% glutaraldehyde solution for two hours and placed in Milloning buffer (pH 7.3) for an equivalent period. Samples were postfixed in 2% osmium tetroxide, dehydrated in a graded acetone series and embedded in Araldite for ultrastructural analysis. Contrast was enhanced with lead citrate. Specimens were observed using a ZEISS 109 transmission electron microscope. For scanning electron microscopy, tissue samples were dehydrated in a graded acetone series reaching the critical point of dehydration in an E-3000 Polaron with CO₂. They were then metal coated with gold-palladium and observed using a ZEISS 950 DSM scanning electron microscope.

HPLC assay

ATP, ADP, and AMP levels were determined in tissue from liver biopsies by HPLC. Total adenine-nucleotide (TAN) levels were calculated by addition of these three values.

Other tests

Plasma aspartate aminotransferase (AST) and bilirubin levels were determined at 24 h post-transplant. Biliary output was collected and recorded for each recipient over the same time period.

Statistical study

Parametric data were analysed using the Student t test. The Mann-Whitney test was used for nonparametric data. Fisher's exact test was used to compare survival rates between groups. A p value of <0.05 was considered to be significant.

Results

Biochemical tests

The HPLC results, graft biliary output and rates of survival are summarized in Tables 1 and 2.

TAN and ATP tissue levels were decreased at the end of preservation (P) in both groups. Only group II livers recovered nucleotide synthesis early after transplant (T).

Plasma AST 24 h post-transplant showed no difference between groups 24-h biliary output was higher in resuscitated livers but did not reach statistical significance. Finally, survival rate was optimal in group

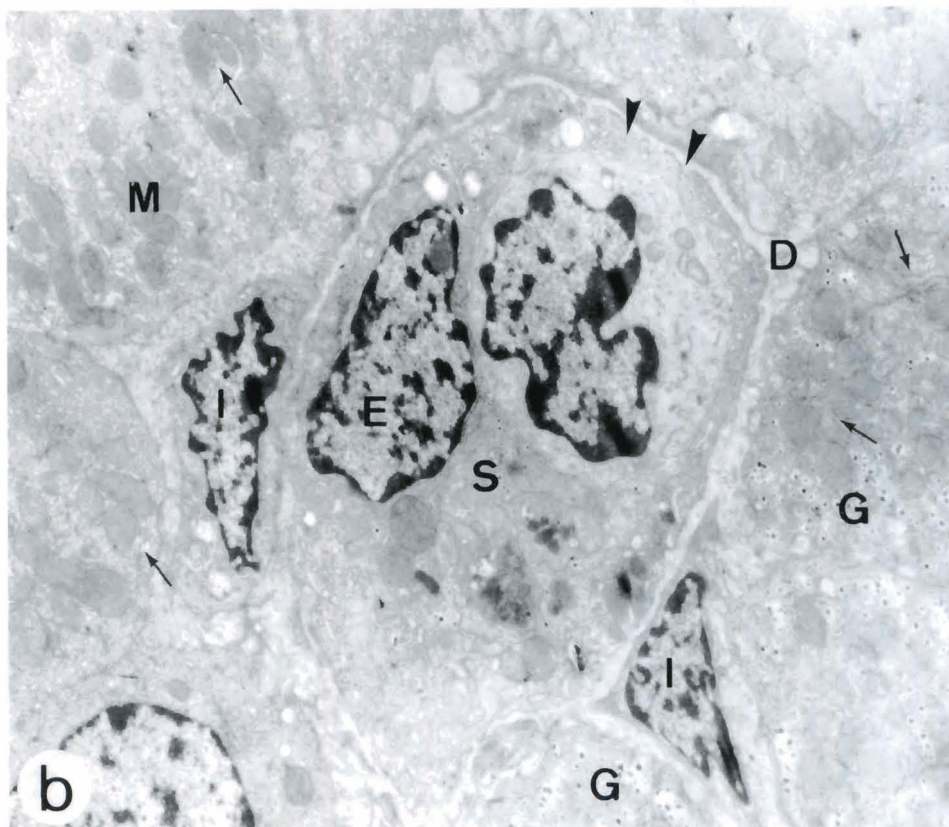
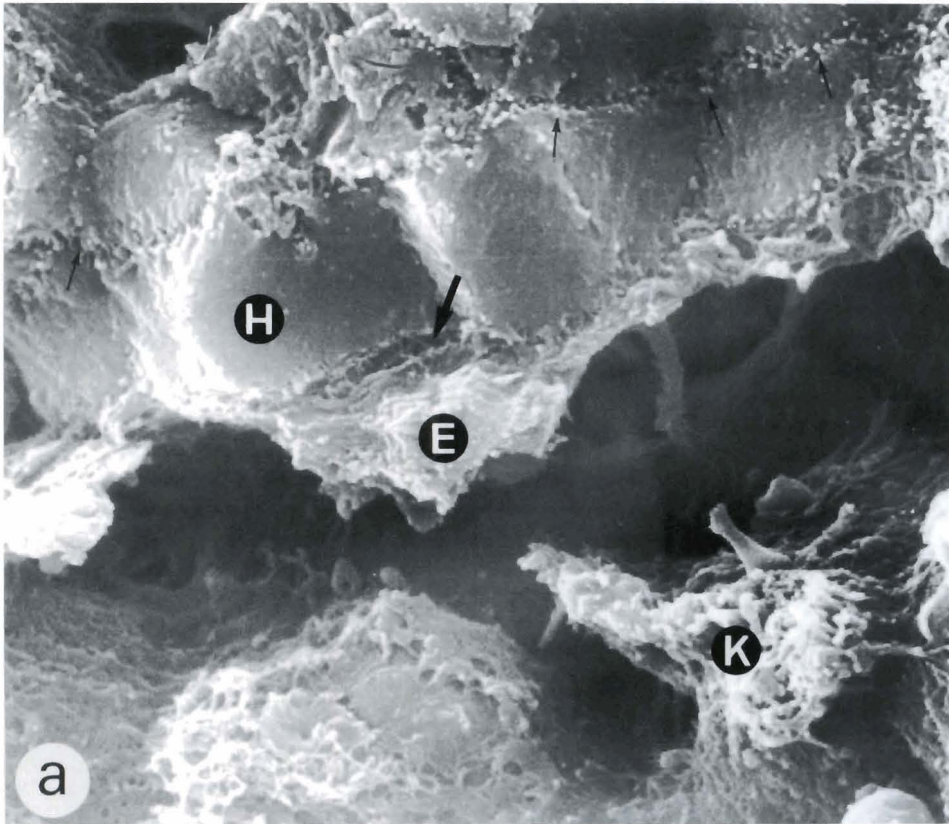


Fig. 1. General morphology of the liver cells. **a.** SEM of a sinusoid endothelium (E) and Kupffer cell (K). Polygonal hepatocytes (H) showing microvilli projecting towards hepatic canaliculi (small arrow) and space of Disse (large arrow). x 3,000. **b.** TEM of hepatocytes showing clear matrix-mitochondria (M) surrounded by abundant RER (arrow) and glycogen granules (G). Hepatocyte microvilli and some Ito cells (I) observed in the space of Disse (D). A sinusoid (S) lined by endothelium (E) with visible fenestra (arrowhead). x 4,400

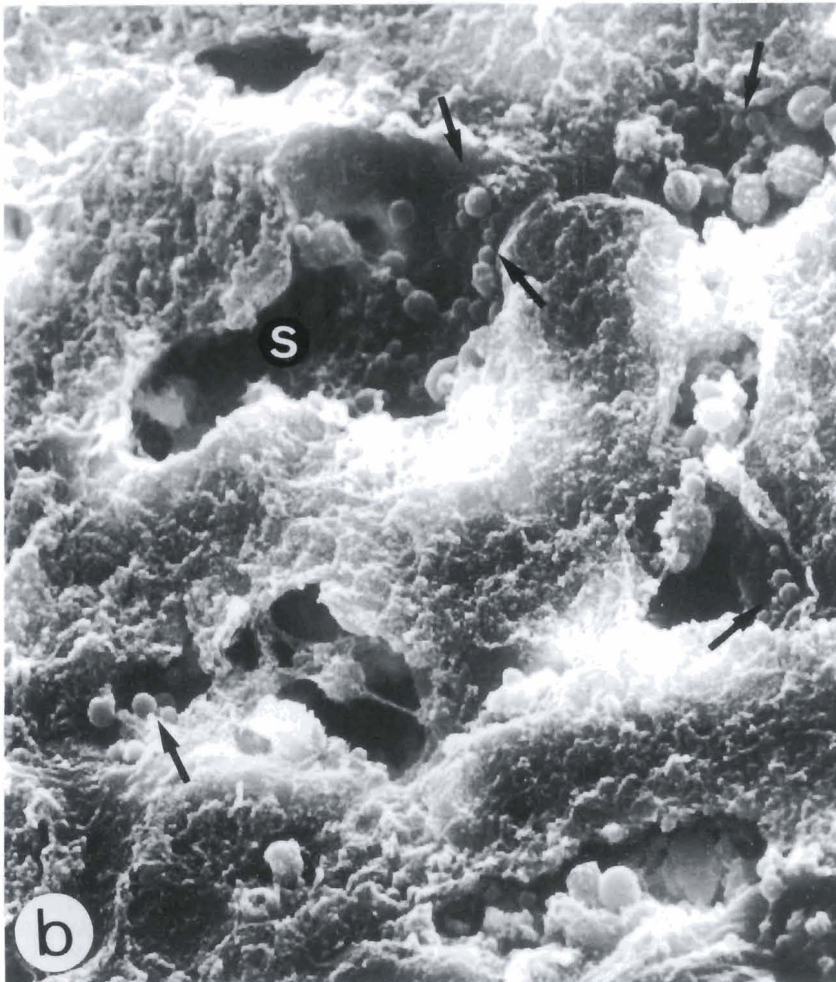
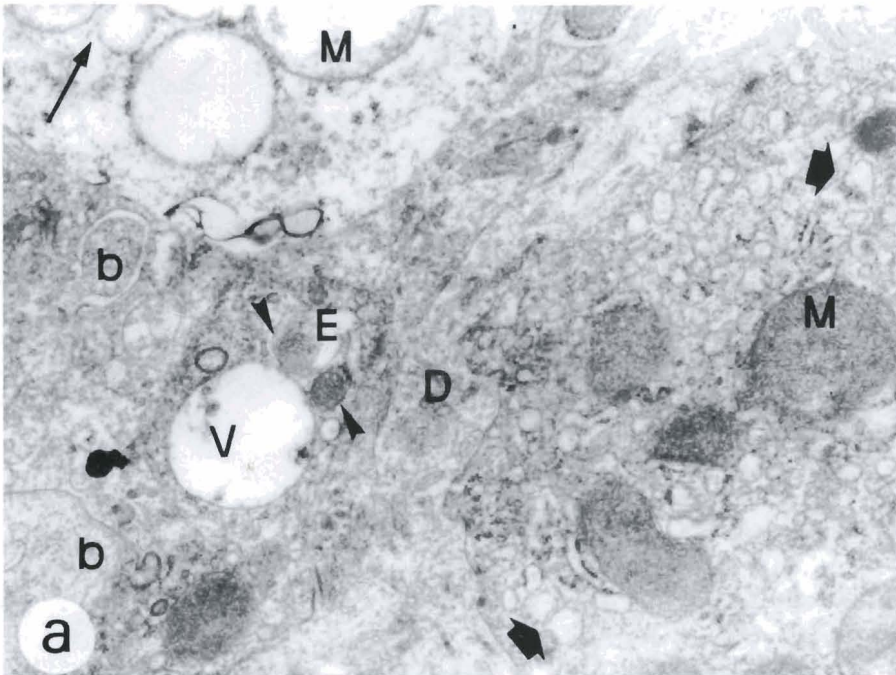
Anoxic-resuscitated liver allografts

Fig. 2. a. Top left: clear hepatocyte showing swollen mitochondria (M) with broken cristae and dilated SER (large arrow); right: a condensed mitochondria hepatocyte with some small vacuole (thick arrow). An endothelial cell (E) with condensed mitochondria (arrowhead) and a vacuole (V). In the luminal space of sinusoid there are some blebs (b). D: Disse space. x 12,000. **b.** Overall image of liver following WI showing numerous blebs (arrow) in the sinusoidal lumen (S). x 1,000

II but poor in group I.

Histological tests

Baseline (B)

At baseline the typical lobular structure of the liver was observed. The sinusoids were lined with fenestrated endothelium intercalated with Kupffer cells. Hepatocyte microvilli projected towards the interior of the hepatic canaliculi and the well-preserved space of Disse. Ito cells (fat-storing cells) with small lipid droplets were occasionally visible in the space of Disse. Hepatocytes were characterised by an elevated number of large mitochondria with dark matrices and clear, radial cristae. The abundant rough endoplasmic reticulum (RER) surrounded the mitochondria and was in close contact

with them. Other visible cell organelles were transversely-sectioned smooth endoplasmic reticulum (SER) and glycogen granules (Fig. 1a,b).

Warm ischaemia (WI)

Two well-defined patterns of cell injury were observed (Fig. 2a). Some hepatocytes contained dense cytoplasm and mitochondria, peroxisomes, multi-vesicular bodies and medium-sized vacuoles. Others had an electronlucent appearance and structural alterations affected both the nucleus and cell organelles. These cells showed breakage of their RER and an increase in SER with very dilated cisternae. The mitochondria were inflated and their cristae fragmented. In the sinusoids some endothelial cells contained micropinocytotic vesicles and very condensed mitochondria, but generally they showed a typical appearance (elongated nucleus,

Table 1. Tissue TAN and ATP levels in anoxic liver grafts.

	WI + R (min)	% Baseline	TAN	% Baseline	ATP
		P	T	P	T
Group I	5 + 0	78±25	76±16	73±41	51±34
Group II	5 + 5	75±18	110±78*	46±19	86±19**

WI: warm ischemia; R: resuscitation; TAN: total adenine nucleotides: $\mu\text{mol}/\text{mg}$ of liver tissue; ATP: adenosine triphosphate; P: end of preservation; T: transplant liver; *: $p < 0.01$; **: $p = 0.09$.

Table 2. Transplant and survival with anoxic donor livers.

	WI + R (min)	AST	BIL	Survival
		24 h	24 h	> 6 days
Group I	5 + 0	1736±774	64±35	1 animal
Group II	5 + 5	1026±332	114±54	5 animals*

WI: warm ischemia; R: resuscitation; AST: aspartate aminotransferase; BIL: bilirubin; *: $p < 0.05$.

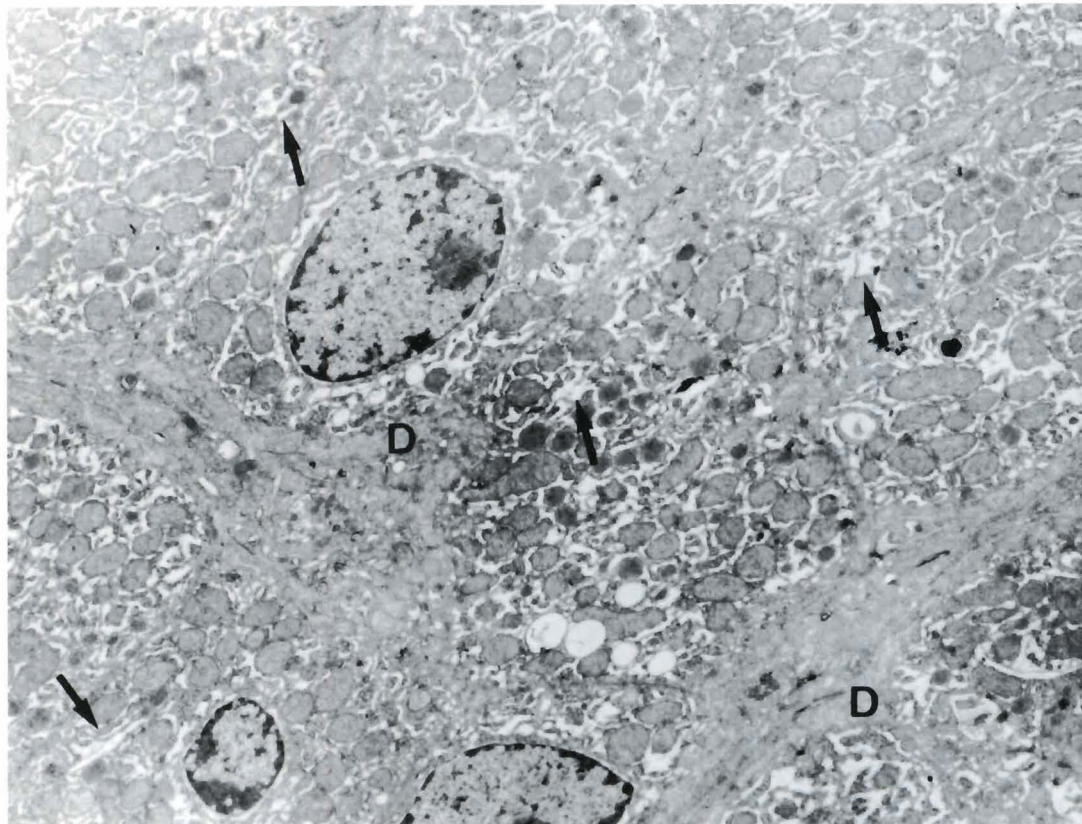


Fig. 3. Hepatocyte seen after 5-min resuscitation showing fusion of their cisternae of the endoplasmic reticulum (arrow) and a scarce number of microvilli projecting towards the space of Disse (D). x 3,000

Anoxic-resuscitated liver allografts

scarce cytoplasm, attachment to the sinusoidal lining). At the end of WI the formation of blebs occurred at the hepatocyte microvilli which eventually became detached from these and appeared in the lumen of the sinusoid (Fig. 2b). The space of Disse was reduced and the hepatic canaliculi were well preserved.

Resuscitation (R)

Immediately after resuscitation of the organ, the cells remained altered and a general inflammation of the tissue was observed. Fusion of SER cisternae had taken place in some cells forming a network of membranous canals in the cytoplasm. Differences in condensation of the cytoplasm were less apparent and very few microvilli projected into the space of Disse (Fig. 3).

Cooling process (C)

After cooling, significant differences between resuscitated and non-resuscitated livers were observed. Cells of the latter group showed a general inflammation of their organelles and their vascular and biliar poles were flattened and lacked microvilli. The morphological alterations observed in earlier stages were still apparent. Dark (dense) and clear hepatocytes were visible. In the dark cells the vesicles seen after WI became fused close

to the sinusoidal cell (Fig. 4). The space of Disse and the hepatic canaliculi were narrow. Blebs of various sizes and a large number of white blood cells were seen in the sinusoidal lumen. Resuscitated livers showed some signs of recovery, the mitochondria with clear matrices having apparently regained their size. They were again surrounded by RER and quantities of SER were reduced. The hepatocytes were still slightly inflamed but the space of Disse was again becoming dilated and the microvilli of some neighbouring hepatocytes were seen to project into it. The hepatic canaliculi were well preserved.

Preservation (P)

After preservation, Group I livers presented similar alterations to those seen earlier, showing some inflamed hepatocytes containing microvesicles and others condensed with numerous lysosomes (Fig. 5a). Oedematous blebs and flocculent material were found in the hepatic canaliculi. Some of the endothelial cells showed vacuolisation. Some white blood cells were found in the sinusoids amongst numerous blebs. The cells of the livers of Group II showed fewer alterations and a clear recovery of typical hepatocyte morphology (Fig. 5b). However, endothelial cells of the sinusoids were vacuolised in some areas and numerous intraluminal blebs were found.

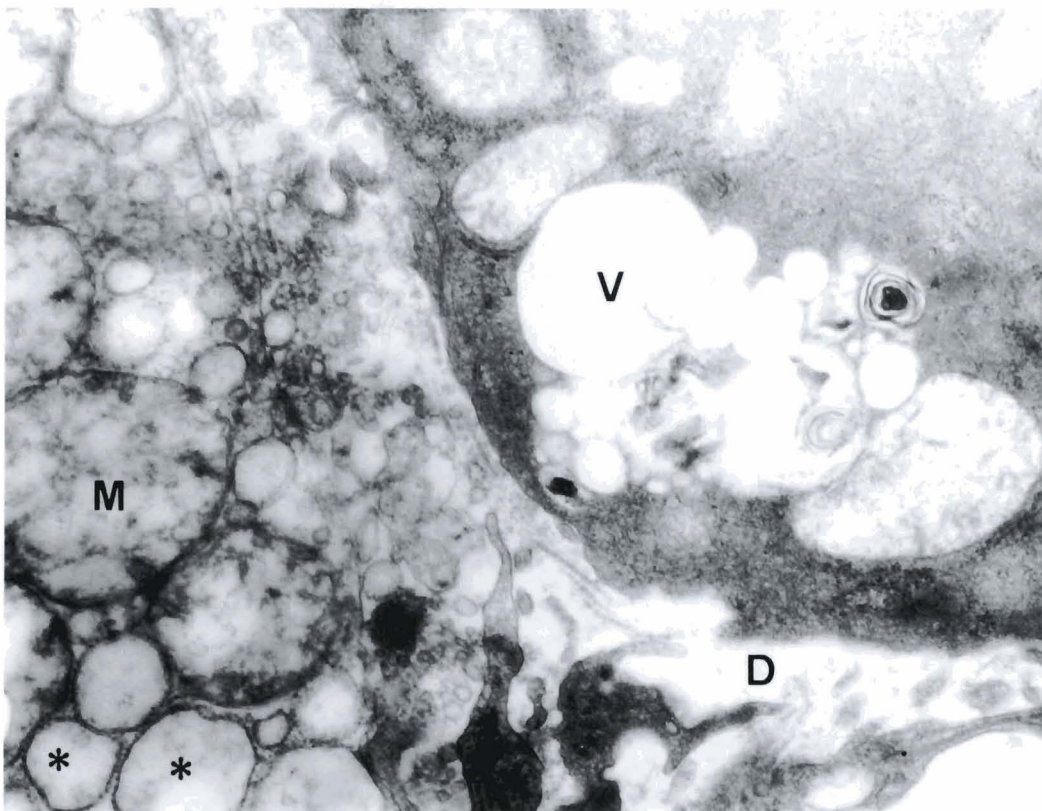


Fig. 4. After the cooling process non-resuscitated livers still show some clear hepatocytes with inflamed mitochondria (M) and dilated RER vesicles (*) and others of condensed cytoplasm and fused vacuoles (V) at the periphery of the hepatocyte vascular pole. There are no microvilli in the space of Disse (D). x 1,200

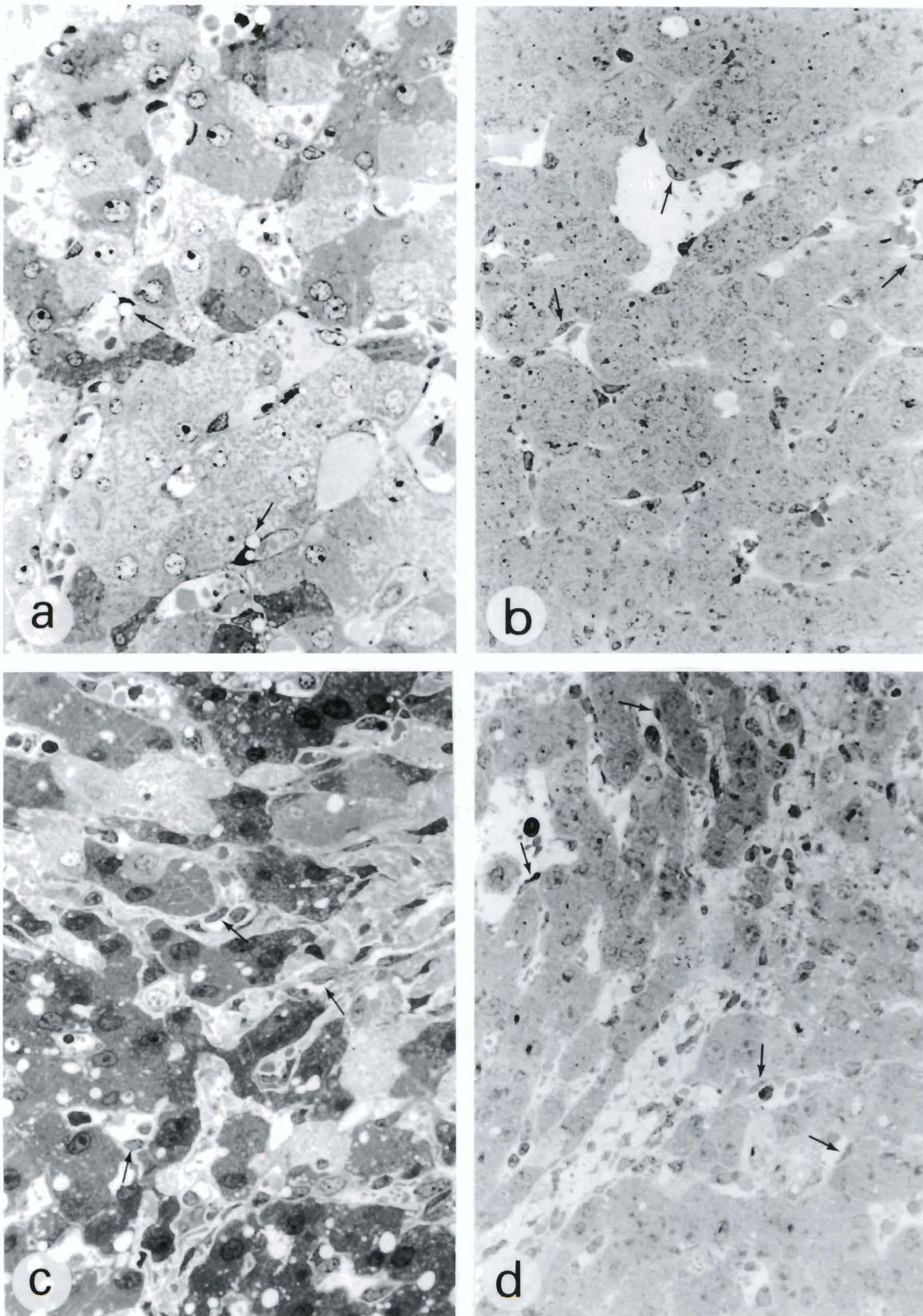


Fig. 5. a. Overall image of a non-resuscitated liver following cold preservation. Hepatocytes at different stages of the condensing process are clearly distinguishable and some vacuolised endothelial cells can be seen. **b.** Hepatocytes of typical morphology in resuscitated livers after cold preservation. Sinusoid endothelial cells are well preserved. **c.** Vacuolised hepatocytes of non-resuscitated livers 1 h after transplant. **d.** Overall image of resuscitated liver 1 h after transplant. The arrow pinpoints an endothelial cell. x 400

Transplanted liver (T)

Transplanted, non-resuscitated livers still showed structural anomalies after 1 h of revascularisation. Dark and clear hepatocytes were still apparent although they seemed to be constricted (Fig. 5c). Microvilli extended into the canaliculi and into the space of Disse which appeared wider. Some of the sinusoid cells were seen to be rounded. Kupffer cells appeared activated (Fig. 6). Neutrophils were present in the sinusoidal lumen and some blebs were still observed.

Resuscitated livers demonstrated noticeable structural recovery. Hepatocytes were apparently normal though in some cases remaining vacuoles were observed (Fig. 5d). The hepatic canaliculi and the space of Disse were well preserved and showed protrusion of microvilli from the hepatocyte. Endothelial cells of the sinusoids showed a typical morphology and Kupffer cells were activated. Sinusoidal lumens were free of blebs and only an occasional blood cell was observed.

Discussion

Structural damage to liver tissue during ischaemia may be the cause of functional failure of the transplanted organ. Maruyama et al. (1995) have suggested that relatively short periods of ischaemia can accelerate hepatic regeneration in the rat. However, after prolonged

periods of ischaemia, damage to the organ may be irreversible and reperfusion may induce additional lesions (Bulkley, 1983).

Fuller et al. (1990) suggested the possibility of recovering hepatic function of rat livers *in vitro* following their long-term cold preservation. The resuscitation of organs following cardiac arrest and the use of xenografts could provide new solutions to the problem of the lack of donors. There exists a general consensus that if the liver to be transplanted is quickly cooled following cardiac arrest; it is not able to perform all its functions correctly in the recipient. It has been shown (Huguet et al., 1978; Harris et al., 1982) that the liver is better able to tolerate WI than cold ischaemia and that damage caused may be reversible. Recently, reversibility of cell injury after WI has been demonstrated in pig liver (Schön et al., 1993). Filipponi et al. (1993) resuscitated pig livers following WI by ex-situ normothermic perfusion and Shirakura et al. (1993) were able to resuscitate different dog organs following cardiac arrest. However, none of these authors reports what happens after transplant of organs.

In this study, alterations in hepatocytes were found after WI similar to those reported by Vasilescu and Tasca (1991) in rat livers. However, these authors also found irreversible early injury to sinusoid endothelial cells, something not seen after WI in the present investigation.

Frederiks et al. (1984), using a temporary ischaemia

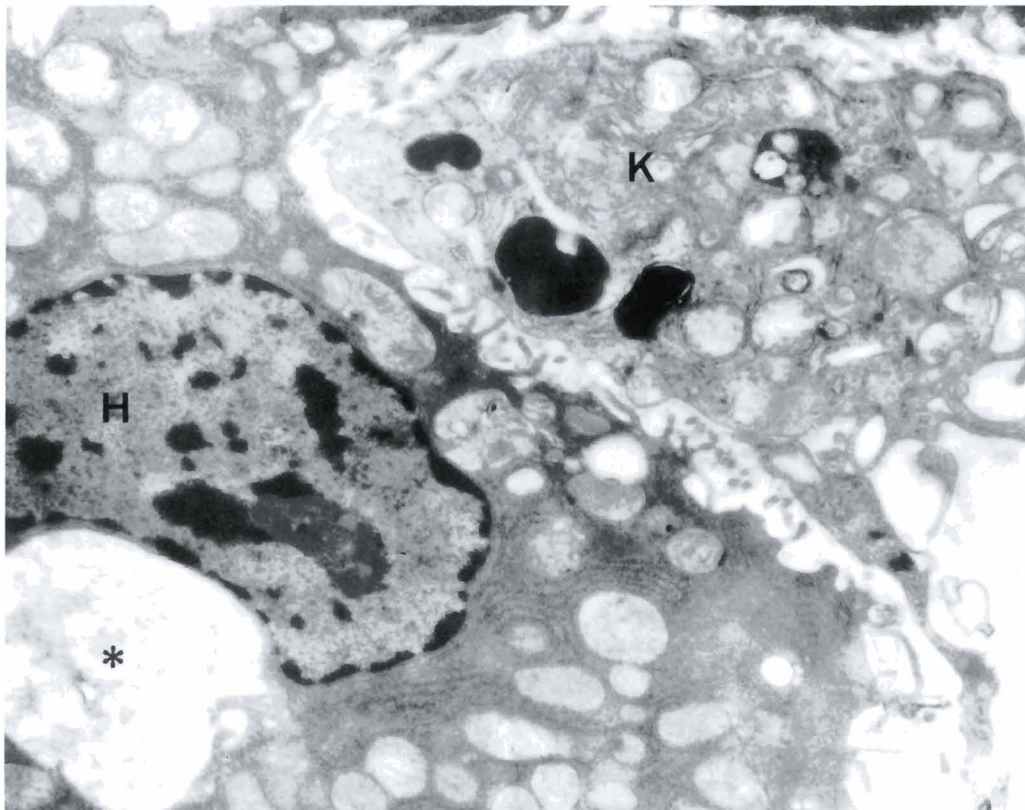


Fig. 6. Non-resuscitated liver. Activated Kupffer cell (K) following reperfusion. Dark hepatocyte (H) with vacuoles (*). x 7,000

model in the rat, found that after ischaemia, the plasma membrane of the biliary canaliculus was multilaminar in appearance with blebs moving towards the sinusoid. This alteration of the hepatocyte plasma membrane was also observed in the present study.

Cold ischaemia causes inflammation of hepatocytes and the formation of blebs at the hepatocyte microvilli which eventually move towards the interior of the sinusoids, via the endothelial fenestra. Bleb formation takes place more rapidly during WI (Lemasters et al., 1995). Similar (but attenuated) alterations are reported here following organ resuscitation. It is well known that bleb formation in the hepatocyte is a reversible process and that even after shedding of blebs the cells will recover if the factor causing injury is removed (Otto et al., 1984).

The extent of cell injury depends on the time and medium used for preservation (Jamieson et al., 1988). However, little is known about how different preservation solutions affect the morphology of hepatic cells during cold preservation. Vasilescu and Tasca (1991) found that rat hepatocyte morphology is well preserved at 4 °C. Sundberg et al. (1990), using the same preservation solution as in the present investigation, obtained very similar results to those reported here. However, when these authors used Wisconsin solution for preservation, they reported condensation of hepatocytes, disruption of the cellular junctions and dilation of the hepatic canaliculi. No alterations to biliary canaliculi were found in the present study. Noshima (1992), using different solutions to preserve rat livers, reported progressive mitochondrial degeneration characterized by inflammation and disorganization of their cristae. The same effects were observed in the present study, though after organ resuscitation and re-establishment of circulation a recovery of mitochondrial morphology was seen. McKeown et al. (1988), who used NaCl and CaCl₂ to preserve rat livers, reported similar alteration of hepatocytes.

Cold preservation selectively injures the endothelium irrespective of the preservation solution or animal species used (Otto et al., 1986). However, under conditions of warm preservation, damage is only observed in hepatocytes (Otto et al., 1986; McKeown et al., 1988). In the present study model, damage continues to be greatest in hepatocytes after 2 h of cold preservation (4 °C). McKeown et al. (1988), working with rat livers and longer preservation times (4 and 8 h), reported irreversible damage to endothelial cells (inflammation and degenerative changes in their nuclei) which eventually became detached and appeared in the lumen of the sinusoid. These effects on endothelial cells were also observed by Vasilescu and Tasca (1991). Sundberg et al. (1990) found oedematous alterations in some endothelial cells. However, in the present model there was no significant damage to the endothelial cells. Some showed small vacuoles in their cytoplasm, but their morphology remained unaltered and they continued to line the sinusoids at all times. The endothelial cells of

large blood vessels suffer less damage than those of the sinusoids due to their structural and metabolic differences (Wisse et al., 1983; Yokota, 1985).

In a recent study, Ohno et al. (1994) described degenerative changes in rat liver Ito cells following 18 h or more of cold ischemia. In the present investigation no such alterations were observed in these cells, though animal species, preservation solution and duration of ischemia were different. Following long periods of cold preservation, reperfusion induces loss of viability of the endothelial cells and loss of the sinusoidal lining (McKeown et al., 1988; Lemasters et al., 1995). These alterations probably result in graft failure.

Damage to hepatocytes undergoing reperfusion is secondary to microcirculatory dysfunction (Nolan, 1981; Myagkaya et al., 1984; Otto et al., 1984). Frederiks and Marx (1989) reported an accumulation of erythrocytes in the sinusoids of irreversibly damaged areas of reperfused rat livers. This indicates poor circulation in these areas. Filippini et al. (1993), working with an *ex-situ* pig liver perfusion model, observed general hepatocyte damage following ischemia. However, following perfusion, this seemed reversible for most cells. Extensive areas of the sinusoid wall lacked endothelial cells although when these were still present they appeared to be unharmed.

In both cold ischaemia (Lemasters et al., 1995) and WI (Jaeschke and Farhood, 1991; Lindert et al., 1992) Kupffer cells are activated shortly after reperfusion. In perfused rat livers, reoxygenation increases phagocytotic activity of the Kupffer cells (Lindert et al., 1992). In the present investigation activated Kupffer cells were not found following the resuscitation period. However, 1 h after revascularisation, livers of both Groups 1 and 2 showed characteristics of activation. The activation of Kupffer cells and the death of endothelial cells induced by reperfusion in the rat occur simultaneously following long cold storage periods (Lemasters et al., 1995). These same effects have been confirmed in human (Carles et al., 1994).

Long periods of reperfusion after ischaemia may cause damage in rat liver as a consequence of infiltration by polymorphonuclear leukocytes (Jaeschke et al., 1990; Koo and Komatsu, 1991). A large number of these cells were observed by Federiks et al. (1992) 3 h after reperfusion in rat liver. Leukocytes contribute to the damage caused by ischaemia/reperfusion by interacting with the endothelial cells of the sinusoid (Grisham et al., 1986; Koo et al., 1991).

Suzuki et al. (1993) demonstrated that the presence and degree of infiltration of neutrophils were important factors in determining the extent of damage caused by ischaemia/reperfusion in rat liver. However, Kim et al. (1994) detected no neutrophils immediately after reperfusion, suggesting that these cells play no part in inducing initial damage. Neutrophils induce secondary injury in the liver at a later time during reperfusion. In the present model few white polymorphonuclear cells were seen although after transplant and revascularisation some neutrophils were observed inside the sinusoids.

Anoxic-resuscitated liver allografts

Immunosuppressant drugs such as cyclosporin A or azathioprine protect against both warm and cold ischemia (Kim et al., 1991a,b). Cyclosporin and FK506 may improve survival rates and hepatic microcirculation by inhibiting the build up of neutrophils (Dhar et al., 1992; Suzuki et al., 1993; Kim et al., 1994).

The present study shows that livers subjected to 5 min of anoxia can fully recover if a 5-min aerobic period is introduced before flushing and cold preservation. All animals from Group II (with the period of resuscitation) survived for more than 6 days, whilst only one from Group I was surviving on the 6th day after transplant. Ultrastructural changes and tissue TAN levels were completely corrected soon after transplantation. The recovery of tissue TAN levels has been suggested as a viability prediction factor in transplant (Hamamoto et al., 1993). The ATP and TAN levels increased after transplant in resuscitated livers but only the difference in the total TAN value between the two groups was significant.

1 to 3 days after surgery, serum transaminase levels increase abruptly. This may be accompanied by a syndrome of functional cholestasis characterized by diminished bile flow, high bilirubin levels, inflammation and slight degeneration of the hepatocyte (Snover et al., 1984). In a more severe form, this phenomenon is called primary graft dysfunction or initial poor function (Ploeg et al., 1993). No significant changes were found in AST or bilirubin levels 24 h after transplant. Neither were any differences detected between the two recipient groups although a better performance of the transplanted organ was achieved in the resuscitated livers.

It may be concluded that 5 min of recovery from anoxia is sufficient to resuscitate livers subjected to 5 min of WI. Trials combining longer WI periods with longer periods of resuscitation should be performed to confirm these findings.

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Anoxic-resuscitated liver allografts

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