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Lipid and protein oxidation in hepatic homogenates and cell membranes exposed to bile acids

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Abstract

Cholestasis occurs in a variety of hepatic diseases and causes damage due to accumulation of bile acids in the liver. The aim was to investigate the effect of several bile acids, i.e. chenodeoxycholic, taurochenodeoxycholic, deoxycholic, taurodeoxycholic, ursodeoxycholic, lithocholic and taurolithocholic (TLC), in inducing oxidative damage. Hepatic tissue of male Sprague-Dawley rats was incubated with or without 1 mM of each bile acid, with or without 0.1 mM $FeCl_3$ and 0.1 mM ascorbic acid for the purpose of generating free radicals. Several bile acids increased lipid and protein oxidation, with TLC being the most pro-oxidative (657% and 175% in homogenates and 350% and 311% in membranes, respectively). TLC also enhanced iron-induced oxidative stress to lipids (21% in homogenates and 29% in membranes) and to proteins (74% in membranes). This enhancement was dose- and time-dependent and was reduced by melatonin. These results suggest that bile acids differentially mediate hepatic oxidative stress and may be involved in the physiopathology of cholestasis.

Keywords: *Bile acid, oxidative stress, cholestasis, lipid peroxidation, protein carbonyls*

Introduction

Cholestatic liver disorders, among the leading indications for liver transplantation [1], are a heterogeneous group of clinical diseases with the common finding of impaired bile flow [2]. Although the various causes of chronic cholestasis include structural, genetic, immunologic and inflammatory processes, one of the putative final common pathways leading to cholestatic liver injury is the intracellular accumulation of toxic bile acids [3,4].

Bile acids are hydroxy steroids, biosynthesized from cholesterol in the liver. Bile acid molecules contain a steroid ring structure and a branched short side chain. The chain terminates in a carboxyl group, which, in physiological terms, can be conjugated with glycine or taurine through an amido moiety. Steroid rings carry hydroxyl groups and common bile acids are differentiated on the basis of the number, position and orientation of these groups.

Bile acids are well-established as being cytotoxic [5,6]. These acids contribute to hepatocellular dysfunction during cholestasis, but the cellular mechanisms by which they cause hepatocellular injury remains unknown. Several studies have demonstrated that oxidative stress plays an important role in the pathogenesis of bile acid hepatotoxicity [7,8]. Furthermore, oxidative stress in cholestatic liver disease is a systemic phenomenon [9,10], probably encompassing all tissues and organs, even those separated by the blood–brain barrier [11].

An estimated 90% of the oxygen organisms breathe is used for energy production by mitochondrial cytochrome oxidase resulting in the formation of two molecules of water. However, 1–4% of the O_2

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taken into cells generates free radicals and other reactive oxygen species (ROS). Free radicals are molecules or atoms characterized by the presence of an unpaired electron in its outer orbital. They are highly unstable, usually very reactive and they often have very short half-lives. Free radicals have a strong tendency to either donate their free electron or pirate an extra electron from an adjacent molecule, thereby completing their molecular structure [12]. It is widely accepted that ROS are harmful to cells; they cause tissue injury by reacting with lipids, proteins or nucleic acids [13].

When free radicals react with the phospholipids in a biological membrane, they initiate a devasting chain reaction, identified as lipid peroxidation (LPO), which leads to loss or suppression of numerous membrane-dependent cellular functions and even to cell death [14]. Exposure of proteins to free radicals leads to gross structural modifications. These oxidatively-modified proteins inhibit the functions of membrane receptors and ionic channels, which have catastrophic consequences on the cells [15].

Collectively, the molecular damage that accumulates is referred to as oxidative stress, which results when there is an imbalance between oxidants and antioxidants in favour of the oxidants. Accumulating evidence indicates that the molecular destruction that is a result of ROS is consequential in the pathogenesis of a number of disease processes [16].

Cells have developed sophisticated mechanisms to maintain redox homeostasis and to cope with the excess of oxygen- and nitrogen-based reactants. These antioxidant protective mechanisms either scavenge and/or detoxify free radicals, block their production or sequester transition metals that are a source of donated electrons.

The aim of the present work was to examine systematically the role of oxidative stress in hepatotoxicity of bile acids. We tested whether individual bile acids *per se* generate oxidative damage and if they can increase the iron-induced oxidative damage. A free radical generator model constituted by ferric chloride and ascorbic acid which simulates the Fenton reaction was used. All studies were performed in two different samples: liver homogenates and hepatic membranes. Liver homogenates retain physiological levels of antioxidants, whereas in hepatic membranes these levels fall dramatically. Since the cellular membrane is constituted, in essence, by lipids and proteins, two different indicators of damage caused by free radicals were used. Malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) and carbonyl contents were quantified as indices of oxidative damage to lipids and proteins, respectively. These biochemical parameters offer an overview of the oxidative damage bile acids can exercise on the hepatic tissue. In order to confirm the pro-oxidative role of bile acids, we also investigated the protective effect of an antioxidant, i.e. melatonin, in preventing bile acid-induced liver injury.

Material and methods

Chemicals

Chenodeoxycholic (QCA), taurochenodeoxycholic (TQA), deoxycholic (DCA), taurodeoxycholic (TDA), ursodeoxycholic (UDA), lithocholic (LCA) and taurolithocholic (TLC) acid, $FeCl_3$, ascorbic acid, ethylenediamine-tetraacetic acid disodium (EDTA-Na_2), Tris (hydroxymethyl) aminomethane (TRIS), 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES) and melatonin were purchased from Sigma-Aldrich (Madrid, Spain). MDA + 4-HDA were measured with the Bioxytech LPO-586 assay kit (Portaland, OR). Other chemicals used were of the highest quality available. Bile acids, $FeCl_3$ and ascorbic acid were diluted in the incubation buffer (Tris-HCl 20 mM; pH 7.4) and prepared fresh just prior to use. Melatonin was diluted in absolute ethanol and incubation buffer; the ethanol concentration was 2% (v/v) in the final mixture.

Animals, liver homogenates and membranes isolation

The handling and animal procedures were made in strict compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals. Sprague-Dawley rats weighing 225–250 g were purchased from Harlan Iberica (Barcelona, Spain), and received standard chow and water *ad libitum*. After being acclimatized for 2 weeks, animals were anaesthetized with sodium thiopental given intraperitoneally (50 mg/kg) and perfused through the heart with 0.9% ice-cold saline, in order to minimize the excess of extra cellular iron and other metallic ions that could artificially increase free radical damage. Immediately after perfusion, the liver was quickly removed, washed in saline solution (0.9% NaCl), frozen and stored at -80°C prior to use. To obtain homogenates, livers were homogenized 1/5 w/v in Tris-HCl buffer (20 mM; pH 7.4). To isolate membranes, livers were homogenized 1/7 w/v in 140 mM KCl/20 mM HEPES buffer (pH 7.4). The resulting suspension was centrifuged at 1000 *x* g for 10 min at 4°C to be cleared of cell debris and nuclei by centrifugation. The supernatant was centrifuged at 50 000 *x* g for 20 min at 4°C. The pellet obtained was re-suspended in 140 mM KCl/20 mM HEPES buffer (pH 7.4) and centrifuged at 10 000 *x g* for 10 min at 4°C. The supernatant and the buffycoat, which contained the membranes, were homogenated and re-centrifuged at 50 000 *x g* for 20 min at 4°C. The resulting pellet was suspended 1/2 v/v in 140 mM KCl/20 mM HEPES buffer (pH 7.4), frozen and stored at -80°C until assay.

Effects of different bile acids

In this experiment, aliquots of liver homogenates and aliquots of hepatic membranes were incubated at 37°C for 2 h with or without 1 mM of each bile acid (QCA, TQA, DCA, TDA, UDA, LCA or TLC), in the presence or absence of 0.1 mM $FeCl_3$ and 0.1 mM ascorbic acid.

Concentration and time-course study with TLC

The aim of this study was to determine the optimal concentration of TLC and incubation time required to induce liver injury. For this purpose, aliquots of liver homogenates and aliquots of hepatic membranes (0.5 mg protein/mL) suspended in 20 mM Tris-HCl buffer (pH = 7.4) were incubated in a water bath with shaking at 37°C for 120 min either in the absence or presence of 0.1 mM $FeCl_3$ and 0.1 mM ascorbic acid and different concentrations of TLC (0.001, 0.01, 0.1, 0.3, 1, 3 mM). The same incubations were performed for 0, 10, 30, 60 or 120 min with 1 mM TLC, in the presence or absence of 0.1 mM $FeCl_3$ and 0.1 mM ascorbic acid.

In subsequent experiments, 5 mM melatonin was added at the same time as TLC to incubation medium. After 120 min, aliquots of liver homogenates or hepatic membranes were assayed to determine melatonin's protective effect against TLC-induced lipid and protein oxidation.

Measurements of protein concentrations

The protein concentrations in incubation media were determined using the method of Bradford [17], in which bovine serum albumin served as standard.

Measurement of malondialdehyde and 4-hydroxyalkenals levels

MDA and 4-HDA are the end-products of peroxidation of polyunsaturated fatty acids. Therefore, MDA + 4-HDA levels are widely used as an index of the oxidative breakdown of lipid in the preparations [18]. Total MDA + 4-HDA levels in incubation media were measured using a spectrophotometric assay [19]. In short, the assay was based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA or 4-HDA yielding a stable chromophore with a peak of maximum absorbance at 586 nm. 1,1,3,3-Tetramethoxypropane was used as standard. Results were expressed as nmol MDA + 4-HDA per mg of protein.

Measurement of carbonyl contents

The carbonyl contents of proteins were quantified by the reaction with 2,4-dinitrophenylhydrazine (DNPH), using the method previously described by Levine et al. [20]. Briefly, 1 mL of samples were mixed with 100 µl of 20 mM Tris-HCl buffer and 200 µl of 10 mM DNPH and reacted for 1 h at 37°C to give a final concentration of 2 mM of DNPH. Next, proteins were precipitated by adding 325 µl of 50% ice-cold trichloroacetic acid. After 10 min of incubation on ice, samples were centrifuged at 3000 *x g* for 10 min. Pellets containing the proteins were washed three times with ethanol/ethyl acetate (1:1 v/v), each wash was followed by centrifugation at 11 000 *x g* for 3 min. Finally, the pellets were dissolved in 700 µl of 6 M guanidine in 2N HCl (pH = 2.0) by vortexing and incubated at 37°C for 15 min. After this interval, samples were clarified by centrifugation at 12 000 *x g* for 10 min and the absorbance of supernatants was read at 375 nm. Protein carbonyl content, expressed in nanomoles per milligram of protein, was estimated by using the molar absorption coefficient of 22 000 M^{-1} cm^{-1} for DNPH derivates. Guanidine-HCl solution was used as a blank.

Statistical analysis

Distribution of the groups was analysed with the Kolmogorov-Smirnov test. When groups showed normal distribution, parametric statistical methods were used to analyse the data. Homogeneity of variance was assessed by the Leven's test. Statistical comparisons among experimental groups were conducted by either one-way analysis of variance (ANOVA) or two-tailed Student's *t*-tests paired as appropriate. Values are expressed as means ± standard error (SEM) and $p \leq 0.05$ was defined for the rejection of the null hypothesis.

Results

After incubating liver homogenates for 2 h, 1 mM concentrations of different bile acids significantly increased LPO (TQA, 214%; TDA, 227%; and TLC, 657%) (Figure 1A) and also caused significant elevations of the carbonyl content (QCA, 55%; TQA, 111%; LCA, 122%; DCA, 155%; TDA, 174%; UDA, 174% and TLC, 175%) (Figure 2A). In hepatic membranes several bile acids increased MDA + 4-HDA concentrations (LCA, 68%; UDA, 72%; DCA, 73%; and TLC, 350%) (Figure 1B) and all bile acids caused elevations in carbonyl content (QCA, 113%; LCA, 133%; TQA, 147%; DCA, 150%; UDA, 175%; TDA, 201%; and TLC, 311%) (Figure 2B).

Some bile acids increased the lipid peroxidation in liver homogenates produced as a result of the Fenton reaction, but the differences were only statistically significant with regard to the control samples oxidized in the presence of TLC, with a 21% increase (Figure 1C). In terms of protein oxidative stress, the only statistically significant increase was observed with TDA (36%) (Figure 2C). In hepatic membranes, bile acids enhanced lipid (UDA, 10%; TQA, 21%; TDA,

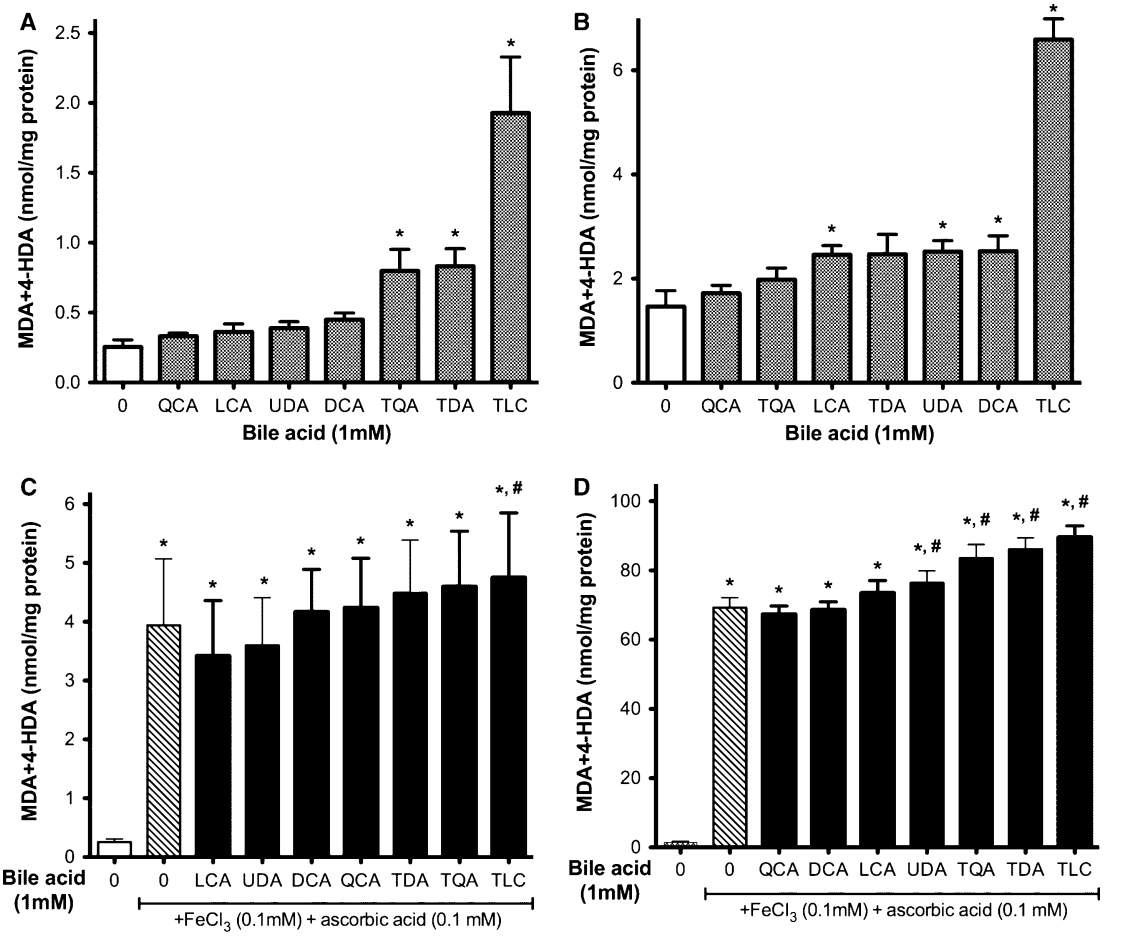

Figure 1. Effect of bile acids on LPO in absence of iron in rat liver homogenates (A) and in rat hepatic membranes (B). The effects of bile acids on LPO induced by $FeCl_3$ and ascorbic acid in rat liver homogenates (C) and in rat hepatic membranes (D). Values are means ± SEM ($n = 6$). $p \leq 0.05$ vs control (*) or vs control + (#).

24% and TLC, 29%) and protein (LCA, 24%; UDA, 27%; DCA, 30%; TDA, 33%; TQA, 41% and TLC, 74%) oxidation due to iron and ascorbic acid (Figure 1D and 2D). TLC was selected for subsequent studies, since it provided the maximal induction of oxidative damage in lipids and proteins.

The second series of studies was performed to determine the optimal time of incubation and concentration of TLC to induce an appropriate amount of LPO and carbonyl contents. Figure 3 shows that 1 mM TLC increased MDA + 4-HDA levels and carbonyl contents in a time-dependent manner in liver homogenates and in hepatic membranes. TLC behaves as an excellent oxidant in terms of LPO and carbonyl contents and its damaging effects increased during the 2 h of incubation. The 2-h incubation period ($p \leq 0.05$) was selected for the subsequent studies since it yielded high levels of LPO products and carbonyl contents.

In TLC concentration kinetic studies, LPO and carbonyl content in liver homogenates were increased by all TLC concentrations studied (Figure 4A and 5A). In membranes, LPO and carbonyl content was elevated in a dose-dependent manner when increasing concentrations of TLC were used (Figure 4B and 5B). Concentrations of TLC greater than 0.3 mM significantly increased the MDA + 4-HDA levels and carbonyl content in hepatic membranes.

In the presence of iron, concentrations of TLC greater than 1 mM and 0.001 mM caused significant elevations of MDA + 4-HDA levels in liver homogenates and hepatic membranes, respectively (Figure 4C and 4D). Incubation of the liver homogenates with $FeCl_3$ and ascorbic acid raised carbonyl contents, but the addition of TLC to the incubation mixture did not further increase levels of carbonyl contents over those induced by $FeCl_3$ and ascorbic acid only (Figure 5C). Hepatic membranes incubated with $FeCl_3$ and ascorbic acid raised carbonyl contents. The addition of TLC to the incubation mixture further increased levels of carbonyls such that the values where significantly elevated over those induced by $FeCl_3$ and ascorbic acid only (Figure 5D). Carbonyl contents increased in a dose-dependent manner with increasing

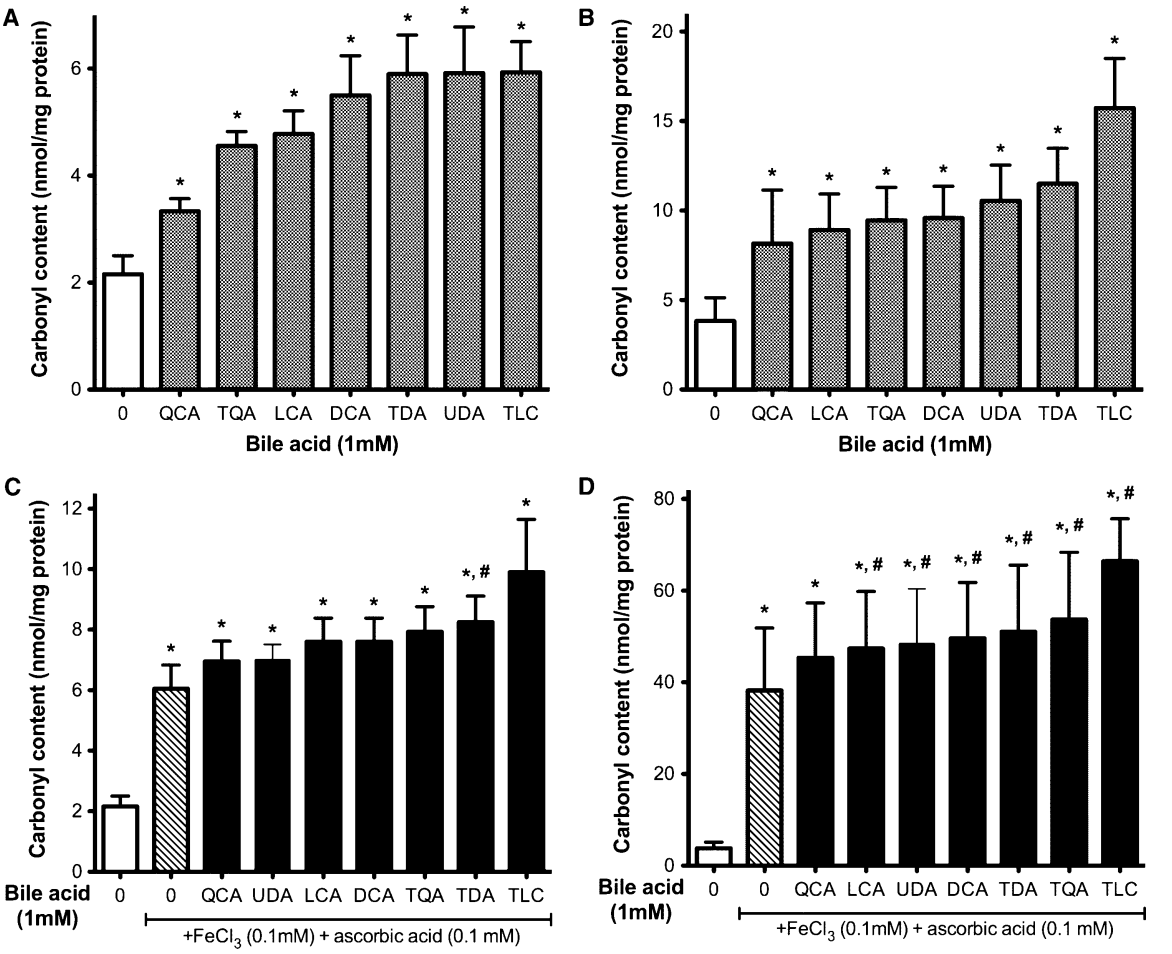

Figure 2. Effect of bile acids on protein oxidation in absence of iron in rat liver homogenates (A) and in rat hepatic membranes (B). Effect of bile acids on LPO induced by $FeCl_3$ and ascorbic acid in rat liver homogenates (C) and in rat hepatic membranes (D). Values are means ± SEM ($n = 6$). $p \leq 0.05$ vs control (*) or *vs* control + (#).

concentrations of TLC. Concentrations of TLC greater than 0.001 mM caused significant elevations in the carbonyl contents of hepatic membranes.

The aim of the third study was to test whether melatonin would prevent TLC-induced oxidative stress. The co-incubation of the liver homogenates and hepatic membranes with melatonin reduced the levels of LPO products which were induced by TLC (Table I). Carbonyl contents in the membranes were also reduced by melatonin in relation to membranes treated with TLC only, and showed no significant differences to those of the control basal samples (no melatonin or TLC) (Table I).

Discussion

In liver tissue of patients with cholestatic liver disease, such as primary biliary cirrhosis, the intra-hepatic concentration of total bile acids can rise as high as 600 nmol/g liver tissue [21]. In the current experiments, homogenates and the hepatic membranes were incubated with 0.001–3 mM TLC. Assuming that 1g liver is equivalent to 1 mL, these concentrations approximate the bile acid concentrations found in cholestatic livers.

The mechanisms by which bile acids are toxic to the liver are not fully understood. Several studies reported in the recent years have suggested a role for ROS in bile acid-mediated toxicity [22–24]. Accumulation of these acids alters essential processes of the cell including energy production by mitochondria, increasing the formation of additional ROS which are capable of oxidizing lipids, proteins and nucleic acids resulting in compromised cellular function [7,8,23–26].

To date, studies have reported that bile acids induce oxidative damage *in vitro* in isolated hepatocytes [7,8], primary cultures of hepatocytes [27], hepatic homogenates [26] and in hepatic mitochondria [7,8,28]. Also intravenous administration of bile acids [26] and bile duct obstruction induce oxidative damage *in vivo* [24,29–31]. It has been hypothesized that the intracellular generation of hydroperoxides by mitochondria is an early event in bile acid-induced hepatotoxicity [31], where interactions of bile acids

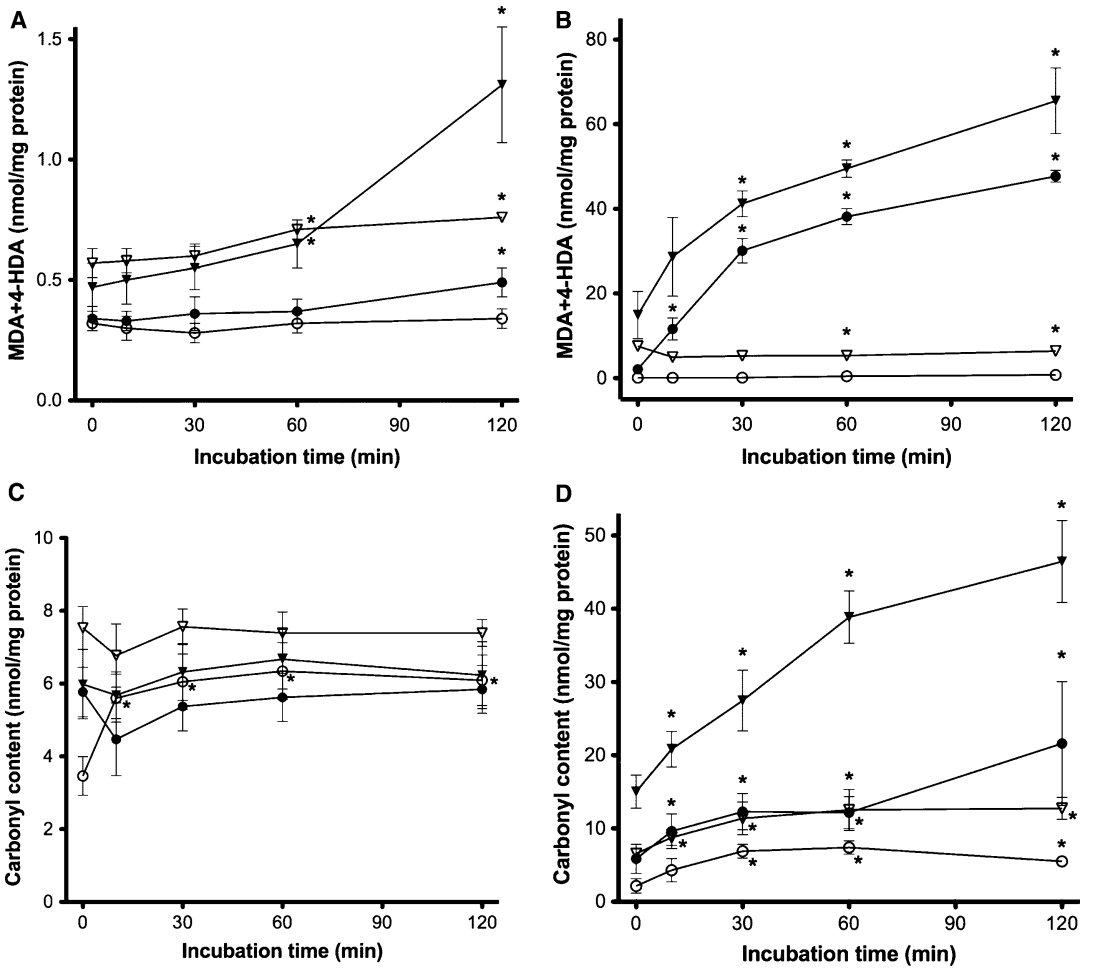

Figure 3. Time-dependent changes in MDA+4-HDA accumulated in liver homogenates (A) and in hepatic membranes (B). Time course study on the induction of protein oxidation in liver homogenates (C) and in hepatic membranes (D). Incubation times were 0, 10, 30, 60 or 120 min in the presence (▼) or absence (▽) of 1 mM TLC, and in the presence of iron and ascorbic acid. Controls were performed in absence of TLC and in the presence (●) or absence (○) of iron or ascorbic acid. Values are means ± SEM ($n = 5$). *$p \leq 0.05$ vs corresponding control at 0 time.

with Complex I and III of the electron mitochondrial transport chain lead to superoxide radical (O_2^-) production.

In the current experiments all the bile acids studied increased the levels of carbonyls both in homogenized tissues and in membranes. Nevertheless, rises in lipid peroxidation were statistically significant only when bile acids conjugated with taurine were used; i.e. TQA, TDA and TLC, in the case of the homogenates and LCA, UDA, DCA and especially TLC in the isolated membranes. Unquestionably, all bile acids studied exhibited pro-oxidant activity.

Bile acids that are conjugated with taurine are more hydrophilic than the free bile acids [32]. Among free bile acids, hydrosolubility also differs, with UDA being the most soluble compared to LCA, DCA and QCA [32]. Hydrophilicity could be important in the induction of the LPO in homogenates, although this may not be true in the case of the membranes. Perhaps by using isolated purified membranes, the more hydrophobic bile acids could accumulate in the hydrophobic core of the bilayer and thereby promote pro-oxidant actions. Protein oxidation was also independent of hydrosolubility of bile acids.

The physiological concentration of bile acids is apparently tolerated without causing damaging effects. However, under conditions in which free iron is available, even low concentrations of bile acids may become cytotoxic [33]. Iron readily generates the highly toxic hydroxyl radical (·OH) from hydrogen peroxide or superoxide via the Fenton and Haber-Weiss reactions [34]. Iron ions are responsible for most of the lipid peroxidation that occurs *in vivo*. In this study, we used $FeCl_3$ and ascorbic acid since ascorbate is a strong reducing agent and it has been classically used to reduce transition metals such as Fe^{3+} or Cu^{2+} and to generate ·OH [35]. The ability of bile acids to enhance ferrous iron-induced LPO is related to the hydrophobicity of individual bile acids [36]. One possible explanation for this effect is an interaction of bile acids with iron ions; it makes iron available at the lipid membrane, which then promotes

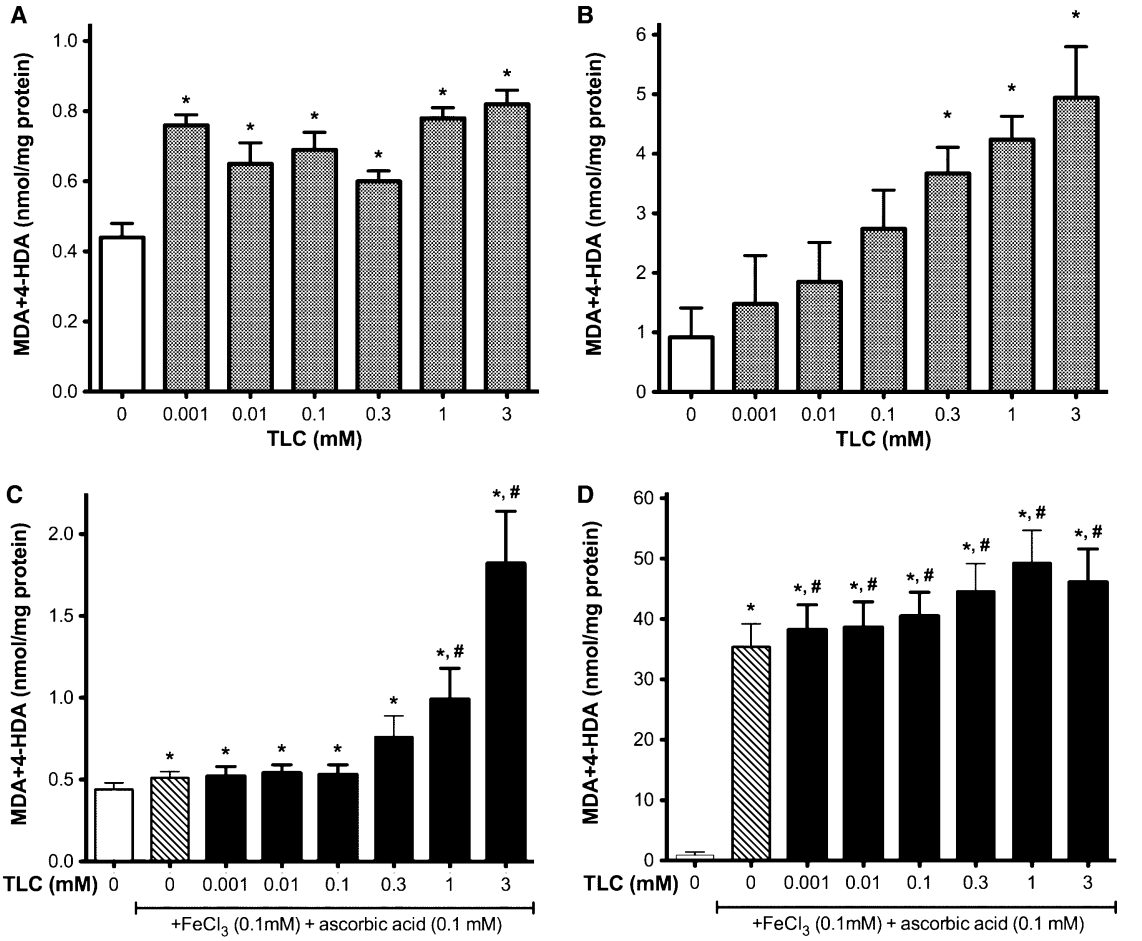

Figure 4. Effect of increasing concentrations of taurolithocholic acid (TLC) on lipoperoxidation (LPO) in absence of iron in rat liver homogenates (A) and in rat hepatic membranes (B). Effect of increasing concentrations of TLC on LPO induced by $FeCl_3$ and ascorbic acid in rat liver homogenates (C) and in rat hepatic membranes (D). Values are means ± SEM ($n = 6$). $p \leq 0.05$ vs control (*) or vs control + (#).

peroxidation. Alternatively, bile acids due to their membrane perturbing properties [37] may render the lipid bilayer more permeable to iron ions, which would also result in the elevated breakdown of lipids. The present results using liver homogenates and hepatic membranes support the idea that bile acids are iron-LPO enhancers and this study is the first to show bile acids as iron-protein oxidation enhancers. While iron induced-carbonyl contents were not increased by TLC in liver homogenates, they were elevated in hepatic membranes. This difference may be due to the remaining physiological antioxidants in liver homogenates that protected the proteins from oxidation. For example, there are high levels of melatonin, a strong oxidative stress inhibitor, in the bile of rats and other mammals [38,39].

These current results are in agreement with those obtained in the absence of iron and reinforce the importance of the solubility of the bile acids. Sreejayan and von Ritter [36] demonstrated that bile acids are capable of promoting LPO induced by iron which is directly related by their hydrophility. This is consistent with the major pro-oxidant effects of TLC in lipids.

Since, under the experimental conditions used herein, TLC was the most pro-oxidant bile acid, it was used in the two kinetic models, one of duration of incubation and the other of concentration. The TLC pro-oxidant effect was time-dependent and a 2 h interval was selected for the subsequent experiments. This is the same incubation period used by Sreejayan and von Ritter [36] to study bile acids in liposomes. Regarding the concentration kinetic, TLC produced an increase in all the studied cases, with the exception of the carbonylation induced by iron in liver homogenates. The existence of a TLC concentration-dependent pro-oxidant effect was evident in the studies with membranes.

This study has shown that bile acids differentially increase the level of cellular LPO and protein carbonyl, both consequences presumed to be due to the increased production of ROS, with oxidative stress being a major component of hepatocyte damage in cholestasis. The protective antioxidant effect of melatonin reported here is consistent with the idea that bile acids cause the generation of reactive oxygen species.

The proposed mechanisms of toxicity of bile acids probably involve free radical generation [40] by

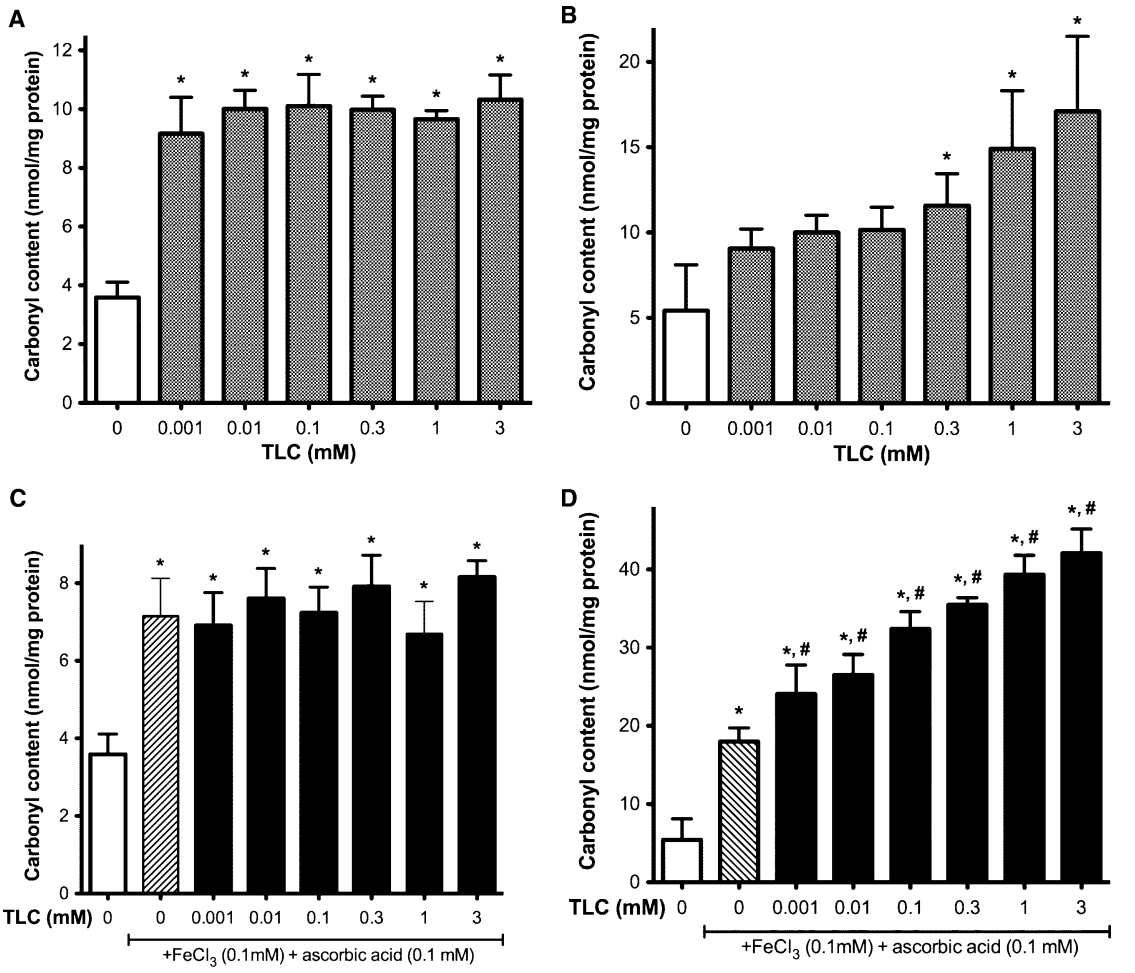

Figure 5. Effect of increasing concentrations of taurolithocholic acid (TLC) on protein oxidation in absence of iron in rat liver homogenates (A) and in rat hepatic membranes (B). Effect of increasing concentrations of TLC on protein oxidation induced by $FeCl_3$ and ascorbic acid in rat liver homogenates (C) and in rat hepatic membranes (D). Values are means ± SEM ($n = 6$). $p \leq 0.05$ vs control (*) or vs control + (#).

mitochondria, a major source of free radicals. Also, a reduction in the endogenous antioxidant mitochondrial defenses including lowered gluthatione and ubiquinone levels, such as observed in bile duct-ligated rats [40]. Finally, reduced Cu, Zn-superoxide dismutase and catalase activities in the liver of patients with diverse hepatopaties have been observed [41], indicating these changes may be contributing to bile acid-induced liver toxicity. Since beneficial effects of antioxidants in the hepatic damage are

Table I. Protective effect of 5 mM melatonin (aMT) against lipid and protein oxidation induced by 1 mM taurolithocholic acid (TLC), in the presence (+) or absence of 0.1 mM $FeCl_3$ or 0.1 mM ascorbic acid (AA) in liver homogenates and in hepatic membranes.

	Homogenates				Membranes			
	MDA+4-HDA (nmol/mg protein)		Carbonyl content (nmol/mg protein)		MDA+4-HDA (nmol/mg protein)		Carbonyl content (nmol/mg protein)	
Without $FeCl_3$+AA								
Control	0.27 ± 0.04		3.47 ± 0.27		0.35 ± 0.18		6.66 ± 1.62	
TLC	0.60 ± 0.07	(*)	8.35 ± 0.59	(*)	5.36 ± 0.58	(*)	10.94 ± 2.45	(*)
aMT	0.44 ± 0.02	(¤)	6.70 ± 0.74	(*)	4.46 ± 0.52	(*,¤)	5.88 ± 1.55	(¤)
With $FeCl_3$+AA								
Control	0.44 ± 0.04	(*)	–		39.98 ± 5.88	(*)	18.14 ± 4.16	(*)
TLC	0.820.14	(*,#)	–		52.06 ± 6.04	(*,#)	43.71 ± 4.67	(*,#)
aMT	0.330.05	(#,§)	–		2.02 ± 0.52	(*,#,§)	2.71 ± 0.94	(#,§)

Values are means ± SEM ($n = 6$). $p \leq 0.05$ vs control (*), vs control + (#), vs TLC (¤) or vs TLC + (§).

typical [42–47], the use of antioxidants to reduce bile acid toxicity at the level of the hepatocyte is suggested.

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References

[1] Neuberger J. Liver transplantation for cholestatic liver disease. Curr Treat Options Gastroenterol 2003;6:113–121.
[2] Javitt NB, Schiff L, Schiff ER. Bile acids and hepatobiliary disease. In: L Schiff, ER Schiff, editors. Diseases of the liver. Philadelphia: Lippincott; 1982. p 119–150.
[3] Greim H, Trulzsch D, Czygan P, Rudick J, Hutterer F, Schaffner F, Popper H. Mechanism of cholestasis. 6. Bile acids in human livers with or without biliary obstruction. Gastroenterology 1972;63:846–850.
[4] Greim H, Trulzsch D, Roboz J, Dressier K, Czyga P, Hutterer F, Schaffner F. Mechanism of cholestasis. 5. Bile acids in normal rat livers and in those after bile duct ligation. Gastroenterology 1972;63:837–845.
[5] Schmuckler DL, Ohta M, Kanai S, Sato Y, Kitani K. Hepatic injury induced by bile salts: correlation between biochemical and morphological events. Hepatology 1990;12:1216–1221.
[6] Scholmerich J, Becher MS, Schmidt K, Schubert R, Kremer B, Feldhaus S, Gerok W. Influence of hydroxylation and conjugation of bile salts on their membrane-damaging properties—studies on isolated hepatocytes and lipid membrane vesicles. Hepatology 1984;4:661–666.
[7] Sokol RJ, Devereaux MW, Khandwala R, O'Brien K. Evidence for involvement of oxygen free radical in bile acid toxicity to isolated rat hepatocytes. Hepatology 1993;17:869–881.
[8] Sokol RJ, Winklhofer-Roob BM, Devereaux MW, McKim JM Jr. Generation of hydroperoxides in isolated rat hepatocytes and hepatic mitochondria exposed to hydrophobic bile acids. Gastroenterology 1995;109:1249–1256.
[9] Ljubuncic P, Tanne Z, Bomzon A. Evidence of a systemic phenomenon for oxidative stress in cholestatic liver disease. Gut 2000;47:710–716.
[10] Lahiri S, Singh P, Singh S, Rasheed N, Palit G, Pant KK. Melatonin protects against experimental reflux esophagitis. J Pineal Res 2009;46:207–213.
[11] Chroni E, Patsoukis N, Karageorgos N, Konstantinou D, Georgiou C. Brain oxidative stress induced by obstructive jaundice in rats. J Neuropathol Exp Neurol 2006;65:193–198.
[12] Cheeseman KH, Slater TF. An introduction to free radical biochemistry. Br Med Bull 1993;49:481–493.
[13] Halliwell B, Aruoma OI. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett 1991;281:9–19.
[14] Kanner J, German JB, Kinsella JE. Initiation of lipid peroxidation in biological systems. Crit Rev Food Sci Nutr 1987;25:317–364.
[15] Davies KJ, Delsignore ME, Lin SW. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. J Biol Chem 1987;262:9902–9907.
[16] Genova ML, Pich MM, Bernacchia A, Bianchi C, Biondi A, Bovina C, Falasca AI, Formiggini G, Castelli GP, Lenaz G. The mitochondrial production of reactive oxygen species in relation to aging and pathology. Ann NY Acad Sci 2004; 1011:86–100.
[17] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72: 248–254.
[18] Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 1990;9: 515–540.
[19] Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-Hydroxynonenal. Methods Enzymol 1990;186:407–421.
[20] Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990;186:464–478.
[21] Poupon R, Poupon RE. Ursodeoxycholic acid therapy of chronic cholestasis conditions in adults and children. Pharmacol Ther 1995;66:1–15.
[22] Dahm LJ, Hewett JA, Roth RA. Bile and bile salts potentiate superoxide anion release from activated, rat peritoneal neutrophils. Toxicol Appl Pharmacol 1988;95:82–92.
[23] Sokol RJ, Devereaux M, Khandwala RA. Effect of dietary lipid and vitamin E on mitochondrial lipid peroxidation and hepatic injury in the bile duct-ligated rat. J Lipid Res 1991; 32:1349–1357.
[24] Sokol RJ, Straka MS, Dahl R, Devereaux MW, Yerushalmi B, Gumpricht E, Elkins N, Everson G. Role of oxidant stress in the permeability transition induced in rat hepatic mitochondria by hydrophobic bile acids. Pediatr Res 2001; 49:519–531.
[25] Krahenbuhl S, Talos C, Fischer S, Reichen J. Toxicity of bile acids on the electron transport chain of isolated rat liver mitochondria. Hepatology 1994;19:471–479.
[26] Sokol RJ, McKim JM, Jr., Goff MC, Ruyle SZ, Devereaux MW, Han D, Packer L, Everson G. Vitamin E reduces oxidant injury to mitochondria and the hepatotoxicity of taurochenodeoxycholic acid in the rat. Gastroenterology 1998;114:164–174.
[27] Rodrigues CM, Fan G, Ma X, Kren BT, Steer CJ. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. J Clin Invest 1998;101:2790–2799.
[28] Rodrigues CM, Fan G, Wong PY, Kren BT, Steer CJ. Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. Mol Med 1998;4:165–178.
[29] Alptekin N, Mehmetcik G, Uysal M, Aykac-toker G. Evidence for oxidative stress in the hepatic mitochondria of bile duct ligated rats. Pharmacol Res 1997;36:243–247.
[30] Tsai LY, Lee KT, Liu TZ. Evidence for accelerated generation of hydroxyl radicals in experimental obstructive jaundice of rats. Free Radic Biol Med 1998;24:732–737.
[31] Sokol RJ, Dahl R, Devereaux MW, Yerushalmi B, Kobak GE, Gumpricht E. Human hepatic mitochondria generate reactive oxygen species and undergo the permeability transition in response to hydrophobic bile acids. J Pediatr Gastroenterol Nutr 2005;41:235–243.
[32] Natalini B, Sardella R, Camaioni E, Gioiello A, Pellicciari R. Correlation between CMC and chromatographic index: simple and effective evaluation of the hydrophobic/hydrophilic balance of bile acids. Anal Bioanal Chem 2007;388:1681–1688.

[33] Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990;186:1–85.
[34] Riederer P, Sofic E, Rausch WD, Schmidt B, Reynolds GP, Jellinger K, Youdim MBH. Transition metals, ferritin, glutathione and ascorbic acid in Parkinsonian brains. J Neurochem 1989;52:515–520.
[35] Buettner GR. Ascorbate autoxidation in the presence of iron and copper chelates. Free Radic Res Commun 1986;1:349–353.
[36] Sreejayan N, von Ritter C. Effect of bile acids on lipid peroxidation: the role of iron. Free Radic Biol Med 1998; 25:50–56.
[37] Heuman DM. Bile salt-membrane interactions and the physico-chemical mechanisms of bile salt toxicity. Ital J Gastroenterol 1995;27:372–375.
[38] Tan D, Manchester LC, Reiter RJ, Qi W, Hanes MA, Farley NJ. High physiological levels of melatonin in the bile of mammals. Life Sci 1999;65:2523–2529.
[39] Koppisetti S, Jenigiri B, Terron MP, Tengattini S, Tamura H, Flores LJ, Tan DX, Reiter RJ. Reactive oxygen species and the hypomotility of the gall bladder as targets for the treatment of gallstones with melatonin: a review. Dig Dis Sci 2008;53: 2592–2603.
[40] Krahenbuhl S, Talos C, Lauterburg BH, Reichen J. Reduced antioxidative capacity in liver mitochondria from bile duct ligated rats. Hepatology 1995;22:607–612.
[41] Togashi H, Shinzawa H, Wakabayashi H, Nakamura T, Yamada N, Takahashi T, Ishikawa M. Activities of free oxygen radical scavenger enzymes in human liver. J Hepatol 1990;11:200–205.
[42] Crocenzi FA, Sánchez Pozzi EJ, Pellegrino JM, Rodríguez Garay EA, Mottino AD, Roma MG. Preventive effect of silymarin against taurolithocholate-induced cholestasis in the rat. Biochem Pharmacol 2003;66:355–364.
[43] Ohta Y, Kongo M, Kishikawa T. Melatonin exerts a therapeutic effect on cholestatic liver injury in rats with bile duct ligation. J Pineal Res 2003;34:119–126.
[44] Ara C, Kirimlioglu H, Karabulut AB, Coban S, Ay S, Harputluoglu M, Kirimlioglu V, Yilmaz S. Protective effect of resveratrol against oxidative stress in cholestasis. J Surg Res 2005;127:112–117.
[45] Gumpricht E, Dahl R, Devereaux MW, Sokol RJ. Licorice compounds glycyrrhizin and 18beta-glycyrrhetinic acid are potent modulators of bile acid-induced cytotoxicity in rat hepatocytes. J Biol Chem 2005;280:10556–10563.
[46] Perez MJ, Castano B, Gonzalez-Buitrago JM, Marin JJ. Multiple protective effects of melatonin against maternal cholestasis-induced oxidative stress and apoptosis in the rat fetal liver-placenta-maternal liver trio. J Pineal Res 2007; 43:130–139.
[47] Catala A, Zvara A, Puskas LG, Kitajka K. Melatonin-induced gene expression changes and its preventive effects on adriamycin-induced lipid peroxidation in rat liver. J Pineal Res 2007;42:43–49.

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