# Dipeptidylpeptidase-IV activity and expression reveal decreased damage to the intrahepatic biliary tree in fatty livers submitted to subnormothermic machine-perfusion respect to conventional cold storage

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## Abstract

Graft steatosis is a risk factor for poor initial function after liver transplantation. Biliary complications are frequent even after normal liver transplantation. A subnormothermic machine perfusion (MP20) preservation procedure was developed by our group with high potential for reducing injury to hepatocytes and sinusoidal cells of lean and fatty livers respect to conventional cold storage (CS). We report the response of the biliary tree to CS or MP20, in lean and obese Zucker rat liver. Dipeptidylpeptidase-IV (DPP-IV), crucial for the inactivation of incretins and neuropeptides, was used as a marker. Liver morphology and canalicular network of lean livers were similar after CS/reperfusion or MP20/reperfusion. CS preservation of fatty livers induced serious damage to the parenchyma and to the canalicular activity/ expression of DPP-IV, whereas with MP20 the morphology and canalicular network were similar to those of untreated lean liver. CS and MP20 had similar effects on DPP-IV activity and expression in the upper segments of the intrahepatic biliary tree of fatty livers. DPP-IV expression was significantly increased after MP20 respect to CS or to the controls, both for lean and obese animals. Our data support the superiority of MP20 over CS for preserving fatty livers. Dipeptidylpeptidase-IV activity and expression reveal decreased damage to the intrahepatic biliary tree in fatty livers submitted to subnormothermic machine-perfusion respect to conventional cold storage.

## Introduction

The critically short supply of normal organs compels that fatty livers (FL) be increasingly accepted for transplantation. Fatty livers are however particularly vulnerable to cold storage preservation by cold storage (CS) at 4°C, commonly employed for organ transplantation.[1-6] Our group has developed an alternative procedure for the preservation of normal and marginal livers (fatty livers, livers from non-heart beating donors) based on subnormothermic (20°C) machine perfusion (MP20).[7-10] In MP20 modality the organ is perfused at 20°C with an oxygenated Krebs-Henseleit (KH)-modified solution enriched with N-acetylcysteine, a glutathione (GSH) precursor, and $Ca^{2+}$.[7] MP20 allowed a better preservation of fatty liver respect to conventional CS in terms of morphology, enzyme release in the perfusate and bile, energy charge, glycogen stores, bile production, production of ROS, nitrites and nitrates and TNF-$\alpha$, and cell death by apoptosis of parenchymal and especially sinusoidal cells.[7-9,11]

Here we report a furthering of the investigation on the response of the various liver cell populations to MP20 and CS, by addressing the biliary tree. The biliary tree is considered the *Achille's heel* even of normal liver transplantation being responsible for long-term graft failure and late re-transplantation.[12-14] Biliary complications include a wide spectrum of functional and anatomical abnormalities and the time requested for the recovery of the biliary tree from preservation is much longer as compared with hepatocytes and sinusoidal cells.[15]

Even for normal liver, the literature regarding biliary complications does not mention the response of bile canaliculi where primary bile is produced.[16] It is plausible that the functional restoration of bile production after transplantation will depend on the integrity of the canalicular network. For evaluating the response of the biliary tree to these two modalities of preservation, we first sought an enzyme marker of the biliary tree of fatty liver demonstrable with enzyme histochemistry for revealing *in situ* the activity and with immunohistochemistry and Western blot for visualizing and quantifying protein expression. Alkaline phosphatase (E.C. 3.1.3.1), one the most commonly used markers of the biliary tree of normal liver, was ruled out, since it demonstrated to be scarcely active in the bile canaliculi of fatty liver;[17] furthermore, no antibody recognizing non-specific alkaline phosphatase of rat (our experimental model) was commercially available. Instead, Dipeptidylpeptidase-IV (DPP-IV; E.C. 3.4.14.5), also known as CD26, demonstrated to be a good *in situ* marker of biliary functionality not only of normal but also of fatty rat liver.[18] DPP-IV cleaves N-terminal dipeptides of proline or alanine-containing peptides including incretins [*e.g.*, glucagon-like peptide 1 (GLP1), gastric inhibitory polypeptide (GIP)], appetite-suppressing hormones [neuropeptide such as P substance, vasoactive intestinal peptide (VIP), neuropeptide Y] and chemokines.[19-23] The activity/expression of DPP-IV are strongly correlated to pathologies such as non-alcoholic fatty liver diseases (NAFLD), non-alcoholic steatohepatitis (NASH) and type II diabetes mellitus (T2D).[23-25] An altered regulation of the DPP-IV substrates incretins and neuropeptides plays an important role in the pathogenesis of obesity and insulin-resistance.[26] Since GLP-1 is a potent anti-hyperglycemic hormone, DPP-IV inhibitors are promising oral hypoglycemic and insulin-sensitizing drugs used in T2D.[27-30] Besides the enzymatic role, DPP-IV also function as a ligand; in particular, it interacts with several extracellular matrix (ECM) proteins, preferentially collagens I and III, and fibronectin.[31-33] DPP-IV can also contribute to ECM degradation,[23] by binding to adenosine deaminase and by activating plasminogen-2, leading to an increase in plasmin levels;[34] this in turn causes degradation of type IV collagen, fibronectin, laminin, and proteoglycan, and matrix metalloproteinases activation.[22,35] DPP-IV is currently been investigated as a target of warm ischemia-reperfusion injury (I/R) in organs such as lungs, heart and kidney.[36]

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Finally, DPP-IV was recently shown to regulate stem/progenitor and more differentiated hemopoietic and other cell behavior by truncating chemokines, colony stimulating factors and interleukins.[37]

We report the effect of MP20 or of CS followed by reperfusion on the activity and expression of DPP-IV and ultrastructure of the biliary tree of obese and lean Zucker rat liver. Our data indicate that MP20 allows a better preservation of the biliary tree respect to CS.

## Materials and Methods

### Chemicals

Unless otherwise stated all reagents were of the highest purity grade available and were purchased from Sigma (Milan, Italy).

### Animals

As liver donors, 11-12 week old obese (*fa/fa*) (375±15 g) and lean (*fa/+*) (300±10 g) male Zucker rats (Charles River, Calco, LC, Italy) were used. The animals were allowed free access to water and food in all the experiments. The use and care of animals in this experimental study was 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 and the liver was washed out with an oxygenated Krebs-Henseleit (KH) medium containing in mmol/L: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 1.25 $CaCl_2$, 25 $NaHCO_3$, 20 HEPES (pH 7.4), 5 glucose and 5 n-acetyl-cysteine (NAC), pH 7.4 (4 mL/min/g of liver) and removed.[38] Samples of control livers were obtained from lean and obese rats immediately after washout.

### Cold storage

After washout with KH, the livers were flushed *in situ* with University of Wisconsin solution (UW) for 2 min and maintained at 4°C in this solution for 6 h. The selected temperature was maintained by a heat exchanger (Julabo-F12, Seelbach, Germany).

### Subnormothermic (20°C) machine perfusion (MP20)

The liver was placed in an organ chamber and connected to a recirculating, constant-flow perfusion equipment containing 200 mL KH medium for 6 h. The KH solution was re-circulated by the roller pump (4 mL/min/g) (Gilson Miniplus-3, Middleton, WI, USA), oxygenated and maintained at 20°C by a heat exchanger (Julabo-F12). 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. At the end of MP20 preservation, normothermic reperfusion was started by switching to a reservoir containing fresh solution at 37°C.[7]

### Normothermic reperfusion

Reperfusion with KH (2 h at 37°C) was performed in the same set up as MP20 both in CS and MP20 preserved livers.

### Morphology

Liver samples were quickly removed, and small fragments were fixed by immersion in 2.5% glutaraldehyde in 0.13 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. Semithin sections (1 µm) were stained with 1% Toluidine Blue. 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 (Carl Zeiss, Oberkochen, Germany) electron microscope operating at 80 kV.

### Demonstration of DPP-IV enzyme activity

Simultaneous azo-coupling method[39] was performed on fixed cryosections by incubation at 37°C for 30 min with a medium containing:

- PVA (polyvinyl alcohol) 18% in TRIS maleate buffer 0.1M pH 8
- Gly-Pro-methoxy-β-naphthylamide 1mM
- NaCl 100mM
- Fast Blue BB 2mM

To stop the reaction and remove the incubation medium, the sections were rinsed with hot (45-50°C) tap water and the slide mounted with glycerin-jelly. With this method the activity of DPP-IV is revealed by an orange-red final reaction product. Control reactions were performed in the absence of substrate or with the selective competitive inhibitor Diprotin-A (Ile-Pro-Ile);[19] in both cases the reaction was negative.

### Demonstration of DPP-IV expression

In order to block aspecific binding of antibodies and fluorochrome, fixed cryosections were pre-incubated for 30 min at room temperature in phosphate buffered saline solution (PBS) containing 10% normal goat serum, 3% bovine serum albumin (BSA) and 1.5% NaCl. Sections were then incubated in the dark for 1 h at room temperature with the primary monoclonal antibody mouse anti-rat CD26/DPP-IV (5E8 clone) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:150 in PBS containing 10% normal goat serum, 3% bovine serum albumin (BSA) and 1.5% NaCl. The slides were then rinsed twice for 5 min in PBS containing 0.2% Triton-X and once for 5 min in PBS. The sections were hence incubated for 30 min in the dark at room temperature with the secondary antibody, Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L) (Molecular Probes Inc., Eugene, OR, USA) diluted 1:700 in PBS. Afterwards, the sections were rinsed 3 times for 5 min in PBS containing 0.2% Triton-X. With this method, DPP-IV expression was revealed by an intense red fluorescence. Control reactions were made by replacing the primary antibody with PBS; in this case, with the green excitation used for revealing Alexa Fluor® 594 red emission, only a faint tissue autofluorescence was observed.

### Microscopy and photomicrography

The slides were observed with a Zeiss Axioskop 2 Plus light microscope (Carl Zeiss Microimaging, Jena, Germany) equipped with Differential Interference Contrast (DIC) system. The emission of Alexa Fluor® 594 was detected using the Zeiss Filter Set 15 (excitation: BP 546/12; beam splitter: FT 580; barrier filter: LP 590). Photomicrography was made using a Canon EOS 1100D digital camera (Tokyo, Japan) set to a resolution of 6 MPixel.

### Image analysis of DPP-IV activity distribution

These were performed with Image-Pro Plus 4.5 software (Media Cybernetics, Rockville, MD, USA) on 3088×2056 pixel microphotographs captured from immunofluorescence images with a Canon EOS 1100D at 20x magnification, corresponding to 303.164 $\mu m^2$ area. The system was previously spatially calibrated with an object-micrometer slide. Measurements were exported in Microsoft Excel™ and then in IBM SPSS 20 software for statistical analysis.

### Western blot

Tissues were lysed with CelLytic™ MT Mammalian supplemented with Protease Inhibitor Cocktail. Lysates were then clarified and centrifuged for 10 min at 4°C. Supernatants were collected in Eppendorf vials. Protein concentration was measured with a spectrophotometer (UV-1601, Shimadsu, Tokyo, Japan) set to 562 nm, using Pierce BCA Protein Assay Kit (bicinchoninic acid - Thermo Fisher Scientific Inc., Rockford, IL, USA). Sample buffer (3X) consisting of 6% SDS, 1.5% DTT, 30% glycerol, 0.03% bromophenol blue, 0.5 M Tris-Gly buffer (1X, pH 8.3 containing SDS 0.1%) was added to the appropriate aliquots of supernatants. After boiling for 3 min at 95°C, equals aliquot of protein (20 µg per lane for a 10 comb gel) were separated by SDS-PAGE on an 10% gel using

Tris-Gly buffer under standard conditions, followed by wet-transfer onto PVDF membrane (BIO-RAD Laboratories, Hercules, CA, USA) at 4°C for 2 h. After blocking with 0.1% Tween-20-TBS and 5% BSA in Tris-buffered saline for 1h at room temperature, membranes were incubated overnight with anti-DPP-IV mouse monoclonal primary antibody (sc-52642, clone 5E8, Santa Cruz Biotechnology) diluted 1:2000 and anti-ß-actin primary antibody Mouse IgG, (BIO-RAD Laboratories) dilution 1:10000 in the blocking solution. Membranes were then washed with PBS containing Tween-20 at 0.5% at room temperature, and then incubated with a mouse HRP-conjugated secondary antibody anti-Mouse Goat IgG (BIO-RAD Laboratories) diluted 1:3000 for 1h at room temperature. After washing 6 times at room temperature in PBS containing Tween-20 at 0.5%, membranes were incubated with MILLIPORE Immobilon Western Chemiluminescent HRP Substrate [SuperSignal West Dura (Pierce, Thermo Fisher Scientific Inc.)] for 5 min and then visualized by ChemiDoc™ XRS+ System (BIO-RAD Laboratories).

### Statistical analysis

The data regarding DPP-IV activity in bile canaliculi were expressed as means ±SD and analyzed by one-way ANOVA with Tuckey test as *post-hoc*. The data concerning DPP-IV expression (evaluated with Western Blot) were analyzed by two-way ANOVA with Tuckey test as *post-hoc*. A two-sided P value <0.05 was considered as significant. Kolmogorov Smirnov/Shapiro Wilk tests and Levene test were applied to evaluate end-point variable normality and variances homogeneity respectively. All the analyses were carried out using IBM SPSS 20 software.

## Results

### Response of lean livers to cold storage or to MP20 followed by normothermic reperfusion: enzyme and immunohistochemistry evaluations

In Figure 1, the catalytic activity of DPP-IV, demonstrated with the azo-coupling method which gives a red-orange reaction product, is illustrated for control lean Zucker rat livers (Figure 1 a,d), for lean rats submitted to CS and reperfusion (Figure 1 b,e) and for lean rat livers submitted to MP20 and reperfusion (Figure 1 c,f). In control lean liver the final reaction product was present at all levels of the biliary tree (Figure 1 a,d). In hepatocytes, it showed the typical *chicken-wire* canalicular pattern (Figure 1a). In Hering canals and bile ductules, the product was present in the cytoplasm and plasma membrane. In larger bile ducts it was present mainly in the apical pole (Figure 1d). The reaction was furthermore present, though much less intense, in sinusoidal endothelial cells. In lean livers preserved by CS and reperfused (Figure 1 b,e) the morphology and canalicular DPP-IV pattern was relatively well preserved in most areas. A few regions were edematous and the canalicular DPP-IV activity was less intense and more blurred respect to the controls. Sinusoidal cells were less positive than in the controls. In lean livers preserved by MP20 and reperfused (Figure 1 c,f), the tissue morphology was in general better-preserved respect to CS-treated lean livers, but a few edematous area was present. The *chicken-wire* pattern of canalicular DPP-IV activ-

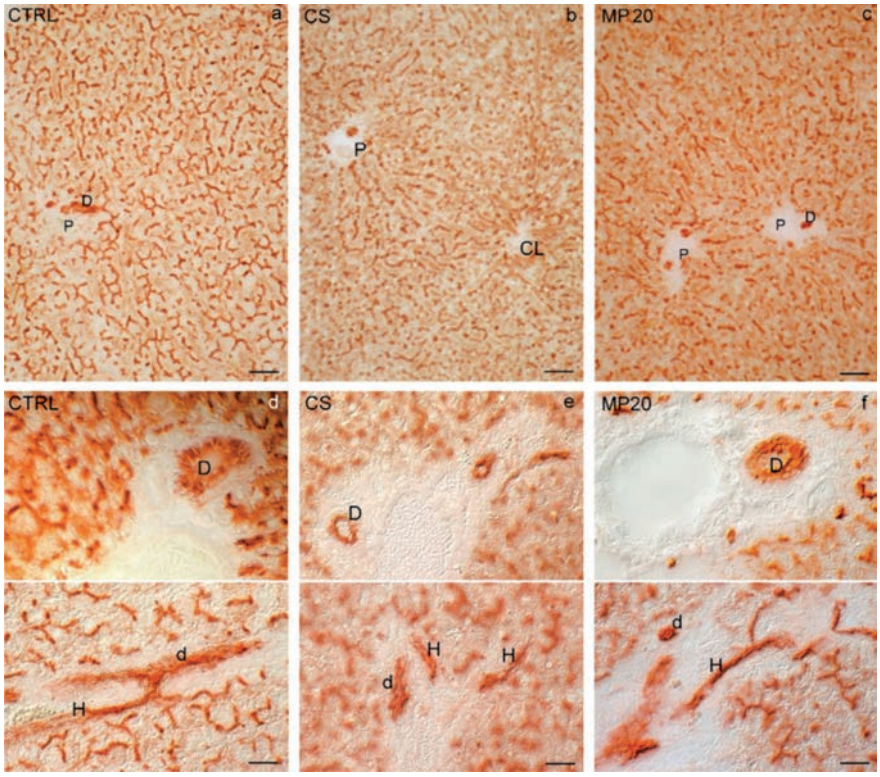


**Figure 1. Enzyme activity of DPP-IV revealed with azo-coupling reaction in a,d) control lean Zucker rat liver; b,e) lean liver submitted to CS/reperfusion; c,f), lean liver submitted to MP20/reperfusion; 8 µm thick cryostat sections. P, portal vein; CL, centrolobular vein; D, bile duct, d, bile ductule; H, Hering canal. Scale bars: a,b,c) 50 µm; d,e,f (Differential Interference Contrast, DIC) 25 µm.**

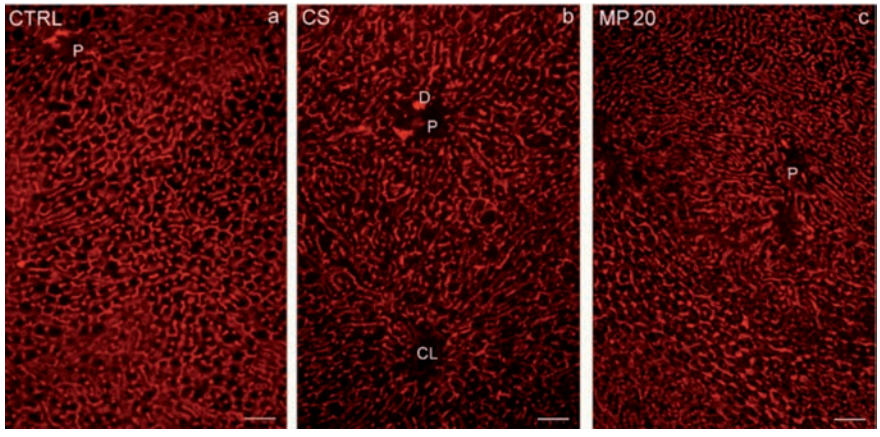


**Figure 2. Expression of DPP-IV revealed by immunofluorescence in a) control lean Zucker rat liver; b) lean liver submitted to CS/reperfusion; c) lean liver submitted to MP20/reperfusion; 8 µm thick cryostat sections. P, portal vein; CL, centrolobular vein; D, bile duct. Scale bars: 50 µm.**

ity was in general well conserved. Sinusoidal cells had low activity, similar to the controls.

In Figure 2 is presented the expression of DPP-IV, revealed by an immunofluorescence technique, in control lean liver (Figure 2a), and in lean livers submitted to CS and reperfusion (Figure 2b) or to MP20 and reperfusion (Figure 2c). The tissue distribution of the red emission corresponded in general, for all three situations, to the pattern of enzymatic activity.

## Response of obese livers to cold storage or MP20 followed by normothermic reperfusion: enzyme and immunohistochemistry

In Figure 3, the catalytic activity of DPP-IV is illustrated for control obese Zucker rat livers (Figure 3 a,d), for obese rats submitted to CS and reperfusion (Figure 3 b,e) and for obese rat livers submitted to MP20 and reperfusion (Figure 3 c,f). In control fatty liver, DPP-IV activity was intense in bile ducts (Figure 3d). A canalicular *chicken-wire* pattern was present only in normal hepatocytes (Figure 3 a,d) whereas in steatotic hepatocytes, particularly abundant in the mid-zone, it was discontinued and occasionally displaced towards the cell periphery, as previously reported.[18] Sinusoidal endothelial cells displayed no activity. In fatty livers submitted to CS and reperfused (Figure 3 b,e) DPP-IV activity was intense in bile ducts, ductules and Hering canals (Figure 3e). The parenchyma was particularly damaged in the mid-zone; elsewhere the *chicken-wire* canalicular pattern was more regular. Endothelial cells were negative. In fatty livers submitted to MP20 and reperfused (Figure 3 c,f) the DPP-IV activity in Hering canals, bile ductules and bile ducts was intense (Figure 3f). The canalicular pattern was much more regular than for CS-treated livers and was similar to that of the control lean liver. Sinusoidal endothelial cells presented low activity. The immunofluorescence determination of DPP-IV expression in controls and treated livers, in keeping with the patterns of DPP-IV activity, is shown in Figure 4.

## Mean area distribution of the canalicular reaction product of the DPP-IV enzyme reaction: comparison of lean and obese rat liver response to preservation

In Figure 5 the areas occupied by the canalicular DPP-IV enzyme activity of control and treated lean and obese rat livers are shown. For lean rat livers no significant differences were found between controls and CS- or MP20 treated livers. By contrast, for fatty livers the canalicular area of CS-treated livers was significantly lower than that of the control fatty liver whereas the area of MP20-treated livers was significantly higher than that of the control liver.

## DPP-IV protein concentration evaluated by Western blot: comparison of lean and obese rat liver response to preservation

In Figure 6, Western blots for DPP-IV expression of untreated (controls), CS-treated and MP20-treated livers are presented; the optical densities of the DPP-IV bands are standardized for the β-actin band and normalized respect to the controls. In Figure 7 an analysis is presented, for each phenotype, of the response to the two different preservation modalities. Both for lean and obese animals, the expression of DPP-IV was significantly increased after MP20 treatment respect to the controls and respect to CS-treated livers. The MP20 type of treatment induced significant differences on the optical density levels.

In Table 1 a statistical analysis of the optical density values is shown as a function of the

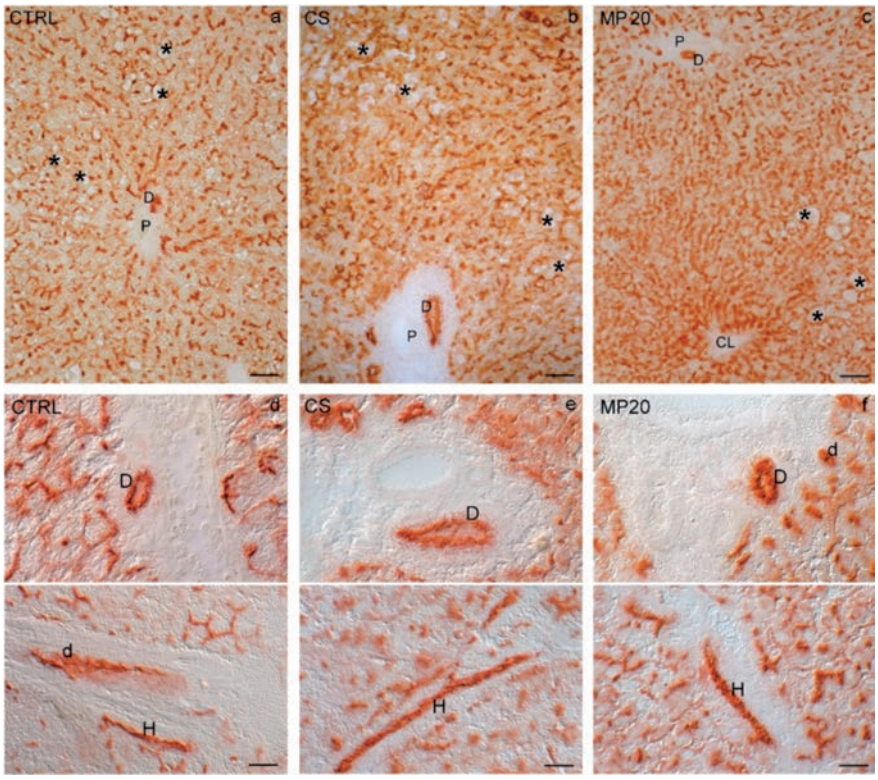


**Figure 3. Enzyme activity of DPP-IV in a,d) control fatty Zucker rat liver; b,e) fatty liver submitted to CS/reperfusion; c,f) fatty liver submitted to MP20/reperfusion; 8 µm thick cryostat sections. P, portal vein; CL, centrolobular vein; D, bile duct; d, bile ductules, H Hering canal; asterisks, lipid droplets. Scale bars: a,b,c) 50 µm; d,e,f (Differential Interference Contrast, DIC) 25 µm.**

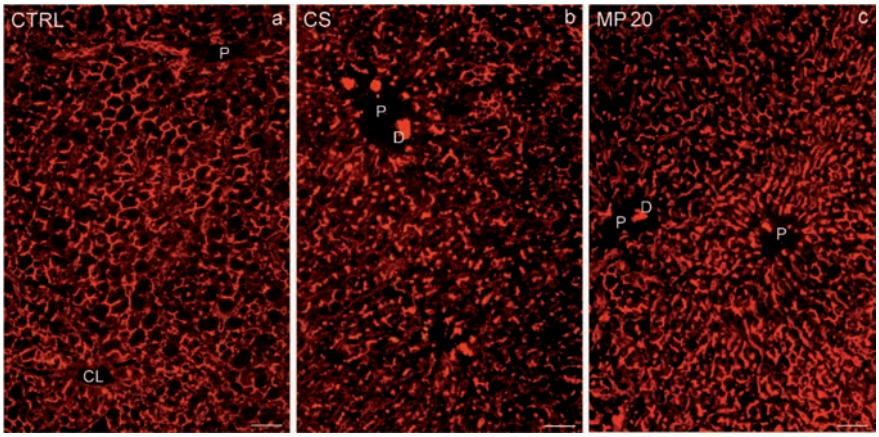


**Figure 4. Expression of DPP-IV in a) control fatty Zucker rat liver; b) fatty liver submitted to CS/reperfusion; c) fatty liver submitted to MP20/reperfusion; 8 µm thick cryostat sections. P, portal vein; CL, centrolobular vein; D, bile duct. Scale bars: 50 µm.**

factors phenotype (lean, obese) and treatment (CS, MP20). The mean optical density level after MP20 was significantly higher than after CS and control, irrespectively of the phenotype factor. As the treatment factor is concerned, the observed differences between cold storage treatment and control were not significant. No significant difference was seen between phenotype levels. In addition, the interaction term between the two factors did not result significant. This implies that MP20 causes a significant enhancement of the DPP-IV expression, independently of the lean or obese phenotype.

## Morphological analysis of obese rat liver response to preservation on semi-thin and ultra-thin sections

In Figure 8, the morphology of control obese Zucker rat liver (Figure 8a), assessed in Toluidine Blue-stained semi-thin sections, is compared to that of fatty livers treated with CS followed by reperfusion (Figure 8b) or with MP20 followed by reperfusion (Figure 8c). Control obese rat liver was characterized by abundant small and large lipid droplets in the cytoplasm of hepatocytes, especially in the mid-zone; blue-stained and unstained droplets indicated the presence of unsaturated and saturated lipids, respectively. Bile canaliculi, easily identified in periportal hepatocytes only, were not evident in steatotic regions likely hidden by lipid droplets, as previously reported.[18] In fatty livers submitted to CS several hepatocytes showed intercellular edema and compressed nearby cells; sinusoids and canaliculi were dilated (Figure 8b). By contrast, in fatty livers submitted to MP20 (Figure 8c), steatosis was much less pronounced and the hepatocytes morphology, in particular their canalicular network, was similar to that of normal lean liver.

Electron microscopy provided further information regarding the liver response. Preservation by CS caused necrosis of several hepatocytes in the mid-zone (the most steatotic region of control fatty liver) (*not shown*). The less damaged hepatocytes still contained lipid droplets but had less glycogen stores than the controls (*not shown*). Figure 9 shows representative transmission electron micrographs at the same magnification of bile canaliculi in the mid-zone of control fatty liver (Figure 9a) and in fatty liver submitted to CS (Figure 9b) or MP20 (Figure 9c) followed by reperfusion. In the control fatty liver, hepatocytes in steatotic regions had bile canaliculi containing abundant microvilli (Figure 9a). In fatty livers submitted to CS, the canaliculi between damaged hepatocytes were dilated, had fewer microvilli and contained cell debris (Figure 9b). In MP20-submitted livers, the canaliculi formed between hepatocytes with normal morphology contained less microvilli respect to control liv-

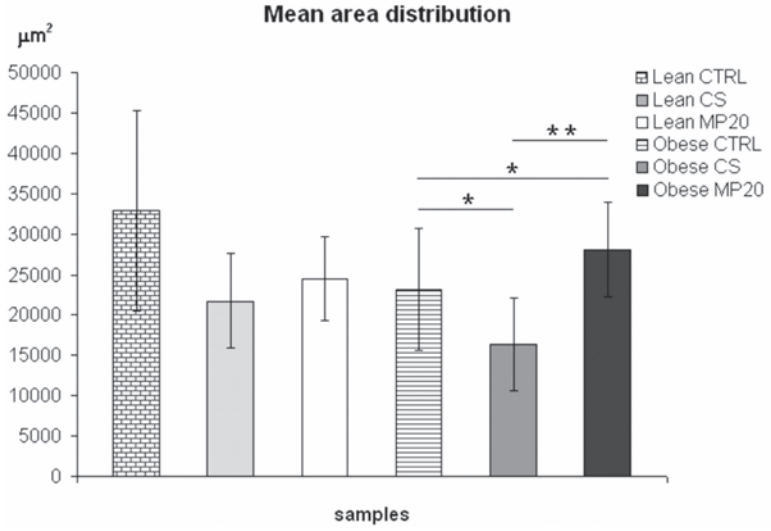


**Figure 5. Image analysis of mean area distribution of DPP-IV expression in lean and obese rat with and without treatments (CS and MP20) visualized at bile canaliculi level. Each bar represents the mean area ± SD of 18 random measurements per different rat model (n=3 for each treatment). The horizontal bars show the statistical significant differences inside obese group (P<0.05), where *P=0.04 and **P=0.01.**

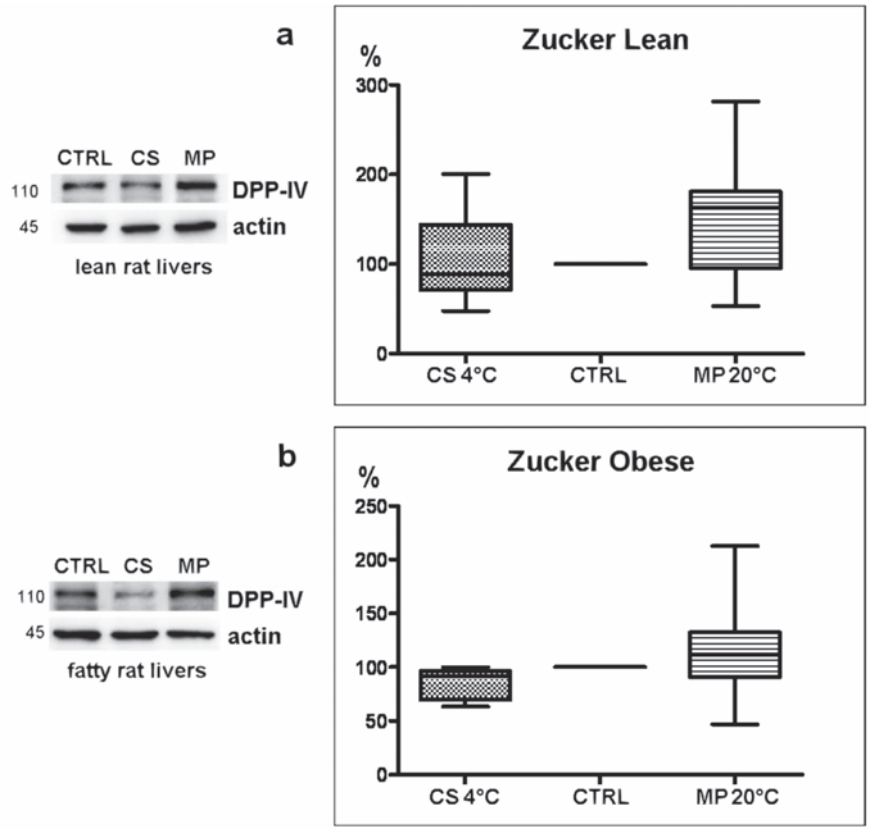


**Figure 6. Western Blot analysis of DPP-IV expression. a) Comparison between control and treated (CS 4°C and MP 20°C) lean Zucker rat livers; boxplot represents the percentage ratios between optical density (OD) values normalized respect to that of control lean livers. b) Comparison between control and treated (CS 4°C and MP 20°C) obese Zucker rat livers; boxplot represents the percentage ratios between optical density (OD) values normalized respect to that of control fatty livers (ß-actin as equal loading).**

ers, but were much better preserved respect to CS (Figure 9c). In MP20-preserved fatty liver most bile ducts and the portal stroma were well-preserved (Figure 10b) and were similar to those of the control fatty liver (Figure 10a). Although CS preservation caused edema in the portal stroma and injury to bile canaliculi, the upper biliary segments appeared less injured (Figure 11).

## Discussion

This paper describes the behavior of the biliary tract of lean and obese rat livers submitted to two different procedures of preservation used for liver transplantation, in both cases followed by normothermic oxygenated reperfusion. The conventional cold storage with University of Wisconsin solution (CS) and subnormothermic machine perfusion (MP20), a modality formerly developed by our group,[7] were investigated. The previous experiments had shown that MP20 afforded a better preservation respect to CS, especially for obese rat livers, as hepatocytes and sinusoidal cells are concerned.[7,9] Few observations suggested a better protection also of the biliary components: livers from obese rats preserved by MP20 produced more bile and released less alkaline phosphatase or glutamyl transpeptidase into the bile than those preserved by CS.[7,9] In the current publication, we present evidence that supports the hypothesis that MP20 is also protective for the functionality of the biliary tree, especially of fatty liver.

DPP-IV is a key enzyme for the truncation, and thus inhibition or alteration of the function, of several bioactive peptides such as incretins, appetite-suppressing hormones (neuropeptides) and chemokines.[20,22] In a previous paper, we suggested that probable substrates of DPP-IV in hepatocytes and cholangiocytes could be the incretins GLP-1 and GIP, and neuropeptides such as vasoactive intestinal peptide (VIP) and substance P, implying a biliary route for the inactivation of these substances.[18] For the lean Zucker rat liver, CS and MP20 gave similar patterns of distribution of enzymatic activity and immunolocalization of DPP-IV in the various tracts of the intrahepatic biliary tree. In keeping with these histochemical observations, no significant differences in the areas occupied by the reaction product of the enzymatic reaction was seen. The quantitative evaluation of protein expression by Western blot revealed a significant increase of the protein after MP20 preservation respect both to the untreated controls and to CS-preserved livers. We speculate that this increase is due to the presence of glucose in the MP20 perfusion medium. In fact, in HepG2 cells, a human hepatoblastoma-derived cell line, high glucose concentration in the medium significantly enhanced DPP-IV expression.[24]

In contrast with lean rat liver, the tissue morphology and the activity/expression of DPP-IV of obese rat liver showed a significantly different response to CS respect to MP20. In the untreated control fatty livers, the canalicular pattern of DPP-IV activity was disarranged, respect to untreated lean liver, by the presence of lipid droplets especially in the mid-zone, as previously described.[18] These patterns are similar to those reported for patients with non-alcoholic steatohepatitis (NASH).[32]

Cold storage of fatty livers caused much more damage to the mid-zone parenchyma than MP20, as previously reported.[7,11] The mid-zone is indeed prone to cold-induced solidification of lipids.[4] As to the reduction of bile canaliculi after CS, this might be due to hypoxia-induced ATP depletion causing actin depolymerization.[40] As MP20 of fatty liver is concerned, several features could explain the better preservation of hepatocytes in general and of canaliculi in particular: i) at 20°C the lipid droplets do not solidify; ii) perfusion in MP20 is made with an oxygenated solution containing glucose, an energy-precursor;[7,8] the ATP/ADP ratio was indeed significantly higher in MP20-preserved fatty livers respect to CS-preserved organs;[8,9] iii) The MP20 perfusion medium contains also NAC, a GSH precursor. Indeed, the intracellular concentration of GSH was previously seen by our group to be significantly higher for MP20 respect to CS.[8] Since the liver of fatty Zucker rats contains less antioxidant substances [*e.g.*, glutathione (GSH), tocopherol, catalase] respect to lean animals,[41] the higher intracellular GSH concentration probably protects the cells from peroxidative damage after reperfusion.

The apparent normalization of the *chicken-wire* patterns of canalicular DPP-IV activity/expression in MP20-preserved fatty livers could explain the significantly larger area of DPP-IV activity in MP20-preserved fatty livers respect either to CS-preserved organs and even respect to the controls. The decrease in lipids induced by MP20 in uninjured hepatocytes, appraised in the semi-thin sections stained by Toluidine Blue, suggests that the lipids have been metabolized at 20°C and/or during normothermic reperfusion. Reduction of steatosis induced by normothermic MP has been occasionally reported.[42,43]

As the upper segments of the intrahepatic

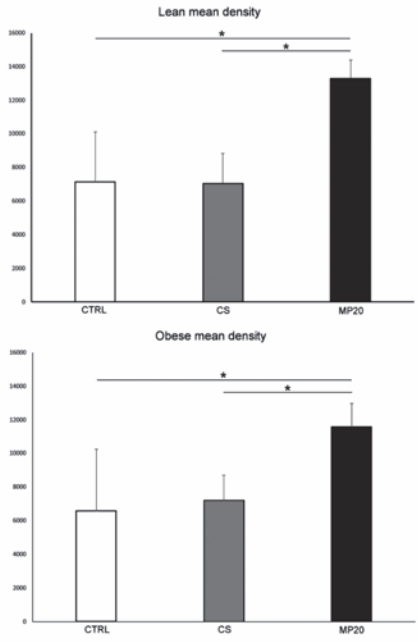


**Figure 7. Comparison between the mean optical density obtained by Western blot of control and treated (cold storage and MP20) lean and obese Zucker rat livers (n=25). Mean ± SD is shown. Statistical analysis performed using 1-way ANOVA test by IBM SPSS 20 software. *P<0.05.**

**Table 1. Comparison of the optical density of the Western blot bands for different conditions of phenotype (lean, obese) and treatment. Two-way ANOVA analysis.**

| Model | N. | Mean ± SD | Test and P value |
|---|---|---|---|
| Phenotype | | | |
| Lean | 11 | 9647.48±3230.56 | f=0.38 P=0.5460 |
| Obese | 14 | 9175.54±3105.42 | |
| Treatment | | | |
| CS | 8 | 7309.6±1962.17 | f=15.07 P<0.001 |
| MP | 12 | 11835.98±1569.89 | |
| Control | 5 | 6814.27± 3060.01 | |
| Phenotype *vs* treatment | | | f=0.05 P=0.9500 |

CS, cold storage; MP, machine perfusion.

biliary tree are concerned, no significant difference in the qualitative patterns of DPP-IV activity/expression in CS- and in MP20-preserved fatty livers was seen respect to the controls. This suggests that these segments of the biliary tree are less vulnerable than bile canaliculi in hepatocytes to the stresses induced by preservation/reperfusion. It is worth recalling that Hering canals (that connect bile canaliculi to bile ductules), and bile ducts are believed to harbor the hepatic stem cell niche.[44,45] DPP-IV has been reported to be a good marker of hepatic stem cells and to play a crucial role in hepatic regeneration.[23]

In the setting of transplantation of fatty livers, the increase of DPP-IV expression induced by MP20 warrants particular attention. In fact, DPP-IV is currently being considered as a therapeutic target for decreasing injury induced by warm or cold ischemia followed by reperfusion to organs such as heart, kidneys and lungs, although with conflicting results.[36] In particular, pharmacological inhibition of DPP-IV is currently being investigated with the aim of preventing the inactivation of incretins, cytokines and neuropeptides (*e.g.*, GLP-1, stromal cell-derived factor-1α and vasoactive intestinal peptide) involved in the control of the inflammatory response and in the mobilization of stem/progenitor cells to sites of injury.[46-50]

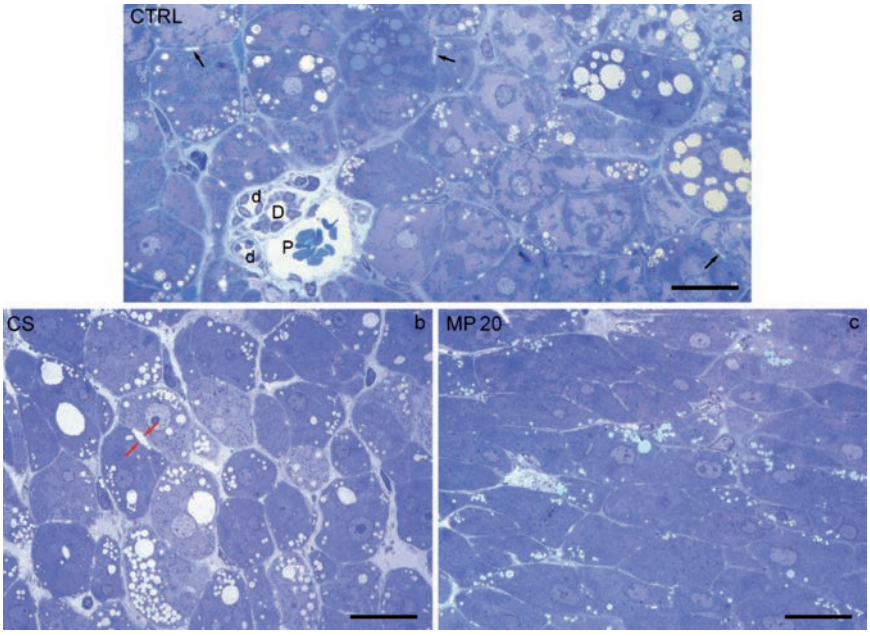


**Figure 8. Semi-thin sections of a) control obese Zucker rat livers; b) treated with cold storage (CS); red arrows, dilated bile canaliculi; c) subnormothermic machine perfusion (MP20). Bile canaliculi are similar to those of control fatty liver; stained with Toluidine Blue, 1 μm thick semithin sections. Black arrows, bile canaliculi; P, portal vein; D, bile duct; d, small bile duct. Scale bars: 20 μm.**

## Conclusions

This study reports for the first time the morphofunctional response of the bile tree to a conventional (CS) and to an innovative (MP20) procedure to preserve fatty livers for transplantation. DPP-IV proved to be sensitive marker of all the bile tree segments. DPP-IV activity and expression in bile canaliculi is shown to be better preserved after MP20/reperfusion than after CS/reperfusion, whereas the upper intrahepatic biliary segments appear unharmed by the two modalities. These observations support and further the previously obtained data showing that MP20 induces significantly less injury to liver cell populations respect to CS and this may be a positive issue.

However, in a potential receiver, the augmented DPP-IV expression might cause a fast inactivation of incretins and neuropeptides, important for the correct functioning of the liver and its interaction with other organs of the gastrointestinal tract and of cytokines involved in the control of inflammation after blood circulation restoring. In this case, pharmacological inhibition of DPP-IV might be necessary after transplantation.

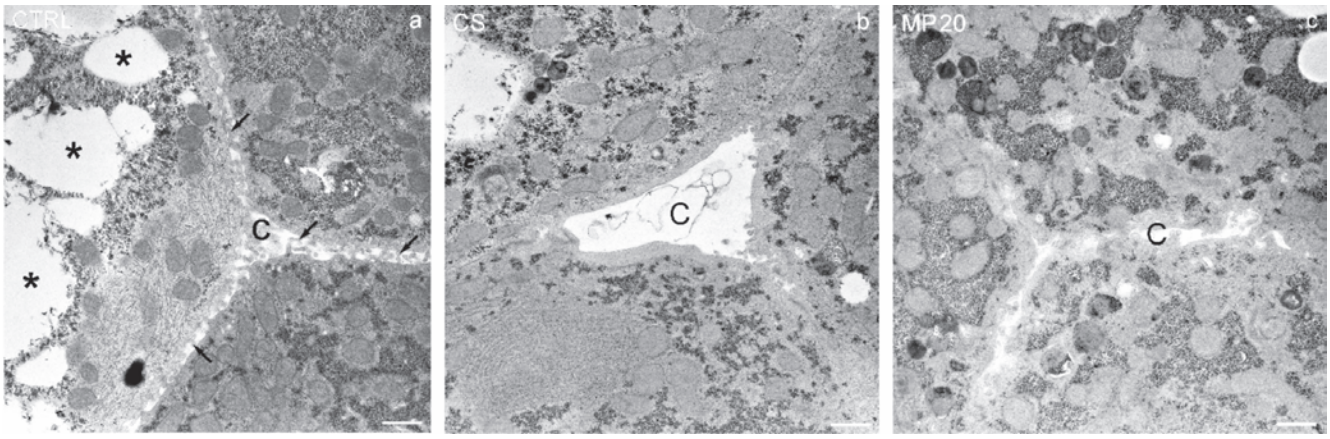


**Figure 9. Electron micrographs of bile canaliculi in a) control fatty livers; b) in cold storage (CS); c) in machine perfusion (MP20) treated fatty livers, taken at the same magnification. C, bile canaliculus; asterisks, lipid droplets; black arrows, microvilli. Scale bars: 1.1 μm.**

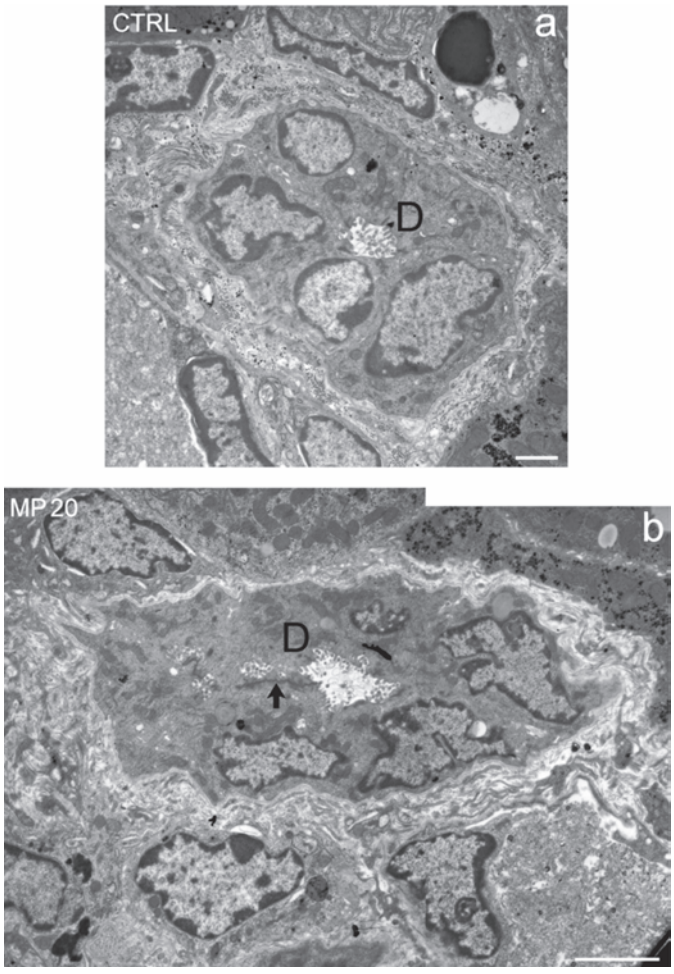


**Figure 10. Electron micrographs of bile ducts in a) control fatty livers; b) in MP20 treated fatty livers. D, bile duct; black arrow, tight junction. Scale bars: a) 1.7 μm; b) 5 μm.**

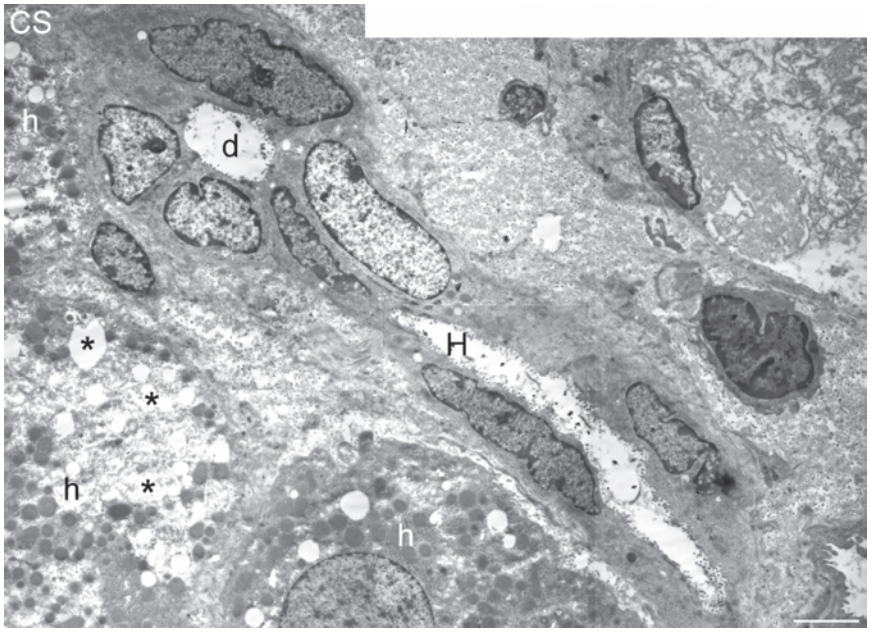


**Figure 11. Electron micrographs of bile ductule in cold storage (CS) treated fatty liver. H, Hering canal; h, hepatocyte; d, bile ductule; asterisks, lipid droplets. Scale bar: 5 μm.**

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