Free Radical Research, Volume 38 Number 7 (July 2004), pp. 697–704

Taylor & Francis
healthsciences

Melatonin Prevents Oxidative Stress and Hepatocyte Cell Death Induced by Experimental Cholestasis

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Accepted by Professor J. Vina

(Received 25 August 2003; In revised form 25 February 2004)

The induction of oxidative stress precedes liver injury during experimental obstructive jaundice (OJ). In this sense, different evidences suggest that melatonin (MEL), as antioxidant, may be useful in the protection against apoptosis and necrosis during experimental cholestasis. In addition, we will also assess if MEL-dependent protection is related to a recovery of antioxidant status disturbances induced by OJ. Cholestasis was achieved by double ligature and sectioning of the principal bile duct. MEL was injected intraperitoneally (500 μg/kg/day). Lipid peroxidation was evaluated by the measurement of malondialdehyde (MDA) content in liver. Different parameters related to antioxidant status, such as reduced glutathione (GSH), glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) were determined in liver. Liver injury was assessed by alanine aminotransferase (ALT) in serum, histological examination, DNA fragmentation and TUNEL assay. The activation of perisinusoidal stellate cells was evaluated by immunohistochemical measurement of α-smooth muscle actin in liver sections. The induction of OJ increased all the parameters related to apoptosis and necrosis in liver. The induction of liver injury was associated with stellate cell activation, as well as an increase in MDA ($p < 0.0001$) and a reduction in GSH, GPx, catalase and SOD content ($p < 0.0001$) in liver. MEL reduced hepatic apoptosis and necrosis ($p < 0.004$) with a significant improvement in all oxidative stress markers. In conclusion, our results showed that MEL recovered the antioxidant status and reduced apoptosis and necrosis induced by experimental cholestasis.

Keywords: Antioxidant status; Apoptosis; Hepatocytes; Melatonin; Necrosis; Obstructive jaundice

INTRODUCTION

The extrahepatic biliary tract obstruction results in the dilatation and cell proliferation of bile ducts with an accumulation of hydrophobic bile acids in hepatocytes.[1] The toxic bile products (glycine conjugates from chenodesoxycholic acid) and neutrophil migration promote the generation of oxygen-free radicals in the hepatic parenchyma.[2–6] In experimental cholestasis models, biliary obstruction has been shown to be characterized by oxidative stress with an increase of lipoperoxides and the severe depletion of antioxidant status.[5,6] Furthermore, increased levels of free radicals have been shown to play a key role in cell death.[7] Hepatocyte apoptosis is currently suspected to be the predominant cell death in cholestasis.[8] Several factors may influence the onset of hepatocyte cell death. Nevertheless, oxidative stress plays a key role in the induction of hepatocyte apoptosis in cholestasis.[9] Consequently, the induction of apoptotic cell death in hepatocytes by obstructive jaundice (OJ) may be blocked by drugs with antioxidant properties. A recent study has demonstrated that some antioxidants such as α-tocoferol, ebselen and idebenone significantly reduce hydroperoxide levels and apoptosis in hepatocytes isolated from rats exposed to hydrophobic bile acids.[7] However, studies have not been carried out to analyze the effect of antioxidants in modulating both types of cell death, apoptosis and necrosis,

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760410001705131

in experimental biliary obstruction models. Among the different molecules used in the treatment of experimental OJ, the administration of melatonin (MEL) has been shown to exert higher antioxidant effect than S-adenosyl-L-methionine or vitamin E.[6,10,11] Another pathological effect of OJ-induced oxidative stress is the activation of stellate cells and enhanced liver fibrosis. It has been observed that addition of different traditional Chinese medicinal herb reduced lipid peroxidation and liver fibrosis induced by bile duct ligation.[12] Nevertheless, Muriel *et al.*,[13] have not found any beneficial effect of different antioxidants, such as vitamin C or E, to prevent lipid peroxidation and liver fibrosis induced by experimental OJ.

The purpose of the present study was to investigate if MEL may prevent oxidative stress, reduction of antioxidant status, stellate cell activation and hepatocyte cell death induced by experimental biliary obstruction.

MATERIAL AND METHODS

Animals

Male Wistar rats (250–330 g) were subjected to controlled conditions of temperature (about 22°C), illumination (12 h light:12 h dark cycle) and provided with food (Purina®, Barcelona, Spain) and water *ad libitum*. Animals were treated according to institutional guidelines and the study was approved by the Research Committee of the Reina Sofia University Hospital.

Experimental Design

Seven animals were included in each of the following groups: (1) sham operated (SO), (2) SO plus MEL, (3) OJ and (4) OJ plus MEL. All the surgical procedures were done with the animals under anesthesia with ketamine (60 mg/kg/i.p.) and midazolam (4 mg/kg/i.p.). Cephazoline (17 mg/kg/i.m.) was used as antibiotic prophylaxis. SO animals were submitted to laparatomy and abdominal closure without bile duct intervention. The procedure for OJ was initiated by a midline ventral incision with exposure of the extra-hepatic bile duct. A double ligature with silk suture was done and the bile duct was sectioned. A two-layer running suture was used for abdominal closure with Dexon (Braun Dexon Barcelona, Spain) and Mersilk (Ethicon, Brussels, Belgium). MEL (Sigma-Aldrich, St Louis, MO, USA) was administered intraperitoneally at a dose of 500 μg/kg/day diluted in 5% ethanol in 0.9% NaCl. MEL was injected daily at 10 h, beginning 1 day before the ligature and for 7 days running. The animals were sacrificed 7 days after OJ. Blood was collected from abdominal aorta and serum was frozen at −20°C until the measurement of alanine aminotransferase (ALT) and direct bilirubin. Several liver specimens were removed and froze at −20°C for the measurement of DNA fragmentation, malondialdehyde (MDA) and antioxidant status. Other liver specimens were fixed in buffered 4% formalin in PBS (PANREAC, Barcelona, Spain) and embedded in paraffin. Thin sections (3 μm) were obtained for evaluation of histological damage, apoptosis and α-smooth muscle actin expression.

Histologic Examination

Liver injury was evaluated in liver fixed sections embedded in paraffin and stained with haematoxylin and eosin.

Biochemical Parameters

ALT and direct bilirubin were measured in serum using an Axon autoanalyzer (Bayer Diagnostics, Tarrytown, NY, USA).

DNA Fragmentation in Liver

The presence of DNA fragmentation in liver is related to apoptosis. Frozen livers (1 g) were homogenized (Ultra-turrax T25, Janke and Kunkel IKA Laboratory) in lysis solution (1% SDS, 10 mM Tris–HCl, 50 mM EDTA), pH 7.4. Samples were incubated with RNAsa (1 mg/ml) (Sigma Chemical Corporation) for 2 h at 37°C and proteinase K (1 mg/ml) (Sigma Chemical Corporation) for 45 min at 48°C. DNA was obtained by phenol–choloform–isoamyl alcohol 25:24:1 (Sigma Chemical Corporation) extraction and precipitated with isopropanol (v/v) and 0.5 M NaCl for 12 h at −20°C. DNA was recovered by centrifugation at 15,000*g* for 20 min, and the pellet was washed with 70% ethanol, dried and resuspended in Tris–EDTA buffer (10 mM Tris, 50 mM EDTA), pH 7.4. Samples (250 μg DNA) were applied and analyzed on 2% agarose gel with ethidium bromide (0.5 μg/ml).

Detection of Apoptosis in Hepatic Sections

Apoptosis was also evaluated in liver fixed sections by immunohistochemistry using a commercial *in situ* apoptosis detection kit (R and D Systems, Minneapolis, USA). In this assay, double-stranded breaks occurred during apoptosis are labeled with biotinylated nucleotides by terminal deoxynucleotidyl transferase (TdT). The biotinylated nucleotides are detected by using streptavidin-horseradich peroxidase conjugate and revealed by diaminobenzidine. The enzyme reaction generates

an insoluble brown precipitate where DNA fragmentation occurred.

Lipid Peroxidation and Antioxidant Status in Liver

MDA was determined in several liver specimens homogenized in 20−mM Tris−HCl buffer pH 7.4 at a ratio of 1:10 (w:v) following the procedure described in a commercial assay (LPO-586, Bioxytec, Portland, USA). Liver for GSH determination was homogenized in 3.75 ml of phosphate-EDTA (0.1 M sodium phosphate, 5 mM EDTA, pH 8.0) with further addition of 1 ml of HPO_3.[14] GSH-400 assay commercial kit (Bioxytec, Portland, USA) was used for measuring Glutathione (GSH) in liver extract. Glutathione peroxidase (GPx) was measured according to Flohe and Gunzler.[15] Catalase and superoxide dismutase (SOD) activities were measured according to Aebi[16] and Sun,[17] respectively.

Measurement of Hepatic Stellate Cell Activation

The activation of hepatic stellate cells was assessed by the evaluation of α-smooth muscle actin expression in fixed liver sections by immunohistochemistry using a commercial assay (IMMH2, Sigma Chemical Corporation). Briefly, deparaffinized sections were incubated with antigen-specific primary antibody and biotinylated secondary antibody. Upon the addition of an extravidin peroxidase reagent included in the assay, a stable avidin-biotin complex is formed with the bound biotinylated antibody. The sites of antibody deposition are visualized by the addition of freshly prepared substrate which contains hydrogen peroxide and the chromogen 3-amino-9-ethyl-carbazole forming reddish-brown insoluble precipitate at the antigens sites.

Statistical Analysis

Results are expressed as means with their corresponding standard errors. Homogeneity of variances was assessed by the Levene's test. Comparisons were made using ANOVA with least significant difference' test (LSD). Statistical significance was set at $p \leq 0.05$.

RESULTS

Induction of Cholestasis

The obstruction of extrahepatic bile duct significantly increased direct bilirubin (4.3 ± 0.68 mg/dl) in comparison with the values obtained in SO (0.1 ± 0.01 mg/dl) group ($p \leq 0.0001$) (Fig. 1). MEL did not modify the concentration of direct bilirubin in plasma from OJ animals.

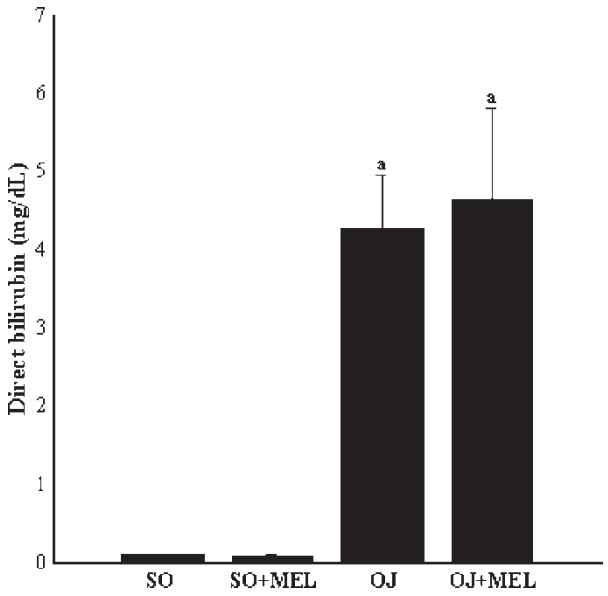

FIGURE 1 Effect of MEL on direct bilirubin concentration in serum from rats with OJ. Bilirubin was measured by routine laboratory methods as described in "Material and Methods" section. The obstruction of extrahepatic bile duct enhanced bilirubin concentration in serum. MEL did not modify the levels of bilirubin in animals with cholestasis. The values are mean ± SE ($n = 7$). The groups with different superscript letters are significantly different ($p \leq 0.05$). (a) $p \leq 0.0001$ in relation to SO group.

Liver Injury during Obstructive Jaundice

Biliary obstruction induced the increase of several biochemical and histological markers of liver injury. In this sense, OJ enhanced different parameters of apoptosis such as DNA fragmentation (Fig. 2) and the presence of apoptotic bodies (Fig. 4C, arrow) in liver. The *in situ* apoptosis detection assay was also positive in liver sections from OJ animals.

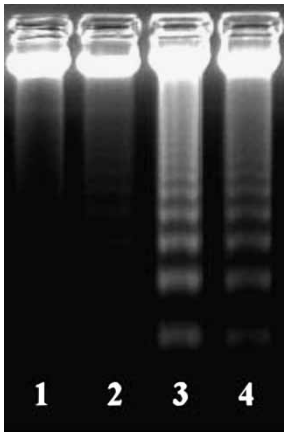

FIGURE 2 Effect of MEL on DNA fragmentation in liver during OJ. Lane 1: SO group, lane 2: SO plus MEL group, lane 3: OJ group, lane 4: OJ plus MEL group. DNA fragmentation in liver homogenates was evaluated in agarose gel as described in "Material and Methods" section. The administration of MEL reduced DNA fragmentation induced by cholestasis. The image is representative of seven different experiments.

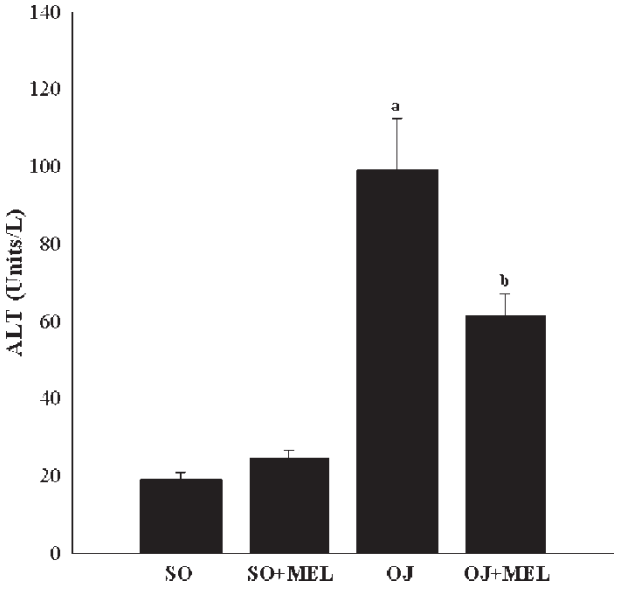

FIGURE 3 Effects of MEL on alanine aminotransferase (ALT) in serum from rats with OJ. ALT was measured by routine laboratory methods as described in "Material and Methods" section. MEL reduced ALT release induced by cholestasis. The values are mean ± SE ($n = 7$). The groups with different superscript letters are significantly different ($p \leq 0.05$). (a) $p \leq 0.0001$ in relation to SO group, (b) $p \leq 0.05$ in relation to OJ group.

In this sense, hepatocyte nucleus and apoptotic bodies from OJ livers exhibited a brown staining, indicating the presence of apoptotic DNA fragments (Fig. 5C). Bile duct ligature also enhanced a biochemical marker of cell necrosis. ALT (113 ± 33.0 U/l) (Fig. 3) rose in serum from OJ animals in comparison with the values observed in SO group (17 ± 2.3 U/l, $p \leq 0.01$). MEL treatment reduced all biochemical and histological signs of apoptosis and necrosis induced by OJ in liver (Figs. 2–5).

Lipid Peroxidation and Antioxidant Status

Bile duct obstruction induced oxidative stress in liver. A marker of lipoperoxidation, such as hepatic MDA, increased significantly in OJ animals (Table I). This enhancement was accompanied by a reduction of GSH (Table I), catalase (Fig. 6A), SOD (Fig. 6B) and GPx (Fig. 6C). MEL reduced all parameters of oxidative stress induced by OJ (Table I, Fig. 6).

Activation of Hepatic Stellate Cell during Obstructive Jaundice

The exacerbation of oxidative stress is accompanied by stellate cell activation and perisinusoidal accumulation of α-smooth muscle actin in different experimental models of liver fibrosis.[13] In fact, the measurement of perisinusoidal α-smooth muscle actin expression is used as an index of stellate cell activation. In our conditions, the obstruction of bile duct was accompanied by an enhancement of α-smooth muscle actin expression in sinusoids and bile ducts (Fig. 7C). The administration of MEL had a general positive impact in the expression of α-smooth muscle actin in SO and OJ-treated rats (Fig. 7B and D).

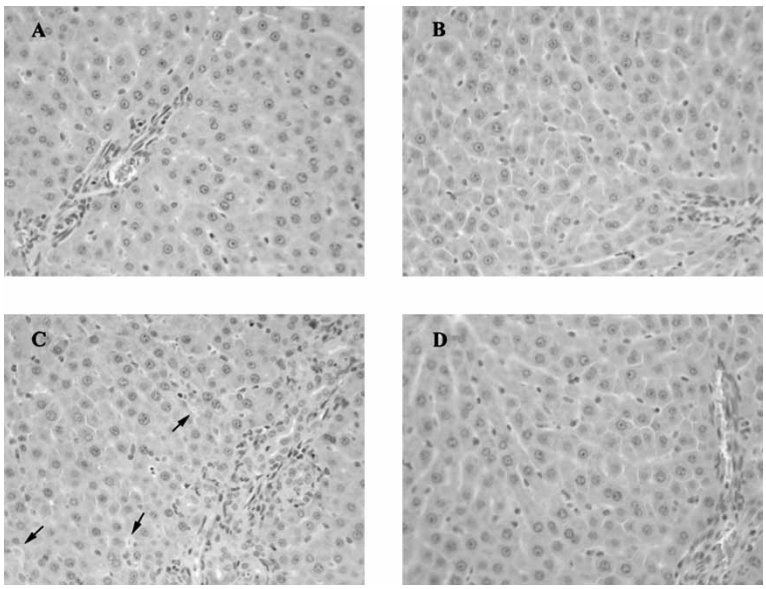

FIGURE 4 Effects of MEL on histological liver injury induced by biliary obstruction observed by light microscopy. (A) SO group, (B) SO plus MEL group, (C) OJ group and (D) OJ plus MEL group. Fixed tissue sections were stained with hematoxylin and eosin as described in "Material and Methods" section. In hepatic sections from animals with biliary obstruction numerous apoptotic bodies exhibiting compacted chromatin (arrow) are shown. MEL reduced all histological signs of necrosis and apoptosis. Original magnification × 400.

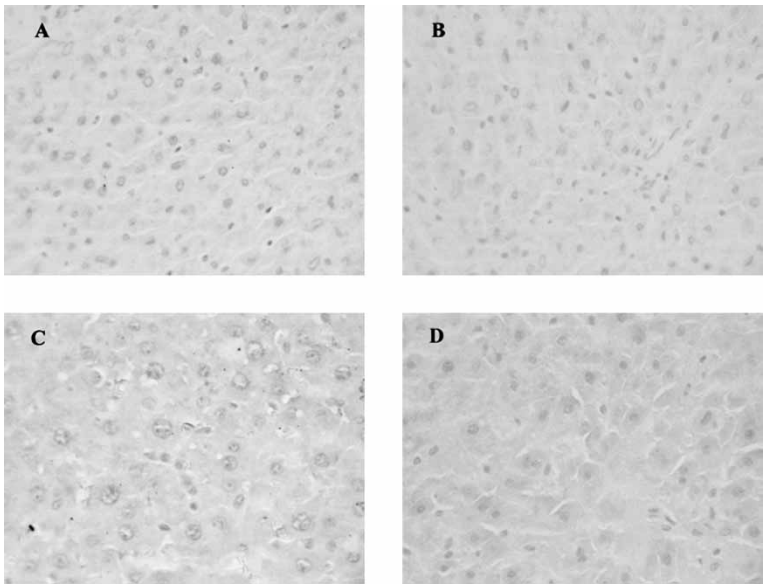

FIGURE 5 Effects of MEL on the presence of DNA fragments in hepatocyte nucleus and apoptotic bodies in liver from rats with biliary obstruction observed by light microscopy. (A) SO group, (B) SO plus MEL group, (C) OJ group and (D) OJ plus MEL group. Fixed tissue sections were processed following the commercial apoptosis detection kit described in "Material and Methods" section. Hepatocyte nucleus and apoptotic bodies in liver from rats with OJ exhibited a brown staining, indicating the presence of apoptotic DNA fragments. MEL reduced the positive nuclear staining induced by cholesasis. Original magnification × 400.

DISCUSSION

In concordance with previous studies, bile duct obstruction induced an intense oxidative stress with a depletion of different molecules and enzymes with antioxidant properties. In these conditions, different markers of apoptosis and necrosis in liver were enhanced in OJ treated animals. The present study also showed that MEL was able to recover the antioxidant status and reduce liver injury observed in experimental cholestasis.

Biliary obstruction is associated with an intense state of oxidative stress. We have previously shown that an important reduction of antioxidant defenses and exacerbation of lipid peroxidation in liver exist during extra-hepatic bile duct ligation in rats.[6,10] Although complete biliary obstruction is considered a systemic disorder, the accumulation of bile acids in liver induces cell death in hepatocytes, which is predominantly apoptotic during obstructive cholestasis.[8,18] In our study, the accumulation of bile acids (Fig. 1) by biliary obstruction induced apoptosis (Figs. 2, 4 and 5) and necrosis (Fig. 3) in hepatocytes. It is generally accepted that oxidative stress is the underlying inducer of apoptosis in hepatocytes.[9] In our conditions, the induction of cell death by OJ was related to an increase of MDA and a reduction of antioxidants status (Table I, Fig. 6). Several mechanisms, such as the direct deleterious effect of oxygen-free radicals,[7–9] activation of activating protein-1 factor (AP-1)[19] or dysfunction of the mitochondrial permeability transition[20,21] have been proposed to explain the induction of apoptosis during exacerbation of oxidative stress. The cytotoxic effect of bile acid has been shown to be related to oxidative stress in cultured hepatocytes. In this sense, the addition of glycochenodesoxycholic acid to cultured hepatocytes induces the generation of free radicals and cell death.[7] This observation has given rise to an intense search of new antioxidant therapies for the management of cholestasic diseases. We have previously shown that pharmacological dose of MEL is more efficient than S-adenosyl-L-methionine and vitamin E against oxidative stress in experimental biliary obstruction.[6,10] In contrast to what occurs with classic antioxidants, such as vitamin E,[22] MEL

TABLE I Effect of MEL on MDA and reduced GSH content in liver from rats with OJ

	SO	SO+MEL	OJ	OJ+MEL
MDA (μmol/g tissue)	6.4 ± 0.04	3.6 ± 0.06*	12.0 ± 0.22**	5.8 ± 0.08***
GSH (mmol/g tissue)	4.6 ± 0.16	6.9 ± 0.31*	3.4 ± 0.16**	5.0 ± 0.23***

OJ enhanced the concentration of MDA and reduced GSH in liver in comparison with the values obtained in SO animals. The induction of oxidative stress by OJ was reduced by MEL treatment. The values are mean ± SE ($n = 7$). The groups with different superscript letters are significantly different ($p \leq 0.05$). *$p \leq 0.0001$ in relation to SO group; **$p \leq 0.0001$ in relation to SO group; ***$p \leq 0.0001$ in relation to OJ group

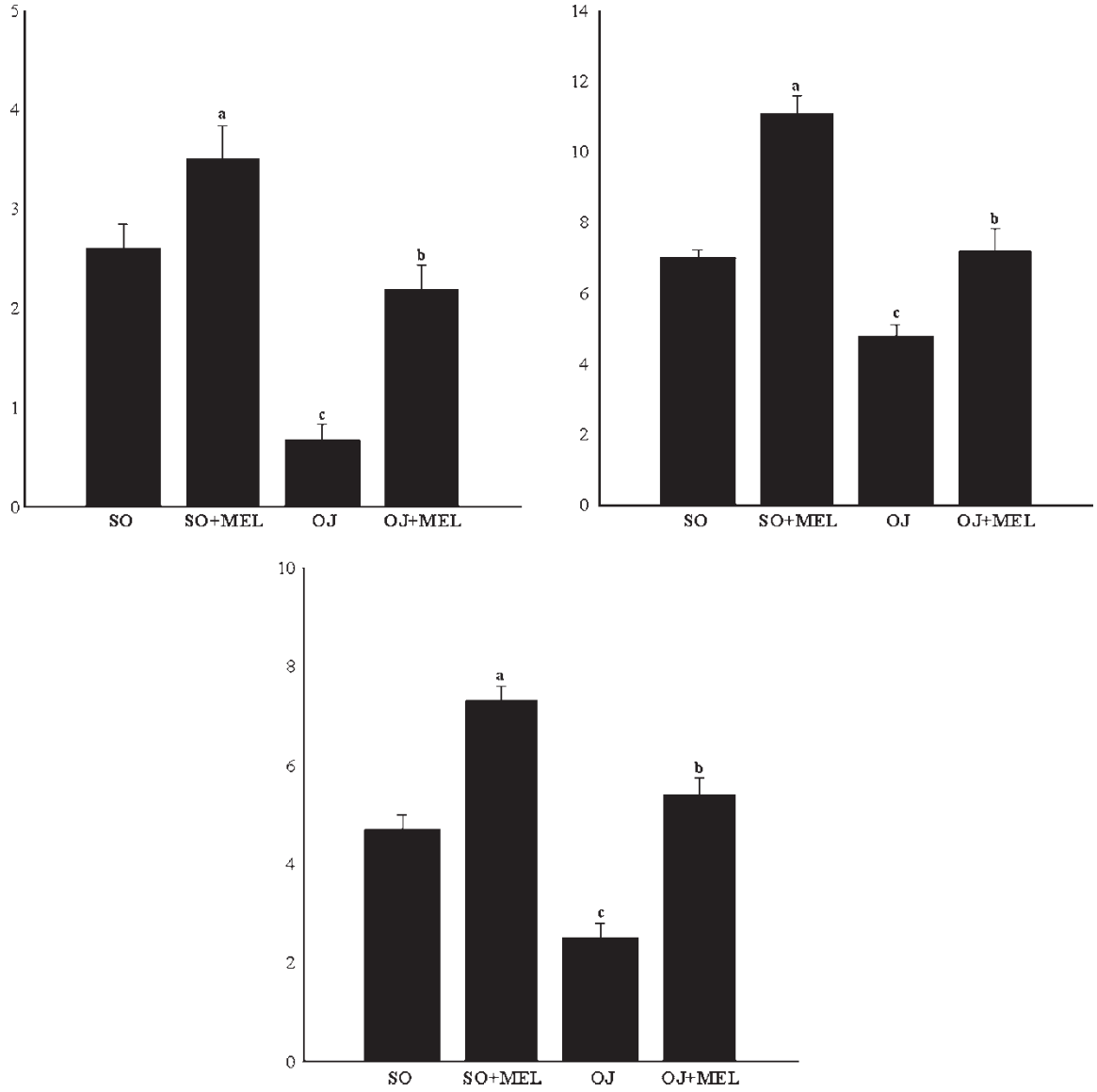

FIGURE 6 Effects of MEL on catalase (CAT) (A), SOD (B) and GPx (C) content in liver from rats with OJ. Antioxidants were measured by routine laboratory methods as described in "Material and Methods" section. MEL was able to abolish the reduction of CAT, SOD and GPx content induced by OJ. The values are mean ± SE ($n = 7$). The groups with different superscript letters are significantly different ($p \leq 0.05$). (a) $p \leq 0.0001$ in relation to SO group, (b) $p \leq 0.0001$ in relation to OJ group, (c) $p \leq 0.0001$ in relation to SO group.

donates electrons through a process whereby the products derived from the reaction of MEL with free radicals can trap new electrons, thus perpetuating the antioxidant action. MEL is able to recover antioxidant status and lipid peroxidation in cholestatic animals submitted to extreme pro-oxidant conditions such as the permanent artificial light exposure.[23] In the present study, the exogenous addition of MEL also exerted a protective effect against oxidative stress and antioxidant status in animals with OJ (Table I and Fig. 6). The improvement of oxidative stress in OJ-treated by MEL was related to a reduction of different parameters of apoptosis (Figs. 2, 4 and 5) and necrosis (Fig. 3) induced by biliary obstruction. The induction of oxidative stress seems to be the underlying mechanism for liver fibrosis in OJ. Nevertheless, previous studies could not demonstrate a beneficial effect of vitamin C or E against lipid peroxidation and liver fibrosis induced by experimental OJ.[13] In our conditions, the reduction of oxidative stress and recovery of antioxidant status by MEL was related to a reduction of α-smooth muscle actin expression in sinusoids and bile ducts (Fig. 7).

In conclusion, the experimental obstruction of the bile duct induced apoptosis and necrosis in hepatocyte, accompanied by an increase of lipid peroxidation, stellate cell activation and a decrease in antioxidant status in liver. The administration of MEL reduced all the parameters associated with oxidative stress and hepatocyte cell death during experimental cholestasis. In this sense, treatment with MEL may be considered an interesting

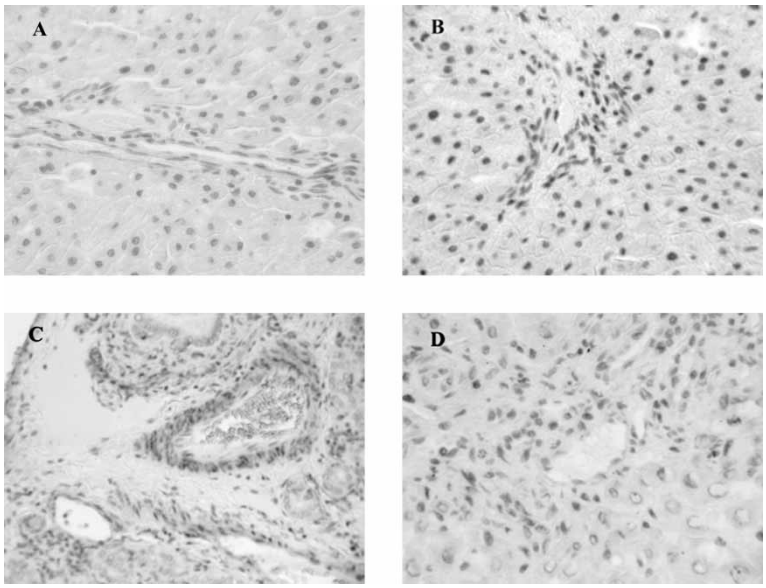

FIGURE 7 Effects of MEL on α-smooth muscle actin expression in fixed liver sections from rats with biliary obstruction observed by light microscopy. (A) SO group, (B) SO plus MEL group, (C) OJ group and (D) OJ plus MEL group. Fixed tissue sections were processed following the commercial kit described in "Material and Methods" section. The obstruction of bile duct was accompanied by an enhancement of α-smooth muscle actin expression in sinusoids and bile ducts. MEL reduced the expression of α-smooth muscle actin induced by biliary obstruction. Original magnification × 400.

therapeutic strategy to block the effect of biliary obstruction on free radicals production and antioxidant status, as well as on stellate cell activation and hepatocyte cell death.

Acknowledgements

This study was supported by the Programa de Promoción de la Investigación en Salud del Ministerio de Sanidad y Consumo (FIS) (99/1087) and by a grant from Fundación Hospital Reina Sofía-CajaSur.

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