Decreased apoptosis in fatty livers submitted to subnormothermic machine-perfusion respect to cold storage

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Abstract

Machine perfusion at subnormothermic temperature (20°C), MP20, was developed by Vairetti et al. and showed to afford a better preservation of fatty livers respect to traditional cold storage (CS) in terms of enzyme release into the perfusate, bile production, glycogen stores, energy charge and oxidative stress. Here we investigated whether it also caused decreased cell death by apoptosis. Fatty and lean Zucker rats were submitted to MP20 or CS for 6 h and reperfused normothermically for 2 h. Apoptotic cells were revealed by immunohistochemistry of activated caspase-3 and M30 (new epitope on CK18 expressed by viable or necrotic cells), but not by sinusoidal lining cells. These data reveal how difficult it is to protect steatotic livers from IR injury. Most strategies for reducing damage during acute stress conditions to FL are still in the experimental stage, and are not clinically applicable. Hypothermic machine perfusion (MP) has shown to improve FL preservation compared with cold storage (CS): bile production, ammonia clearance, urea production, oxygen consumption, and ATP levels were significantly higher after MP, compared with CS. Our group has developed a machine perfusion system with recirculation of an oxygenated medium at 20°C (MP20): ATP levels, energy charge, ATP/ADP ratio and bile production, were higher and nitrate/nitrite (NOx) concentration lower, in FL submitted to MP20, respect to CS. Moreover, oxidative stress, tumour necrosis factor (TNF-α), caspase-3 activity, and biliary alkaline phosphatase release, were lower in FL preserved by MP20. We have also shown a better preservation of FL by MP20, respect to CS, in terms of morphology, glycogen stores and reactive oxygen species (ROS) production.

A key feature of IR in the liver is apoptosis, but little is known about the mechanisms of cell death in FL after CS, and the few available information is controversial. Some authors observed predominant necrotic forms of hepatocyte death after warm IR. Baskin-Bey et al. found that hepatocyte apoptosis predominated in steatotic grafts after cold ischemia-warm reperfusion, whereas Fernandez et al. using a different animal model, did not observe apoptosis in steatotic livers undergoing transplantation, after cold preservation-warm reperfusion. As cold IR is concerned, disagreeing observations have been made, that might be related to different experimental settings, such as the duration of cold ischemia or the different animal models. In particular, cathespin-dependent hepatocyte death by apoptosis was determined in cathespin B knockout mice, fed with a methionine/choline deficient diet, after 24 h of cold preservation with University of Wisconsin (UW) solution and 1 hour warm reperfusion. By contrast, no apoptosis was identified in Zucker rat liver preserved with UW solution for 6 h and reperfused for 4 h, whereas both apoptotic and necrotic cell death was identified in experiments where fatty Zucker rat livers preserved for 6 h with UW solution were transplanted in lean animals.

We investigated the response of FL to MP20, respect to CS, in terms of cell death by apoptosis. We used the terminal deoxy nucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, which identifies apoptosis in all kinds of cells, and is not limited by the occurrence of DNA strand-breaks. Therefore, we also investigated the expression of activated caspase 3 and of the neo-epitope M30, specific to the Asp396 caspase cleavage site of cytokeratin 18 (CK18) not expressed by viable or necrotic cells. Proteolytic cleavage of cytokeratin 18 during apoptosis takes place before the disruption of cell membrane asymmetry, and before the occurrence of DNA strand-breaks. CK18 is expressed by hepatocytes and bile duct cells, but not by sinusoidal lining cells (SLC).
Materials and Methods

Chemicals

Unless otherwise mentioned, all chemicals were of the highest purity available and were purchased from Sigma (Milano, Italy).

Animals

Homozygous (fa/fa) obese male Zucker rats (11-12 week old, 375±15 g; Charles River, Italy) were used as models of F32 and heterozygous (fa−), lean animals (300±10 g) were used liver donors. The animals had free access to water and food. The use and care of animals in this study were approved by the Italian Ministry of Health and by the University Commission for Animal Care. Rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.) and received 250 units of heparin via the inferior vena cava. The bile duct was cannulated (PE-50), an intravenous catheter (16-gauge) was inserted into the portal vein. The catheter (EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM 900 electron microscope operating at 80 kV. EM

Machine perfusion

The liver was placed in an organ chamber, and was connected to re-circulating standard perfusion equipment containing 200 mL KH medium for 6 h. The KH solution was recirculated by a roller pump (Gilson Minipuls-3), oxygenated and maintained at 20°C by a heat exchanger (Jalabo-F12). The perfusion solution was oxygenated by a glass oxygenator, resulting in a pre-hepatic oxygen concentration of 500 to 600 mmHg, and in post-hepatic concentration of about 120 mmHg at 37°C. During MP at 20°C the pre-hepatic oxygen concentration was 550 to 650 mmHg, and the post-hepatic concentration about 450 mmHg at 37°C. The perfusate ran freely via the suprahepatic caval vein into the organ chamber, and was immediately re-circulated by the roller pump into the reservoir. Air emboli were removed from the system by a bubble trap. The portal venous pressure was continuously measured throughout the perfusion by means of a water column connected to the portal vein inflow catheter. Pre-calibration was performed each time just before connecting the liver to the circuit. At the end of MP preservation we started normothermic perfusion by switching to a reservoir containing fresh solution at 37°C. Cold storage

After washout with KH, the livers were flushed in situ with UW for 2 min and maintained at 4°C in this solution for 6 h. After CS, livers were washed out of the storage solution by discarding the initial effluent in a non-circulating system with 20 mL KH at 37°C then subjected to recirculating perfusion with KH.

Normothermic perfusion

Reperfusion with KH (2 h at 37°C) was performed in the same set up as MP, both in MP and CS preserved livers. The portal venous pressure (in mmHg) was monitored by a water column connected to the portal vein inflow catheter. Pre-calibration was performed each time just before connecting the liver to the circuit.

Morphology and immunohistochemistry

Liver pieces were rapidly removed after normothermic reperfusion, fixed in 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2-7.4 for 24 h and processed routinely until they were embedded in Paraplast wax.

Electron microscopy

Samples of the liver were quickly removed, and small fragments were fixed by immersion in 2.5% glutaraldehyde in 0.1 M Millonig buffer (pH 7.2-7.4) at 4°C for 4 h, rinsed, post-fixed with 1% osmium tetroxide at 4°C for 2 h, washed, dehydrated through graded concentrations of alcohol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate for 7 min and lead citrate for 2 min, coated with carbon, and observed with a Zeiss EM 900 electron microscope operating at 80 kV.

Activated Caspase-3

Paraffin sections of 6 μm thick were cut and deparaffinized in xylene and rehydrated with graded ethanol and water. Antigen retrieval was performed by microwave treatment with a pre-heated 10 mM citric acid buffer (pH 6) by incubation in a microwave oven at 100 W for 20 min, according to the manufacturer’s instructions. After cooking, aspecific antibody binding was blocked for 1 h in PBS with 10% normal goat serum, 3% bovine serum albumin, and 1.5% NaCl. Primary antibody for 5 μm and stained with Hematoxylin and Eosin (H&E) for histological examination.

Immunohistochemical sections were examined using a Zeiss EM 900 electron microscope operating at 80 kV.

Immunostaining for H2O2

The slides were counterstained with Mayer’s Hematoxylin.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling method (TUNEL)

Para formaldehyde of 6 μm thick were collected on poly-L-lysine-coated glass slides, and the nuclear DNA fragmentation of apoptotic cells was labeled in situ with the ApoTag Peroxidase in situ Apoptosis Detection Kit (Intergen Co. Purchase, NY, USA). Briefly, the sections were deparaffinized and treated with 20 mg/mL proteinase K (Boehringer, Mannheim, Germany) for 15 min. After rinsing with distilled water, the sections were treated with 3% hydrogen peroxide in 10% methanol for 15 min. The sections then were washed with distilled water and incubated in the provided equilibration buffer for 10 min. The sections were incubated with terminal deoxynucleotidyl transferase (TdT) in the provided reaction buffer with digoxigenin-dUTP in a humidifier chamber at 37°C for 1 h. The incorporated digoxigenin-dUTP was detected by peroxidase-conjugated antidigoxigenin antibody and the signal developed by incubation with 3,3-diaminobenzidine (DAB) in the presence of H2O2. The slides were counterstained with Hematoxylin.

Apoptosis quantification and statistical analysis

Immunohistochemical sections were exam-
ined by light microscopy (Zeiss Axioskop 2 Plus light microscope (Carl Zeiss Microimaging, Jena, Germany)) and apoptotic cells counted. Ten high-power fields (x 400) for each animal were selected on the basis of the best preserved tissue areas (n=5 each treatment). Peripheral areas of tissue sections were not considered. Immunoreactivity was expressed as percentage of stained cells, calculated by dividing the number of stained nuclei by the total number of hepatocytes in any given zone. Statistical analysis was performed using SPSS 13.0 statistical software. General Linear Model, univariate, was used to compare sets of data, with significant differences at P<0.05.

Results

Morphology - light and electron microscopy

Morphological observation of control Zucker lean rat liver showed no evidence of steatosis. There were no substantial differences in morphology after CS compared with MP20, and the parenchyma was well-preserved after both treatments (not shown).

Control obese animals showed severe micro- and macrovesicular fatty infiltration in hepatocytes, mainly in the midzone (MZ) (not shown). In FL submitted to CS, the parenchyma was highly damaged, especially in the MZ and centrilobular region (CL) where sinusoids appeared markedly dilated and hepatocyte ballooning and necrosis were frequent (not shown). By contrast, when FL were submitted to MP20, the parenchyma was much better preserved, the sinusoidal structure was normal, macrosteatosis and vacuolar degeneration of hepatocytes much less abundant, and necrosis was never observed. The analysis of liver ultrastructure further supported the observations. In particular, for FL submitted to MP20, most endothelial cells appeared normal with well-developed cytoplasmic processes lining the sinusoid, and without any morphologic evidence of apoptosis. Apoptotic cell morphology was rare in control lean and fatty livers.

On the contrary, CS-preserved FL liver showed frequent SLC apoptosis, in particular of cells with the typical morphology of endothelial, stellate and Kupffer cells (Figure 1). Hypothermic injury to sinusoidal endothelial cells, detachment from the basal lamina, rounding and loss of cytoplasmic processes, were observed. Kupffer cells phagocytizing apoptotic bodies were recorded. By contrast, when FL was preserved by MP20, the hepatocytes ultrastructure was less damaged and fewer apoptotic cells were observed.

Figure 1. Representative transmission electron micrographs of apoptotic parenchymal and sinusoidal cells in the liver of obese Zucker rats submitted to conventional cold storage (CS) or to Machine Perfusion at 20°C (MP20). (a), MP20: ultrastructure of an apoptotic binucleated hepatocyte (bHep) with condensed chromatin margination surrounded by dilated nuclear envelope in both nuclei; well-preserved mitochondria, endoplasmic reticulum and storage material (glycogen rosettes and lipid droplets); scale bar: 1.1 µm. (b), CS: ultrastructure of an early apoptotic cell with typical sinusoidal endothelial cell morphology (End). In sharp contrast with MP20 preservation (a), the nearby hepatocyte (Hep) contains swollen, poorly-contrasted mitochondria; scale bar: 1.7 µm. (c), CS: ultrastructure of an apoptotic sinusoidal cell containing lipid droplets and thus presumed to be a stellate cell (Stel). As in b) the neighboring hepatocytes (Hep) contain packed and swollen mitochondrial, lipid droplets and intracytoplasmic edema; scale bar: 1.7 µm. (d), CS: ultrastructure of an advanced-stage (karyorrhexis) apoptotic Kupffer cell (Kupf); a nearby sinusoidal cell with abundant rough endoplasmic reticulum and lipid-rich cytoplasm (Stell) and the cytoplasm of a hepatocyte (Hep) with abundant microvilli in the interhepatocyte recess, can also be recognized; scale bar: 1.7 µm.

Figure 2. Hepatocytes and SLC death quantified as the percentage of caspase-3 positive cells/ total cells, in 10 random microscopic high-power fields (x40 objective). Statistically significant decrease of apoptotic hepatocytes and SLC after MP20 respect to CS was observed for obese Zucker rat liver (a), but not for the lean one (b). Values (%) are reported as mean (5 rats each group) ± standard error; *P<0.05.
Immunodetection of cleaved caspase-3

A statistically significant decrease of apoptotic hepatocytes and SLC (P<0.05) after MP20 respect to CS was observed for obese Zucker rat liver, but not for the lean one (Figure 2 a,b). In fatty and lean control livers only few hepatocytes and SLC were positive (Figure 3 a,d). Single positive cells were located mainly in the MZ area both after CS or MP20 (Figure 3 b,c,e,f).

Immunodetection of M30 antigen

A statistically significant decrease of early apoptotic hepatocytes (P<0.05) after MP20 respect to CS was observed, for both lean and obese Zucker rats (Figure 4 a,b). In fatty and lean control livers minimal M30 staining was present (Figure 5 a,d). After both CS and MP20, M30 positive hepatocytes were mainly located in the periportal (PP) and MZ regions. They showed a granular cytoplasmic staining pattern (Figure 5 b,c,e,f). Most M30-positive cells did not present obvious apoptotic nuclear morphological features, thus supporting the suggestion that immunohistochemical detection of M30 marks an early event in the apoptotic process.

TUNEL reaction

A statistically significant decrease of apoptotic hepatocytes and SLC (P<0.05) for obese Zucker rat was observed after MP20 respect to CS (Figure 6a). In lean animals a similar decrease was observed only for SLC (Figure 6b). Few hepatocytes and SLC were positive to TUNEL staining in control lean and lean livers (Figure 7 a,d). Both after CS or MP20, TUNEL-stained cells were located mainly in PP and MZ area (Figure 7 b,c,e,f).

Portal pressure

Steatotic livers preserved by CS exhibited a gradual and time-dependent pressure increase (Figure 8a). Conversely, FL preserved by MP at 20°C even at the end of reperfusion showed lower pressure values, similar to those observed in livers obtained from lean rats, for which the time-dependent increased pressure was negligible (Figure 8b).

Discussion

In this study we further strengthen the working hypothesis that MP20 is a better strategy to protect steatotic livers than conventional CS. We have shown in previous reports that not only normal livers but especially FL submitted to subnormothermic machine perfusion (MP20) exhibit a marked damage reduction, evaluated as enzyme and cytokine release, excretory function, energy recovery and oxidative stress. Here we show that MP20 also protects FL from apoptotic cell death. In particular we found a significant reduction of apoptosis of hepatocytes and especially of SLC in FL preserved for 6 h with MP20 and reperfused respect to livers preserved by CS. Apoptotic SLC and hepatocytes were more numerous in the PP zone of both CS and MP20 treated livers. The mechanism of the predominance of apoptosis in the PP zone is still unclear. Although PC regions have lower oxygen tensions than PP areas, which may suggest that they would be more susceptible to hypoxic damage, IR injury is different from hypoxic injury and it has been shown that hepatocytes with lower resting oxygen tensions may be more resistant to reperfusion injury. Oxidative stress was demonstrated to cause hepatocyte apoptosis mainly in PP and MZ areas. Our group too has previously presented evidence for ROS reaction with a lobular zonation, decreasing from PP to PC hepatocytes. This could explain the prevalence of apoptosis in the PP zone of both CS and MP20 treated livers. The mechanism of the predominance of apoptosis in the PP zone is still unclear. Although PC regions have lower oxygen tensions than PP areas, which may suggest that they would be more susceptible to hypoxic damage, IR injury is different from hypoxic injury and it has been shown that hepatocytes with lower resting oxygen tensions may be more resistant to reperfusion injury. Oxidative stress was demonstrated to cause hepatocyte apoptosis mainly in PP and MZ areas. Our group too has previously presented evidence for ROS reaction with a lobular zonation, decreasing from PP to PC hepatocytes. This could explain the prevalence of apoptosis in the PP zone of both CS and MP20 treated livers.
apototic hepatocytes in the PP area. A further explanation could be the different mitochondrial components and enzyme contents of PP and PC zones.\(^{37}\)

Damage to SLC may be a major component of graft dysfunction after IR injury, and since apoptosis is a major cause of SLC loss after IR, limiting apoptosis may provide one therapeutic approach.\(^{38}\) Several studies established that in non-steatotic liver SLC are more injured than hepatocytes by cold preservation, also in terms of apoptotic cell death.\(^{39-43}\) The SLC remain viable upon oxygenated reperfusion of the graft but die rapidly thereafter in reimplanted grafts.\(^{44}\) Low temperature preservation causes injury to SLC, such as alteration of the extracellular matrix and cytoskeleton,\(^{40,45}\) detachment endothelial cells with loss of cytoplasmic processes lining the sinusoids,\(^{46,47}\) and possibly Kupffer cell activation.\(^{48}\) SLC of FL are more prone to lose their viability after CS than those of normal liver.\(^{49}\) Damage of SLC can lead to impaired sinusoidal flow through adhesion and activation of circulating leukocytes,\(^{9}\) platelet activation,\(^{50}\) and ultimately a secondary injury to hepatocytes causing organ non-function or dysfunction after transplantation.\(^{51}\)

In our study, apoptosis was observed mainly in SLC (sinusoidal endothelial cells, Kupffer cells, and stellate cells, as demonstrated by electron microscopy) and it was significantly higher in FL after CS respect to MP20. Probably, after CS apoptosis occurs during reperfusion, since it cannot occur in a milieu with low oxygen tension.\(^{52}\) Indeed, although some components of SLC injury already occur during the preservation process,\(^{53}\) SLC apoptosis is an active process that requires ATP as energy supply and the release of mediators such as TNF-\(\alpha\),\(^{54,55}\) or the elevation of ATP levels; the latter are lowered in ischemic livers.\(^{56}\) The reduction of SLC apoptosis revealed by caspase-3 expression and by the TUNEL assay, is in keeping with our previous biochemical data. Indeed, we have previously shown that MP20 greatly reduced TNF-\(\alpha\) release as compared to CS, suggesting Kupffer cell protection when using subnormothermic temperature.\(^{17}\) The relevance of this protection is correlated to a reduction in the first source of ROS during reperfusion, thus limiting oxidant stress to hepatocytes. A further factor recognized as major mediator of apoptosis is oxidative stress.\(^{37,38}\) We indeed reported a higher abundance of ROS-positive sinusoidal cells for CS than for MP20.\(^{17}\) Finally, we also previously reported that caspase-3 activity, evaluated by \(p\)-nitroaniline release, was lower with MP20 than with CS, thus confirming the reduction of apoptotic cell death.\(^{17}\)

The reduction of apoptotic cells is important, since damaged SLC can exacerbate microcirculatory disturbances of FL and microcirculatory impairment has been implicated as an important mechanism in steatotic liver injury after transplantation.\(^{7,50}\) Reduced SLC apoptosis likely improves sinusoidal flow during reperfusion, decreases no-reflow phenomenon and leads to a reduction in secondary ischemic insults produced by flow disturbances.\(^{49}\) During reperfusion after MP20, we found a statistically significant decrease of portal pressure, respect to CS-preserved livers. The improvement of the flow in the sinusoids for FL preserved by MP20 can also be ascribed to the action of N-Acetylcysteine (NAC) included in the perfusion medium. NAC is not only a precursor of glutathione, therefore contributing to the protection against oxidative damage,\(^{61,62}\) but it also has vasorelaxant effects.\(^{63,64}\) Adding NAC to the liver before CS caused an amelioration of sinusoidal microcirculatory injury after cold IR in steatotic rat liver.\(^{5}\)

It is not possible to establish from the present data whether the damage to sinusoidal cells was caused by increased portal pressure or vice versa. It can be speculated that the IR

Figure 5. Representative light photomicrographs of M30 stained sections from liver of lean (a-c) and obese (d-f) Zucker rats. In lean (a) and obese (b) control livers minimal M30 staining was present. Both in lean and obese livers after CS (b,e) or MP20 (c,f), anti-M30 positive hepatocytes were mainly located in the PP and MZ regions and they showed a granular cytoplasmatic staining pattern. CL, centrolobular vein; P, portal vein. Scale bar: 50 \(\mu\)m.

Figure 6. Hepatocytes and SLC death were quantified as the percentage of TUNEL positive cells/total cells, in 15 random microscopic high-power fields (x40 objective). Statistically significant decrease of apoptotic hepatocytes and SLC after MP20 for obese Zucker rat was observed respect to CS (a). The same was observed in lean animals only for SLC (b). Values (\%) are reported as mean (5 rats for each group) \(\pm\) standard error; \(^*P<0.05\).
damage after CS/reperfusion is the triggering cause for SLC apoptosis, and that the morphological changes of apoptotic cells hinder flow through the sinusoids thus contributing to increased portal pressure and initiating a vicious cycle of damage to the parenchyma.

Furthermore, limiting apoptosis is important, since apoptotic cells can be thrombogenic. Indeed, it has been demonstrated that apoptotic cells express phosphatidylserine on the cell surface membrane, essential for both thrombin generation and Tissue Factor (a procoagulant) activation. Finally, the decrease of apoptotic SLC is important, since the regeneration of these cells is more difficult than that of hepatocytes because it needs the mobilization of bone marrow precursors. Although the most relevant effect is the reduction of SLC apoptosis after MP20 respect to CS, also the decrease in hepatocyte death deserves mentioning since CS caused anyway early apoptotic features (expression of activated caspase 3 and M30) of parenchymal cells. Reducing both SLC and hepatocytes apoptosis is crucial in cases of mild or moderate steatosis. Results from recent clinical studies in humans and in animal models of steatosis suggest that the inhibition of apoptosis by ischemic preconditioning or by pharmacological strategies protect against I/R injury.

In conclusion, MP20 provides protection to FL, not only by significantly reducing hepatic enzymatic leakage, hepatic steatosis, and by increasing energy metabolism recovery during reperfusion, but also by reducing significantly SLC and hepatocyte apoptosis, thus potentially improving the quality of liver grafts without need of further protective strategies. These findings have important clinical implication since MP20 can render steatotic donor livers useable for transplantation.

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