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Effects of Silymarin and Vitamins E and C on Liver Damage Induced by Prolonged Biliary Obstruction in the Rat

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Abstract: Oxidative stress, in particular lipid peroxidation, induces collagen synthesis. Thus, we administered various antioxidants to bile duct-ligated rats for 28 days and lipid peroxidation, glutathione content, fibrosis, necrosis and cholestasis were evaluated. Extrahepatic cholestasis was induced by double ligation and section of the common bile duct. The study included eight groups (n=6), four groups were bile duct-ligated and received either vitamin C (50 mg/kg/day, orally), vitamin E (400 IU/rat/day, orally), silymarin (50 mg/kg/12hr, orally) or vehicles; four groups were sham-operated controls. Collagen content was determined by measuring hydroxyproline in liver samples; malondialdehyde was used to estimate lipid peroxidation levels; reduced and oxidized glutathione were determined fluorometrically; alanine aminotransferase and bilirubins colorimetrically. Bilirubins increased several times, alanine aminotransferase once, reduced/oxidized glutathione ratio decreased three times, lipid peroxidation and collagen increased about three-times by biliary obstruction ($p<0.05$). Silymarin, vitamin E or C failed to prevent these effects significantly. It is not possible to clarify the role of oxidative stress in the fibrotic process induced by chronic biliary obstruction with the present results. Therefore, it seems reasonable to propose that a wide mixture of antioxidants, administered by the parenteral route (because cholestasis decreased the absorption of lipophilic compounds), is needed to counteract the oxidant stress produced by cholestasis.

Free radicals, oxidative stress and lipid peroxidation are frequently associated with several types of liver diseases (Muriel 1997). However, the question is whether these factors contribute to the development of liver damage or are consequences or parallel events of the pathological process. While in some models of liver damage it is clear that oxidative stress plays an important role, like in acetaminophen (Muriel *et al.* 1992) or CCl_4 intoxications (Muriel & Mourelle 1990a & b; Sotelo-Félix *et al.* 2002), in other models it is not so clear. In particular, in liver damage produced by bile flow obstruction the role of oxidative stress and lipid peroxidation processes in the development of liver injury is not fully understood. Several studies have demonstrated that free radicals are present in cholestatic damage. Bile acids enhance the release of reactive oxygen species from activated rat polymorphonuclear cells (Dahm *et al.* 1998), inflammatory cells which are present in both experimental and human cholestatic liver lesions (Kountaras *et al.* 1984). High concentrations of plasma lipid peroxides have been observed in humans with cholestasis (Lemonnier *et al.* 1987) and in bile duct-ligated (BDL) rats (Muriel 1996). A reduced antioxidative capacity in hepatic mitochondria and reduced glutathione (GSH) levels in the liver were reported after 28 days of biliary obstruction in the rat (Krähenbühl *et al.* 1995).

We have shown that lipid peroxidation increased three days after bile duct-ligated rats while liver injury was evident after one day of bile flow obstruction (Muriel & Suárez 1994), suggesting that oxidative stress (which actually occurs) is rather a consequence than a cause of liver damage. Moreover, treatment of seven day-bile duct-ligated rats with lipophilic (vitamin E) and/or hydrophilic (trolox) antioxidants completely prevented the increase in lipid peroxidation and effectively maintained the normal GSH/oxidized GSH ratio both in liver and plasma, but failed to prevent liver damage measured by histology and by plasmatic enzyme activities (Barón & Muriel 1999). This strongly suggests that oxidative stress does not play an important role in the development of acute liver damage produced by bile duct ligation. However, the role of oxidant stress in chronic cholestatic injury is still a matter of study.

Prolonged biliary obstruction for 28 days or more is associated with considerable increases in liver collagen content. It has been postulated that products of lipid peroxidation (aldehyde-protein adducts for example) modulate collagen gene expression and may be a link between liver injury and fibrosis (Chojkier *et al.* 1998). Chojkier *et al.* (1989) have previously shown that, in cultured cells, enhanced lipid peroxidation stimulates collagen gene transcription whereas basal lipid peroxidation regulates the constitutive collagen gene transcription (Houglum *et al.* 1991). In the CCl_4-cirrhosis model, Bedosa *et al.* (1994) found colocalization of increased collagen α_1 (I) mRNA with lipid peroxidation by means of *in situ* hybridization and immunohistochemical study for malondialdehyde and 4-hydroxynonenal protein adducts. These experiments suggest that hepatocyte lipid peroxidation may play a major role in the regulation of collagen gene expression and that it may be a link between hepatocyte injury and hepatic fibrosis.

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Therefore, despite oxidative stress is not the cause of acute cholestatic liver injury (Muriel & Suarez 1994; Barón & Muriel 1999), it seems reasonable to investigate if lipid peroxidation produced by cholestasis may induce collagen accumulation with the final result of liver fibrosis. To test this hypothesis, vitamin E (a lipophilic antioxidant), vitamin C (a hydrophilic antioxidant) and silymarin, a wide range oxygen radicals scavenger (Pascual *et al.* 1993), were administered daily to bile duct-ligated rats during 28 days. Lipid peroxidation, glutathione content, fibrosis, necrosis and cholestasis were evaluated.

Materials and Methods

Materials. Vitamin C (L(+)-ascorbic acid) was obtained from Merck Co., Darmstadt, Germany. Silymarin, vitamin E ((±)-α-tocopherol acetate), GSH, oxidized GSH, o-phthaldehyde, chloramine-T, methyl cellosolve, sodium thiosulfate, p-dimethylaminobenzaldehyde, thiobarbituric acid and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, USA. Sodium acetate, sodium hydroxide, glacial acetic acid, hydrochloric acid, sodium chloride, toluene, and potassium hydroxide were obtained from J. T. Baker Co., Xalostoc, Mexico City, Mexico. The other reagents were of the best quality commercially.

Treatment of animals. Male Wistar rats weighing around 300 g were used. The animals had free access to food (standard Purina chow diet; Purina, St. Louis, MO, USA) and water. Extrahepatic cholestasis was induced by double ligation and section of the common bile duct (bile duct ligation). Eight groups (n=6) were performed. Four groups were bile duct-ligated and received vitamin C (50 mg/kg/day, orally), vitamin E (400 IU/rat/day, orally), silymarin (50 mg/kg/12 hr, orally) or vehicles; four groups were sham-operated and received vitamin C, vitamin E, silymarin or vehicles as indicated before. Blood was collected by cardiac puncture and the liver was rapidly removed. All of the samples were kept on ice until analysis. Animals received human care and the study complied with the institution's guidelines and the Mexican official regulation (NOM-062-ZOO-1999) regarding technical specifications for production, care and use of laboratory animals.

Alanine aminotransferase and bilirubins determinations. Plasma was obtained for the determination of alanine aminotransferase (ALT) (Reitman & Frankel 1957) activity and for bilirubin content (kit from Bioxon®, Becton Dickinson, Mexico city, Mexico).

Assessment of lipid peroxidation. The extent of lipid peroxidation was estimated in liver homogenates by measurement of malondialdehyde formation using the thiobarbituric acid method (Okawa *et al.* 1979). Protein was determined according to Bradford (1976) using bovine serum albumin as standard.

Reduced and oxidized glutathione determinations in liver. GSH and oxidized GSH were prepared in 0.1 M sodium phosphate 0.005 M EDTA buffer (pH 8.0) and kept on ice until used. O-Phthaldehyde solution was prepared in reagent-grade absolute methanol just prior to use. For tissue pareparation 250 mg of liver were homogenized on ice using a polytron homogenizer. The solution used for homogenization consisted of 3.75 ml of the phosphate-EDTA buffer and 1 ml of 25% HPO3, which was used as a protein precipitant. The total homogenate was centrifuged at 4° at 100,000 ×g for 20 min. to obtain the supernatant for the assay of GSH and oxidized SGH (Hissin & Hilf 1976).

Collagen quantification. Collagen concentrations were determined by measuring hydroxyproline-containing liver samples after digestion with acid (Prockop & Undenfriend 1960) as described previously (Muriel 1996).

Statistics. For statistical analysis, ANOVA followed by the Tukey test was used to compare groups. In all cases a difference was considered significant when $P<0.05$.

Results

Sham-operated animals treated with vehicles, silymarin, vitamin C or E gained around 20 g weight per week, while bile duct-ligated animals, regardless of the pharmacological treatment, did not gain or loose weight during the 4 weeks of treatment. Livers from sham-operated rats weighed around 12 g, while livers from biliary obstructed rats weighed about 20 g. Pharmacological treatments were not capable of modifying liver weights.

Bile duct ligation in the rat is a model of complete mechanical extrahepatic cholestasis. Thus, as expected, in rats with bile flow obstruction (bile-duct ligated rats) total, conjugated and unconjugated bilirubins increased several times ($P<0.05$) as compared with sham-operated controls (fig. 1). Since conjugated bilirubins increased considerably, it can be assumed that hepatocytes were still able to conjugate bilirubins. Antioxidant treatments with silymarin, or vitamins C or E was not capable of preventing cholestasis induced by bile duct ligation as determined by bilirubin concentrations.

Fig. 2 shows the plasmatic enzyme activity of alanine aminotransferase under the various experimental conditions used in this study. Alanine aminotransferase is a cytosolic enzyme of the hepatocyte and an increase in serum of this enzyme reflects an increase in plasma membrane permeability, which, in turn, is associated with cell death (Kaplan 1993). Biliary obstruction for 28 days led to significant increases in plasma alanine aminotransferase activity regardless of the antioxidant treatments, indicating that these antioxidants, at the doses tested, were not able to prevent necrosis measured by plasmatic alanine aminotransferase activity.

As an indicator of oxidative stress at the hydrophilic level, we measured GSH and oxidized GSH in the liver of animals subjected to bile duct ligation and treated with antioxidants. Fig. 3 depicts GSH (upper panel), oxidized GSH (middle panel) and GSH/oxidized GSH ratio (lower panel) in the liver. Reduced glutathione decreased slightly but significantly by chronic cholestasis, whereas oxidized GSH increased considerably, and as a consequence, the GSH/oxidized GSH ratio decreased almost three times in the liver of bile duct-ligated rats. Interestingly, vitamins C, E or silymarin treatments failed to prevent this important decrement in the GSH/oxidized GSH ratio. Sham-operated rats treated with antioxidants showed normal GSH and oxidized GSH contents.

Because lipid peroxidation has been associated with collagen synthesis induction (Chojkier *et al.* 1989 & 1998; Houglum *et al.* 1991; Bedosa *et al.* 1994), both parameters are depicted in fig. 4. The extent of lipid peroxidation

(upper panel) was expressed as the liver content of malondialdehyde, one of its end-products which has been associated with the induction of collagen synthesis (Bedosa 1994). A very important increase (about six-times) in lipid peroxidation was observed after 4 weeks of bile flow obstruction. Vitamins C and E showed inhibition of the lipid peroxidation process, however, the difference did not reach statistical significance probably due to interindividual variations (S.E.). The bile duct-ligated group treated with silymarin depicted the same malondialdehyde concentration in the liver as the untreated bile duct-ligated group. Collagen content (fig. 4, lower panel), determined by hydroxyproline quantification, increased three times by bile duct ligation for 28 days. Neither silymarin, vitamin C or E were capable of preventing collagen accumulation. It is worth noting that the antioxidants failed to (significantly) prevent both lipid peroxidation and collagen accumulation (fig. 4 both panels) induced by bile duct ligation.

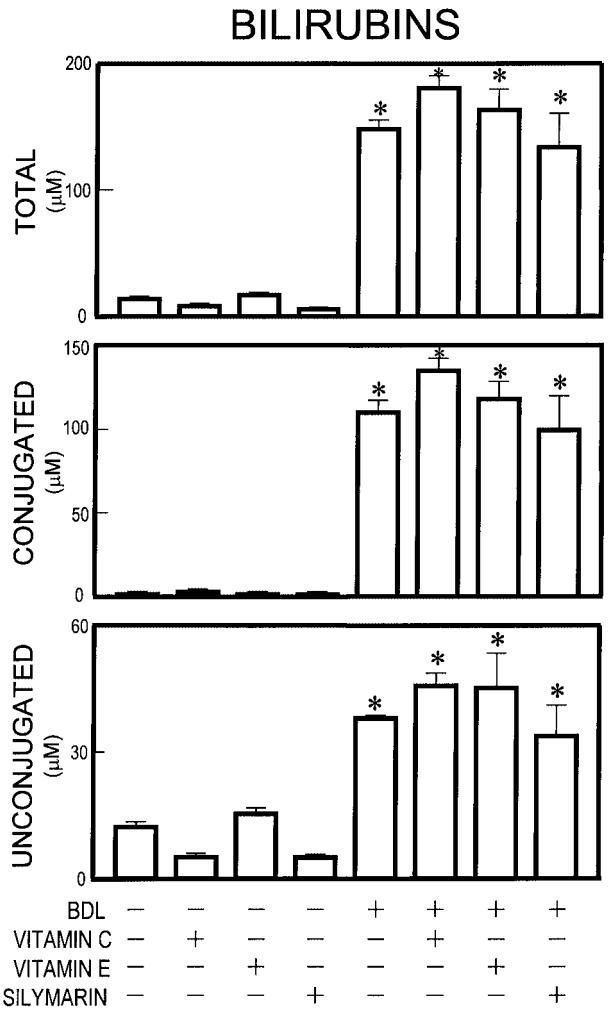

Fig. 1. Total (upper panel), conjugated (middle panel) and unconjugated (lower panel) bilirubins, determined in plasma from sham-operated or bile duct ligation-operated rats treated with vitamin C (50 mg/kg/day, orally), vitamin E (400 IU/rat/day, orally), silymarin (50 mg/kg/12 hr, orally) or vehicles. Each bar represents the mean value of experiments performed in duplicate assays with samples from 6 animals±S.E.M. (*) Means different from the control group, $P<0.05$.

Discussion

Neither silymarin nor vitamin E nor vitamin C had considerable effect on bilirubin, transaminases, glutathione, lipid peroxidation or collagen contents in the liver of rats subjected to biliary obstruction.

Despite the fact that lipid peroxidation and reduced glutathione depletion do not seem to participate importantly in acute cholestasis induced by bile duct ligation in the rat (Muriel & Suárez 1994; Barón & Muriel 1999), these processes may induce collagen synthesis (Chojkier *et al.* 1989 & 1998; Houglum *et al.* 1991; Bedosa *et al.* 1994), linking liver damage with a fibrotic process. However, treatment with high doses of hydrophilic (vitamin C) or lipophilic (vitamin E) antioxidants or a wide range oxygen radicals scavenger, silymarin (Pascual *et al.* 1993) failed to protect the liver from cholestasis, necrosis, oxidative stress or fibrosis. With the present results it is not possible to clarify the role of oxidative stress in the fibrotic process induced by chronic biliary obstruction. A more potent antioxidant therapy may be needed to study if oxidative stress is important for the development of fibrosis in the bile duct-ligand model.

The dose of vitamin C was chosen according to a report by Halim *et al.* (1997) in which 50 mg/kg prevented oxidative stress, necrosis and fibrosis induced by CCl_4 intoxication. We have previously reported that 400 IU/kg of vitamin E prevented lipid peroxidation and glutathione depletion (Barón & Muriel 1999). The dose of silymarin employed has been widely used by us (Mourelle *et al.* 1989; Muriel & Mourelle 1990a & b) and others (Boigk *et al.* 1997).

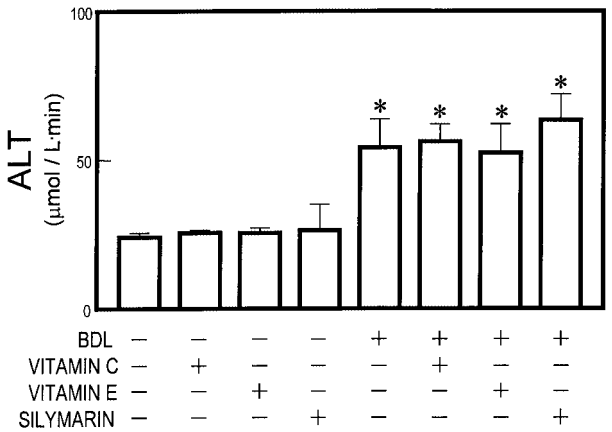

Fig. 2. Enzymatic activity of alanine aminotransferase (ALT) determined in plasma from sham-operated or bile duct ligation-operated rats treated with vitamin C (50 mg/kg/day, orally), vitamin E (400 IU/rat/day, orally), silymarin (50 mg/kg/12 hr, orally) or vehicles. Each bar represents the mean value of experiments performed in duplicate assays with samples from 6 animals±S.E.M. (*) Means different from the control group, $P<0.05$.

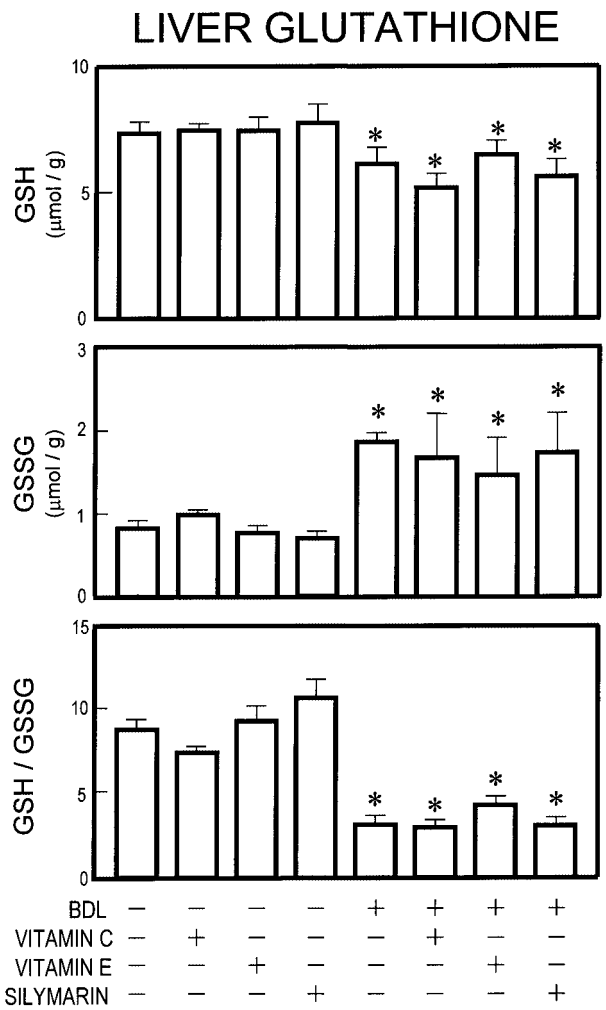

Fig. 3. Reduced (GSH; upper panel), and oxidized GSH (GSSG; middle panel) glutathione, and GSH/GSSG ratio, determined in livers from sham-operated or bile duct ligation-operated rats treated with vitamin C (50 mg/kg/day, orally), vitamin E (400 IU/rat/day, orally), silymarin (50 mg/kg/12 hr, orally) or vehicles. Each bar represents the mean value of experiments performed in duplicate assays with samples from 6 animals±S.E.M. (*) Means different from the control group, $P<0.05$.

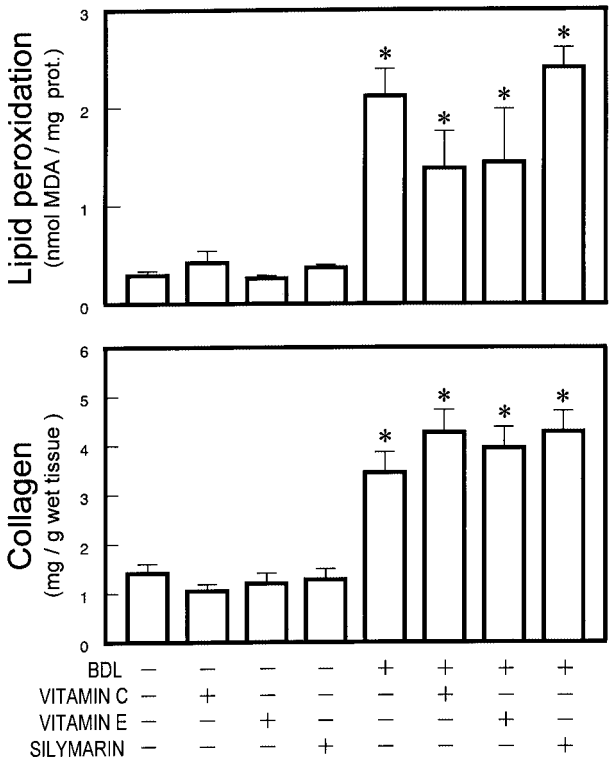

Fig. 4. Lipid peroxidation, expressed as malondialdehyde (MDA) content (upper panel) and collagen, measured as the hepatic hydroxyproline content, determined in livers from sham-operated or bile duct ligation-operated rats treated with vitamin C (50 mg/kg/day, orally), vitamin E (400 IU/rat/day, orally), silymarin (50 mg/kg/12 hr, orally) or vehicles. Each bar represents the mean value of experiments performed in duplicate assays with samples from 6 animals ± S.E.M. (*) Means different from the control group, $P<0.05$.

In addition to its antioxidant properties, ascorbic acid (vitamin C) has a well documented role in collagen metabolism as a direct requirement for prolyl and lysyl hydroxylases (Peterkofsky 1972). Ascorbic acid also stimulates collagen production in cultured chick tendon (Schwarz & Bissell 1977) and human skin (Murad *et al.* 1981) fibroblasts. Ascorbic acid increases procollagen mRNA levels in cultured fibroblast (Murad *et al.* 1981; Lyons & Schwarz 1984), and this effect is the result of the stimulation of procollagen gene transcription and the decreased degradation of the procollagen mRNA (Lyons & Schwarz 1984). Thus, these effects may explain the lack of an antifibrotic effect of vitamin C on bile duct-ligand fibrosis. In fact, the bile duct-ligated group receiving vitamin C showed higher concentrations of collagen than untreated bile duct-ligated rats (fig. 4), although this difference did not reach statistical significance.

Boigk *et al.* (1997) tested the effect of silymarin in a model of bile duct occlusion in which, instead of ligation of the bile duct, injection of Na-amidotrizoate to the common bile duct was performed to induce total bile flow obstruction. They found a partial but significant prevention of fibrosis by silymarin treatment (50 mg/kg/day) whereas we did not, even when silymarin was administered more frequently (50 mg/kg/12 hr). The antifibrotic effect of silymarin observed by Boigk *et al.* (1997) may be attributed to inhibition of Kupffer cells that are known to be involved in the development of acute (Muriel *et al.* 2001) and chronic liver damage (Muriel & Escobar 2003). The main difference between the two studies is that the bile duct was obstructed for 6 weeks, producing a larger amount of collagen (nine times). Interestingly, the antifibrotic effect of silymarin was evident when the rats were treated from week 4 to 6 of bile duct occlusion no matter if rats were treated the previous 3 weeks. In the present work, only 4 weeks of obstruction of bile flow was performed, and only mild fibrosis was obtained (three times), and silymarin was administered for 28

days only. We have previously reported important beneficial effects of silymarin in acute (Muriel & Mourelle 1990a) and chronic (Mourelle *et al.* 1989; Muriel & Mourelle 1990b) CCl_4 intoxications and in paracetamol overdose (Muriel *et al.* 1992). However, in those liver damage models, oxidative stress clearly plays a key role in liver pathogenesis (Muriel 1997) and cannot be compared with the bile flow obstruction model in the rat (Muriel & Suarez 1994; Barón & Muriel 1999).

The lack of antioxidant effect of the compounds evaluated in this work may be due to the following factors. Cholestasis is associated with the retention of copper and bile acids, both are prooxidants. Not one, but a wide array of enzymatic and non-enzymatic antioxidant defences are needed to maintain the normal state of the cell. These defences include superoxide dismutase, glutathione peroxidase, catalase, β-carotene, ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, vitamin A, NADPH, adenosine, coenzyme Q, urate, methionine, cysteine, phenols and flavonoids (Mates *et al.* 1999). In this work we tested the effect of only three antioxidants individually. Moreover, due to the lack of bile salts in the gut, a consequence of cholestasis is malabsorption of fat soluble factors (for example vitamins A, D, E and K) and other free radical scavengers such as carotenoids. In this work, antioxidants were administered orally, and it seems reasonable to propose that a wide mixture of antioxidants, administered by the parenteral route, may be needed to counteract the oxidant stress produced by cholestasis. Furthermore, given the very low values of intrahepatic GSH/oxidized GSH ratio observed herein, N-acetylcysteine (a precursor of GSH), should be included in the list of antioxidants for the treatment of the increased oxidative stress observed in cholestasis.

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