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In Situ Demonstration of Improvement of Liver Mitochondria Function by Melatonin after Cold Ischemia

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Abstract. *In a previous investigation, reperfusion with a melatonin-containing medium was demonstrated to enhance bile production and tissue ATP levels in rat livers, cold-preserved with University of Wisconsin (UW) or Celsior solutions, with respect to melatonin-free reperfusion; lipid peroxidation products in the perfusate were not influenced by the indole. This was ascribed to an increased efficiency of the hepatocyte mitochondria induced by melatonin. Reactive oxygen species (ROS) normally leak from the electron transfer chain in mitochondria and excessive ROS production is presumed to mediate ischemia-reperfusion (I/R) damage. A histochemical reaction was used to demonstrate ROS on the same model. Compared to the lobular zonation of ROS in control livers, the stained area of cold-preserved livers reperfused without melatonin was restricted to a narrow portal region, in keeping with the much lower ATP content. When reperfusion was performed with melatonin, the liver morphology was improved and the ROS reaction in hepatocytes more intense, though not reaching the control liver pattern. Sinusoidal cells were poorly-stained in both cases. In conclusion, with this different approach, melatonin was confirmed to improve mitochondrial performance and to discriminate parenchymal from sinusoidal cell behavior. Our observations confirm that melatonin mitigates I/R injury and support its potential in liver transplantation.*

Cold ischemia/reperfusion (I/R) injury plays a critical role in the occurrence of primary non-function and delayed graft function, which are major limiting factors in liver transplantation (1-4). Re-oxygenation of the ischemic liver causes the generation of numerous reactive oxygen and nitrogen species (ROS and RNS, respectively) including superoxide anion ($O_2^{\bullet -}$), oxydryl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite ($ONOO^-$). Although low concentrations of ROS and RNS play an important role as mediators in normal cell metabolism and signal transduction, in higher concentrations these species can be harmful (5-8). The oxidative stress induced by I/R is worsened by the concomitant depletion of endogenous antioxidants (*e.g.,* glutathione, vitamin C, superoxide dismutase, glutathione peroxidase) (9). The sources of ROS generation in liver I/R are not well-defined yet; the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine/xanthine oxidoreductase (XOR) reactions and the electron transfer chain (ETC) in mitochondria are believed to play key roles (10). The products of ATP breakdown formed during the hypothermic/ischemic phase may contribute to the formation of ROS once oxygenation is restored through the XOR reaction (11), but recent evidence suggests that the role of XOR is less important than that of mitochondria (9, 12). The low ATP levels are a further risk factor as they cause intracellular edema, and calcium-dependent activation of phospholipases, triggering inflammatory cascades and impairing the functioning of the ETC (11). Minimizing the adverse effects of I/R and, in particular, controlling the acute hepatobiliary dysfunction elicited by cold I/R, might significantly increase the number of patients that can successfully undergo liver transplantation. In particular, various attempts are being made to reduce post-ischemic tissue injury by using free radical scavengers or substances that might enhance the endogenous antioxidant systems (13). Melatonin (N-acetyl-5-methoxytryptamine), used pharmacologically, appears particularly attractive for this purpose as discussed below,

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not only because it meets the most important prerequisites to protect tissues against I/R damage, but also, and most importantly, because locally secreted melatonin appears to modulate the function of the gastrointestinal tract (GIT), including the liver.

Melatonin, secreted by the pineal gland and by several other non-pineal sources (14-16), modulates pleiotropic physiological functions which comprise the regulation of the circadian and endocrine rhythms and aging, enhancement of immune function and the capacity to act as a potent wide-spectrum free radical scavenger and antioxidant and, at least when given at a pharmacological level, as a stimulator of the endogenous antioxidant system (17-20). Furthermore, melatonin was shown to play a direct role in mitochondria homeostasis, by scavenging ROS and RNS, improving mitochondrial respiration, increasing ATP synthesis and reducing the harmful reduction in the mitochondrial membrane potential that may trigger the apoptotic cascade (21-24). The indole protection of polyunsaturated fatty acids in membranes, proteins and DNA by direct scavenging action upon short-lived radicals implies that melatonin is widely distributed in the cell. Indeed, melatonin is lipophilic (25) but also slightly hydrophilic (26), and such amphiphilicity allows it to reach, by diffusion or receptor-mediated mechanisms (27), all cell compartments, especially the nucleus and mitochondria where it is present in high concentration (28).

The GIT contains several hundred times more melatonin as compared to the pineal gland (14, 29-30). In particular, melatonin is present in high concentrations in the portal circulation, in the liver and in the bile (29, 31). In the GIT, melatonin is believed to synchronize the digestive processes, to regulate liver metabolism and to protect the intestinal epithelium, the liver and its annexes from the oxidant effects of biliary salts (14, 29-31). As a drug, melatonin can be administered in high doses without causing significant side-effects: more than 1200 mg/kg of melatonin have been administered to humans without signs of toxicity and no LD_{50} has not been identified to date (17, 32). Pharmacologically, melatonin has been found to limit I/R damage especially in experimental models of neuronal (33, 34), cardiac (35), renal (36, 37), hepatic (38-40) and gastrointestinal (41-42) injury. The capacity of melatonin to protect the liver from I/R was similar to that of N-acetyl-cysteine (NAC), a widely used antioxidant in clinical practice; the best results were obtained when melatonin and NAC were used in combination (43). A similar synergistic effect was observed between melatonin and a prostaglandin E1 analog (44).

Recently, we undertook an investigation to test the potential of melatonin to improve liver function using the isolated and perfused rat liver model. In normothermic perfusion of the liver, exogenous melatonin was demonstrated to cause an increase in bile production, in bile-bilirubin and in hepatic-ATP levels (45). Hence, the effects of reperfusion were studied with a medium containing melatonin of livers cold-preserved for 20 h with 2 of the most commonly used preservation solutions, the University of Wisconsin (UW) and the Celsior solutions. When livers cold-preserved for 20 h with UW or Celsior solutions were reperfused normothermically with a medium lacking melatonin, their ATP levels were much lower than those of the control liver (not submitted to I/R). In contrast, melatonin, added to the reperfusion medium, increased bile production, bile-bilirubin and ATP levels and reduced the damage to bile duct cells for both preservation solutions. Melatonin had no effect on the uptake of hyaluronic acid (an index of damage to sinusoid endothelial cells), nor on the formation of thiobarbituric acid reactive substances (TBARS) (index of lipid peroxidation) in the perfusate. These experiments showed that reperfusion with a melatonin-containing medium significantly improved the restoration of liver function after cold storage. While the mechanisms whereby melatonin augmented hepatic physiology in these studies remain unknown, it was presumed that positive effects at the level of the mitochondria accounted for the restored function (45), in agreement with the current speculations concerning melatonin effects at the mitochondrial level (24).

Our above-mentioned experiments were conducted using a biochemical approach, that has the disadvantage of not providing information regarding the response of the single cell types of the liver within their topological context in the hepatic lobulus, known by its metabolic zonation (46) and by lobular zone-dependent liver cell vulnerability to I/R (47). This kind of information is provided by a histochemical approach. The working hypothesis for the present research was that a melatonin-induced improvement of bile production and the enhanced ATP levels implied less damage to the tissue and better performance of hepatocyte mitochondria, with corresponding higher ROS leakage (10). The TBARS levels in the perfusate were expected to reflect ROS production by sinusoidal cells.

The presence of ROS *in situ* can be investigated with the DAB-Mn^{2+} method, devised originally by Briggs *et al.* to demonstrate superoxide anion in phagocytic cells (48) and improved in sensitivity and subcellular site of reaction detection by Kerver and co-workers, who included Co^{2+} and a tissue protectant (polyvinyl alcohol, PVA) in the incubation medium (49). In order to assess which enzyme(s) produced the ROS detected by the DAB-Mn^{2+}-Co^{2+} method, Kerver *et al.* (49) tested various scavengers. As sodium azide prevented the reaction completely, cytochrome C oxidase was held responsible for part of the generation of the final reaction product. The reaction was also prevented by specific inhibitors of NADH:coenzyme Q reductase and aldehyde reductase, suggesting that the

production of the colored product was also due to superoxide anion production by NADH:coenzyme Q reductase and aldehyde oxidase, followed by spontaneous singlet oxygen formation.

The DAB-Mn^{2+}-Co^{2+} method was employed to demonstrate ROS on frozen liver sections from the samples used for biochemical determinations. Since the Kerver *et al.* (49) experiments were conducted on the same rat liver model, their controls with enzyme inhibitors were not repeated here.

Materials and Methods

Reagents. Unless otherwise stated, all reagents were of the highest purity grade available and were purchased from Sigma (St. Louis, Mo, USA).

Cold storage model. Male Wistar rats (250-300 g) were allowed free access to water and food. The use and care of the animals in this experimental study was approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council. The rats were anesthetized with sodium pentobarbital (40 mg/kg *i.p.*) and, after opening the abdomen, received 250 units of heparin *via* the inferior vena cava prior to liver isolation. An intravenous catheter (16-gauge) was inserted into the portal vein and the liver was perfused *in situ* with Krebs-Henseleit bicarbonate (KHB) with 5 mM glucose under a continuous flux of 95% O_2 and 5% CO_2 mixture (4 ml/min/g) followed by the preservation solution (either ViaSpan™: DuPont Pharmaceuticals, Wilmington, DE, USA (UW) or Celsior: Pasteur Merieux Serums et Vaccins, Lyon, France) at 4˚C. An identical catheter was introduced into the thoracic inferior vena cava and was closed above the right renal vein. The liver was excised, immersed in 50 ml of preservation solution and stored for 20 h at 4˚C. After the cold ischemia period, the liver was reperfused at 37˚C with KHB buffer with 5 mM glucose for 2 h in a non-recirculating fashion under a continuous flux of 95% O_2 and 5% CO_2. Melatonin (100 μM) was included in the KHB solution during the reperfusion period of some livers. Flow rates remained stable for the duration of the perfusion and were not different between the 2 groups. Five animals were used for each experimental condition. For each, the liver was carefully and rapidly dried with a paper cloth, small pieces were cut and frozen immediately in liquid nitrogen and stored at –80˚C until use. For the control experiments (n=3), the liver, without further treatment, was cut into small pieces (about 5 mm thick), frozen immediately in liquid nitrogen and stored at –80˚C until use.

Histochemical test to demonstrate ROS. Sections 8 μm thick were cut on a manually-driven Leica CM 1850 cryostat at a cabinet temperature of –27˚C. The sections were placed onto clean glass slides and used immediately for the ROS tests.

The DAB-Mn^{2+}-Co^{2+} technique was used (49). In brief, unfixed cryostat sections were incubated for 30 min at 37˚C with a medium containing 12.5 mM DAB, 5 mM $MnCl_2$ and 40 mM $CoCl_2$ dissolved in 10% w/v PVA average mol. wt. 70,000-100,000), in 100 mM Tris-maleate buffer (pH 8.0). After incubation, the sections were rinsed in hot distilled water (60˚C) to stop the reaction immediately and to remove the viscous incubation medium. The sections were mounted in glycerol jelly. Since the colored product is light-sensitive, the slides were kept in the dark at 4˚C until observation. Observation and microphotography were carried out within 24 h of the experiments.

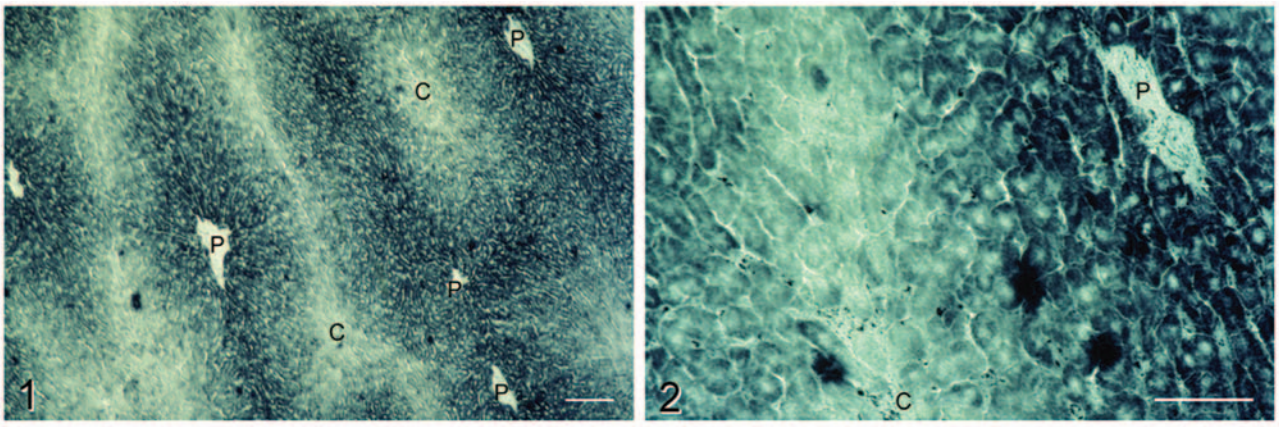
Microscopy and photomicrography. The staining ascribed to ROS was best evaluated by improving the optical contrast with Nomarski differential interference contrast (DIC); this allows referral of even scarce positive cells to their topological position within a poorly-stained or negative tissue area (39). The slides were observed with an Axioscop 2 *plus* light microscope. The control samples were photographed with a Zeiss MC63 system with Kodak 64T film; in this case, the color slides were digitized with an Epson SU1640 scanner at 1200 dpi resolution. Images of samples from cold-preserved, reperfused livers were captured with an Olympus 4.1 megapixel C-4040 Zoom digital camera. The digital images were processed with Paint Shop Pro 7.02.

Results

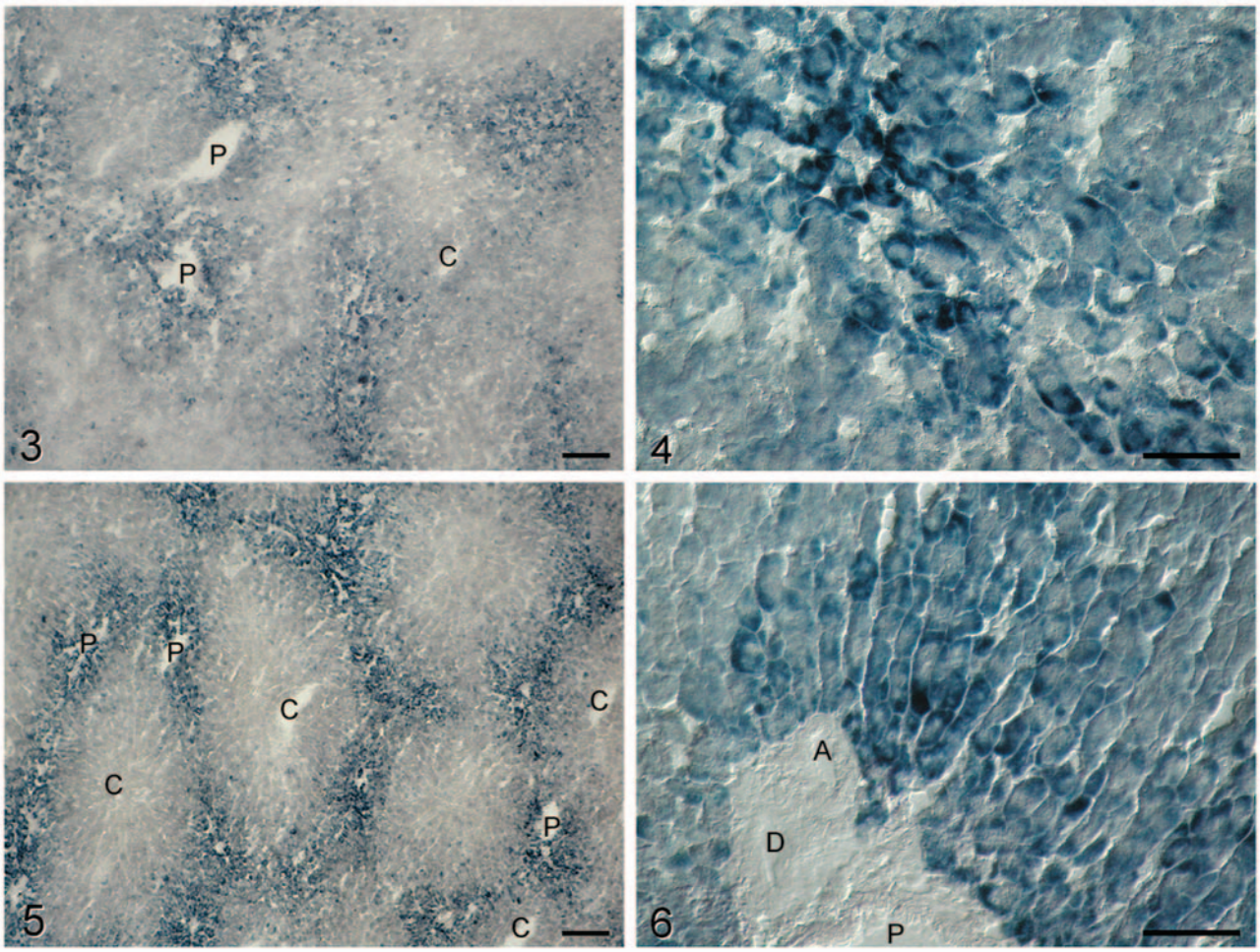
With the histochemical method employed to demonstrate ROS, the final reaction product is generated as a blue DAB-cobalt polymer.

Control livers. The reaction intensity was very high in the periportal area (PP), decreasing progressively through the mid-zone (MZ) to the pericentral (PC) region of the hepatic lobulus (Figure 1). In hepatocytes, the product was distributed in the cytoplasm, with an apparent polarization towards the sinusoids (Figure 2). Several sinusoidal cells displayed vesicular reactivity (Figure 2). The stromal cells and smooth muscle cells of the portal artery wall were also moderately-positive; bile duct cells were negative (not shown).

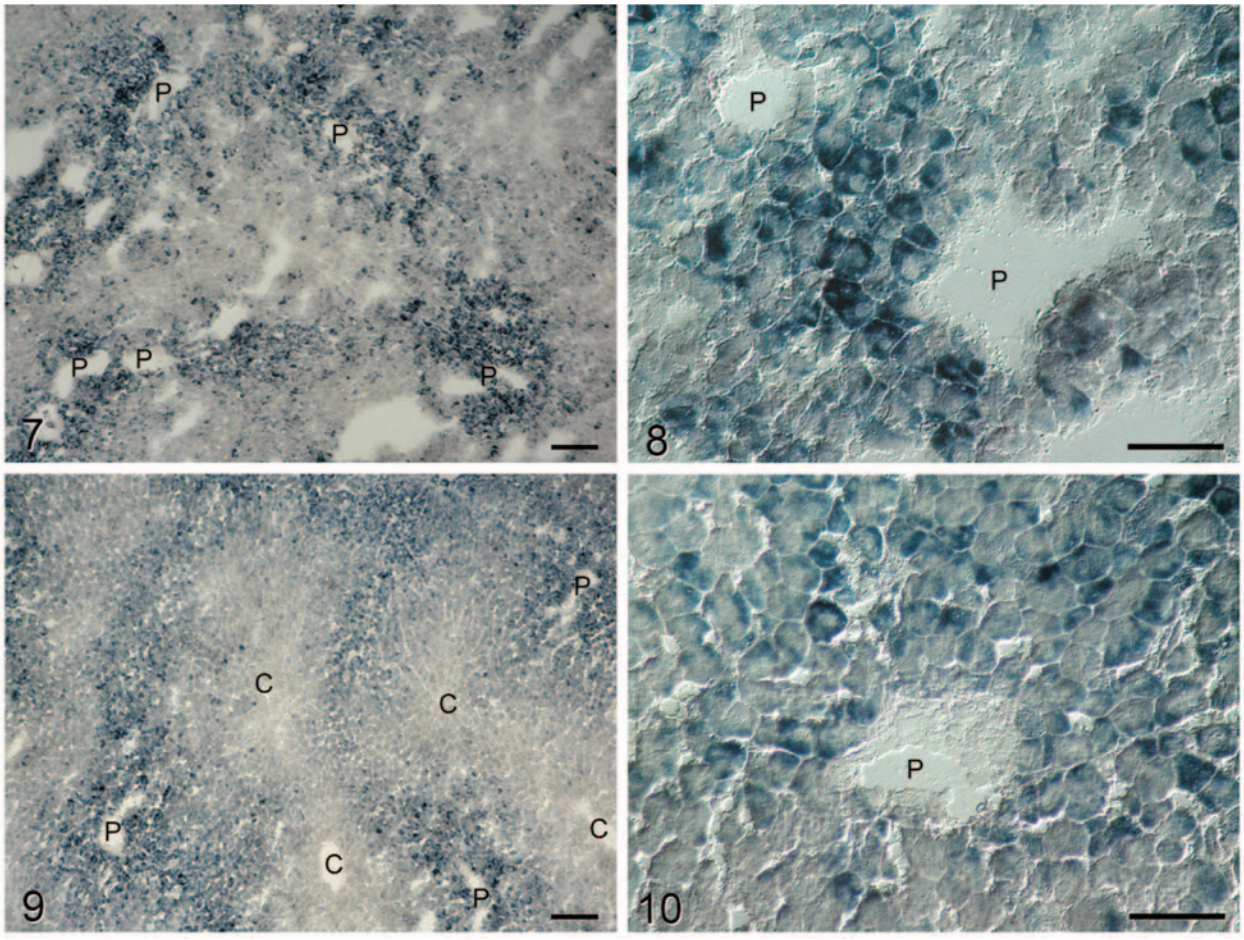
University of Wisconsin-preserved livers. In livers cold-preserved with UW solution and reperfused *without* melatonin, the parenchyma appeared highly disorganized and edematous (Figure 3). The sinusoids were dilated, especially in the MZ and PC regions. The ROS reaction was heterogeneous, and present almost exclusively in PP hepatocytes; in a few of these the staining was very intense and polarized towards sinusoids oriented parallel to the portal areas (Figure 4). When reperfusion of UW-preserved livers was made *with* melatonin, there was a marked improvement of the morphology with less edema and sinusoid dilatation (Figure 5). Though the intensity of the ROS reaction was not as high as in the control liver, a trend towards lobular staining zonation was again apparent (Figure 6). Intracellularly, the ROS reaction product was polarized toward sinusoids draining into the centrolobular vein and, in some cells, towards bile canaliculi (see Figure 11). Staining was also observed in a few sinusoidal cells.



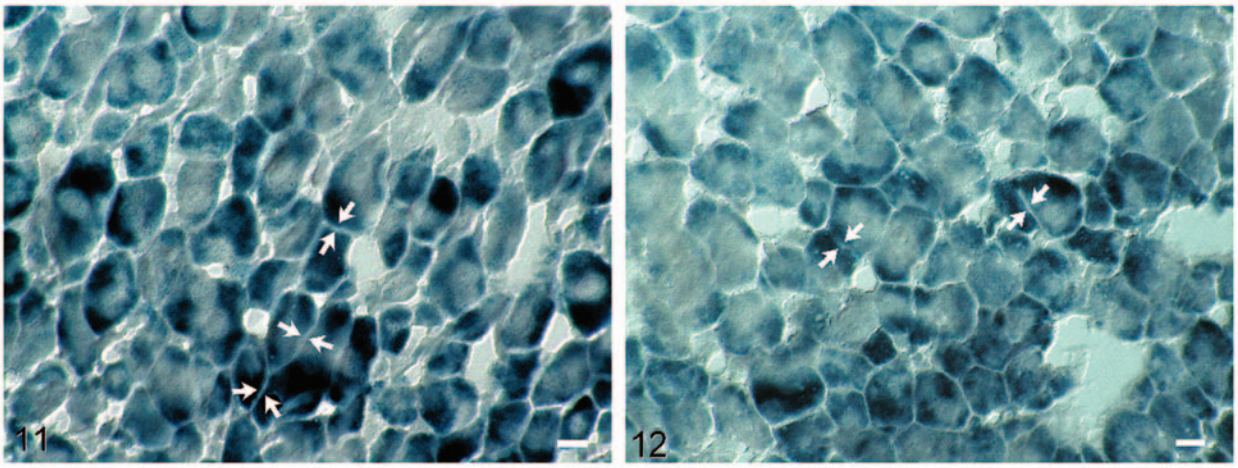
Figures 1-2. *Photomicrographs of the ROS reaction on control rat liver under low (Figure 1) and moderately-high (Figure 2) magnification. The zonation of the reaction is apparent. Figure 1: x10 objective (scale bar: 100 μm). Figure 2: x40 objective (scale bar: 50 μm). Differential interference contrast (DIC). P: portal vein; C: central vein.*



Figures 3-6. *ROS reaction of livers cold-preserved with University of Wisconsin solution and reperfused without (Figures 3, 4) and with (Figures 5, 6) 100 μM melatonin. When reperfusion was conducted without melatonin, the ROS reaction was present only in a narrow region of portal hepatocytes and the parenchyma was edematous. With melatonin, the morphology of the hepatocyte plates returned to normal; there was less edema and the ROS reaction extended to a wider area of portal hepatocytes. Figures 3 and 5: x10 objective (scale bar: 100 μm). Figures 4 and 6: x40 objective (scale bar: 50 μm). Differential interference contrast (DIC). P: portal vein; C: central vein; A: portal artery; D: bile duct.*



Figures 7-10. *ROS reaction of livers cold-preserved with Celsior solution and reperfused without (Figures 7, 8) and with (Figures 9, 10) 100 μM melatonin. Compared to livers preserved with University of Wisconsin solution, the parenchyma was more edematous when reperfusion was performed without melatonin. As with University of Wisconsin solution, when reperfusion was conducted without melatonin, the ROS reaction was present only in a narrow region of portal hepatocyte. A remarkable improvement of tissue morphology was seen when reperfusion was performed with melatonin. Figures 7 and 9: x10 objective (scale bar: 100 μm). Figures 8 and 10: x40 objective (scale bar: 50 μm). Differential interference contrast (DIC). P: portal vein; C: central vein.*



Figures 11-12. *Details under high magnification of the ROS reaction in portal areas of livers cold-preserved with University of Wisconsin (Figure 11) or with Celsior (Figure 12) solutions and reperfused with melatonin. Intracellularly, the reaction appeared extremely intense in some areas of hepatocytes and was often polarized towards bile canaliculi (arrows), suggesting that mitochondria therein were particularly active. Differential interference contrast (DIC). x63 objective (scale bar: 10 μm).*

Celsior-preserved livers. In livers subjected to cold preservation in Celsior solution and reperfused *without* melatonin, the parenchyma was even more edematous than for the liver preserved with UW. The ROS reaction was heterogeneously distributed in PP hepatocytes; a few positive sinusoid cells were also observed in the mid-zone and centrolobular region (Figure 7). The ROS reaction in hepatocytes was more polarized towards bile canaliculi than to sinusoids (Figure 8). When reperfusion was made *with* melatonin, there was a marked improvement of the structure of liver parenchyma (Figure 9). As with UW, the ROS reaction was more homogeneously distributed and extended to a larger number of PP cells (Figures 10 and 12). Compared to the control liver, the staining intensity was less and corresponded to a narrower portal area.

Discussion

The investigation herein described was intended to demonstrate ROS production *in situ* in cold-preserved livers reperfused normothermically with an oxygenated solution, either containing or lacking melatonin. Two preservation solutions (UW and Celsior) were compared. The previously reported biochemical data obtained on samples from the same experiments (45), and pertinent to the interpretation of the present histochemical data, are summarized. The ATP content of livers preserved with either UW or Celsior solutions and reperfused without melatonin had ATP levels about 7 times lower than the control livers (not submitted to cold-preservation). Reperfusion with melatonin caused significant increases of liver ATP and of bile production (indices of liver functionality after I/R) with respect to melatonin-free reperfusion, for livers preserved with both solutions. The ATP values obtained with a melatonin-containing medium were, nevertheless, much lower than the ATP levels of control livers (about 4 times lower for UW-preserved livers and 5 times lower for Celsior-preserved livers). Melatonin had no effect on the release of lactate dehydrogenase (LDH; index of hepatocellular damage), nor on the levels of TBARS (index of lipid peroxidation) evaluated into the perfusate. Melatonin also did not alter the uptake of hyaluronic acid (HA; index of damage to sinusoid endothelial cells), nor the balance of tissue levels of glutathione (GSH/GSSG ratio); the latter were, however, decreased compared to the controls. The biochemical data suggested that melatonin enhanced mitochondrial performance, but this caused no effect on oxidative stress nor injury to the hepatocytes and sinusoidal cells.

By using a histochemical approach, we sought not only a confirmation with a different technique other than the biochemical indications, but also for topological information regarding the behavior of hepatocytes and sinusoidal cells in the various lobular zones.

Control livers. The strong ROS reaction in the PP and MZ hepatocytes of control livers is in keeping with data published by Kerver *et al.* (49), who demonstrated that staining was mainly due to superoxide anions ($O_2^{\bullet -}$) and singlet oxygen. At the electron microscopic level, they found an electron dense reaction product both at the inner and outer membranes and at the intermembrane space of mitochondria. It is well-known that approximately 2-5% of consumed oxygen normally undergoes one-electron reduction with the formation of $O_2^{\bullet -}$ (50), mainly at the level of complex I (NADH coenzyme Q reductase) and complex III (ubiquinol cytochrome c reductase) (51). The leaked ROS are kept under control by endogenous antioxidants, such as Mn-superoxide dismutase and GSH (6) and, presumably, also by melatonin (52). The presence of ROS reaction in the PP and MZ hepatocytes thus indicates the intense flow of electrons through the ETC. The staining polarization towards the sinusoidal regions reflects higher energy production in this region, probably related to energy-dependent membrane transport processes. The lobular zonation of ROS production is consistent with oxidative energy metabolism predominating in the PP zone where oxygen tension is much higher than in the PC region (46). We also detected ROS in portal sinusoidal cells and in a few Kupffer-like cells in the MZ and PC regions. The ROS reaction in the sinusoidal cells might be due to NAD(P)H-oxidase activity in endothelial and Kupffer cells (53).

Cold-preserved livers submitted to I/R. Interstitial edema is a well-known drawback of reperfusion after cold preservation that all clinically-used organ preservation solutions attempt to overcome, though with different strategies and rates of success (54, 55). In our case, when reperfusion was carried out without melatonin, the livers cold-preserved with Celsior were more edematous than those preserved with UW. Reperfusion with melatonin improved the situation. Melatonin has been shown to prevent the edema caused by increased permeability of capillaries induced by warm ischemia-reperfusion in the hamster cheek pouch model of microcirculation and also to preserve capillary perfusion (56). This effect was ascribed to the detoxification by melatonin of superoxide anions produced by endothelial cells submitted to I/R. Furthermore, melatonin was reported to suppress cerebral edema caused by artery occlusion/reperfusion (57). In our model of cold I/R, the ROS reaction was not very intense in sinusoidal cells even when reperfusion was performed without melatonin and the indole had no effect on hyaluronic acid uptake by sinusoidal endothelial cells. The sinusoidal cells in our model, thus, did not appear to be suffering from oxidative stress. Therefore, the identified edema decrease might be due to melatonin effects other than its antioxidative capacity and, in particular, to its anti-inflammatory action (58).

Compared to the controls, the ROS reaction in hepatocytes was much less intense even when reperfusion was conducted with melatonin when livers were submitted to cold I/R. This is in keeping with the much lower ATP levels of I/R-submitted livers. Recalling that a low staining reaction did not correspond to lower levels of lipid peroxidation products in the perfusate, it can be presumed that the low production of ROS was not related to decreased oxidative stress, but rather to diminished metabolic activity due to the stress induced by cold-preservation followed by reperfusion.

In comparison with melatonin-free reperfusion, reperfusion with the indole caused an extension of the area occupied by ROS-positive hepatocytes, for livers cold-preserved with UW or Celsior solutions. This result is in agreement with the reported melatonin-induced increase in tissue ATP levels and in bile production, with respect to melatonin-free reperfusion. The biochemical analyses proved that there was neither a correspondent increase in LDH leakage from the parenchyma, nor in the levels of lipid peroxidation products in the perfusate. Therefore, the results of the present histochemical study suggest that increased ROS production did not enhance oxidative stress to the parenchyma. The presumed improved performance of the mitochondria might be explained by an increase in the activity of the ETC induced by melatonin, which was demonstrated to significantly increase the activity of the ETC complexes I and IV from brain and liver mitochondria (21, 23, 52). In addition, melatonin has been proven to scavenge H_2O_2 (18, 59), the most important ROS produced in mitochondria from $O_2^{\bullet -}$, thus avoiding the consumption of the intra-mitochondrial GSH pool and consequent mitochondrial damage. Interestingly, the reaction products of melatonin with H_2O_2 (*e.g.,* N-acetyl-N-formyl-5-methoxy kynuramine and N-acetyl-5-methoxy kynuramine) are also effective ROS scavengers (22, 60). Furthermore, since melatonin is highly lipophilic, it was thought to increase the fluidity of the inner mitochondrial membrane, protecting protein from oxidative damage (61). In addition, it has been reported that melatonin inhibited the production of NO within mitochondria and the formation of $ONOO^-$ (22). This effect could be relevant to the melatonin-induced increased ATP levels determined in our previous work, since NO has been demonstrated to be a potent reversible inhibitor of cell respiration and, if persisting in the cell environment, to induce impairment of ATP synthesis and, eventually, the opening of the membrane transition pore, followed by apoptosis and cell death (62). Research to document the effect of melatonin upon the expression and catalytic activity of NO synthases is currently under way in our laboratory.

It is necessary to stress that the efficacy of melatonin in neutralizing $O_2^{\bullet -}$ has been described as modest or poorly-defined (59, 63). Moderate concentrations of $O_2^{\bullet -}$ are believed to possess important signaling functions (7) that include the stimulation of strategies for responding and surviving in hypoxic conditions, such as vasodilatation, induction of glycolytic enzymes, erythropoiesis and eventually angiogenesis (64, 65). It may indeed be significant that melatonin, so efficient in quenching most ROS, seems to be ineffective upon $O_2^{\bullet -}$ and that physiological I/R events well-tolerated by several animal species (*e.g.,* arousal from hibernation, initiation of autonomic respiration in newborns or re-surfacing from diving) correspond to elevated production of endogenous melatonin (66).

In conclusion, by means of a different analytical approach, the published biochemical data that melatonin improves mitochondrial function were confirmed. The melatonin-induced improvement of morphology of the liver parenchyma and the trend towards a lobular zonation of ROS similar to that of the control liver support the conclusion, drawn from our former biochemical analyses, that melatonin in the reperfusion medium improves the restoration of liver function after cold preservation stress. Therefore, our histochemical data support the hypothesis that melatonin should be taken into serious consideration as a pharmacological strategy for allowing the liver to overcome I/R stress associated with transplantation (45, 67).

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