Liver International 2003, 23, 262–268
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Liver International
ISSN 1478-3223

Fibrosis and glycogen stores depletion induced by prolonged biliary obstruction in the rat are ameliorated by metadoxine

Muriel P, Deheza R. Fibrosis and glycogen stores depletion induced by prolonged biliary obstruction in the rat are ameliorated by metadoxine. Liver 2003: 23: 262–268. © Blackwell Munksgaard, 2003

Abstract: *Background/Aims:* To evaluate liver-beneficial properties of metadoxine, not related with alcohol metabolism, bioactivation of external toxins or antioxidant mechanisms, the chronic bile duct ligation (BDL) model was used and results were compared with colchicine. *Methods:* Seven groups ($n = 6$) of male Wistar rats were used. Four groups were BDL and received metadoxine (60 mg/kg/12 h i.p.), colchicine (10 μg/rat/day/p.o.), both or vehicles; three groups were sham-appropriate controls. Collagen content was determined by measuring hydroxyproline in liver samples; malondialdehyde (MDA) was used to estimate lipid peroxidation levels; glycogen was determined utilizing the anthrone reagent; gomory's trichromic stains of liver sections were performed. *Results:* Collagen increased four-fold by BDL, metadoxine, colchicine or both prevented fibrosis partially; MDA levels increased three-fold by BDL and no treatment had any significant effect; glycogen was almost depleted in the cirrhotic group, metadoxine preserved glycogen; bilirubins, and alanine aminotransferase and γ-glutamyltranspeptidase activities increased several-fold in the BDL group, and both drugs prevented these effects partially. The histopathological analysis correlated with biochemical data. *Conclusions:* Both compounds showed similar antifibrotic properties; metadoxine was more effective in preserving glycogen. Besides its antioxidant effects and its ability to induce alcohol metabolism, metadoxine possesses important antifibrotic and antinecrotic properties, and maintains energy stores efficiently.

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Key words: fibrosis – liver injury – necrosis – glycogen – cholestasis – biliary obstruction – cirrhosis –metadoxine

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Received 15 October 2002,
accepted 4 March 2003

Metadoxine (pyridoxol L,2 pyrrolidone-5-carboxylate) is a combination of pyridoxine and pyrrolidone carboxylate. Experimental studies have demonstrated that this drug induces an increase in hepatic adenosine triphosphate (ATP) concentration and restores the hepatic levels of reduced glutathione (1, 2). In addition, metadoxine accelerates the plasma clearance of ethanol and acetaldehyde, and reduces the time exposure of the liver and other tissues to the toxic effect of ethanol and its metabolites (3). These properties explain its beneficial effects on alcohol liver diseases and others caused by free radicals and oxidative stress (4). Interestingly, metadoxine has also shown antifibrotic effects in the CCl_4-chronic administration model of fibrosis (5, 6). However, it is known that the action mechanism of CCl_4 is due to free radical generation, lipid peroxidation induction and depletion of reduced glutathione (7), making it difficult to separate the antioxidant properties of the compound from a direct antifibrotic effect. In addition, CCl_4-induced liver injury depends on the production of trichloromethyl radical by CYP2EI which can be modulated by several drugs (7). The bile duct ligation (BDL) model of fibrosis and cirrhosis in the rat is not caused by oxidative stress, since lipid peroxidation occurs later than liver damage (8), and restoration of normal redox status by administration of antioxidants does not ameliorate liver injury (9). These studies suggest that the oxidative stress observed in BDL, does not play a causative role in this model of liver disease. Moreover, no bioactivation of any toxin is required in the BDL model to produce damage. Therefore, in order to evaluate liver-beneficial properties of metadoxine not related with alcohol metabolism, bioactivation of an external toxin or

antioxidant mechanisms, the chronic BDL model was used. The ability to preserve liver energy stores, and the antinecrotic and antifibrotic properties of metadoxine were evaluated in this model of liver damage and compared with a well-known antifibrotic compound, colchicine (10).

Materials and methods

Materials

Metadoxine (Metasin®, Eurodrug laboratories, Fustery laboratories, Mexico City, Mexico) was purchased from the drugstore. Colchicine, chloramine-T, methyl cellosolve, sodium thiosulfate, p-dimethylaminobenzaldehyde, anthrone, thiobarbituric acid and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Citric acid, sodium acetate, sodium hydroxide, glacial acetic acid, hydrochloric acid, sodium chloride, toluene, sulfuric acid, iodine, ethanol, xylene, potassium hydroxide and formaldehyde were obtained from J. T. Baker (Xalostoc, Mexico). The other reagents were of the best quality commercially available.

Treatments 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) and water. Extrahepatic cholestasis was induced by double ligation and section of the common bile duct (BDL). The rats were categorized into seven groups ($n = 6$). Four groups were BDL and received metadoxine (60 mg/kg/12 h i.p.), colchicine (10 μg/rat/day/p.o.), both or vehicles; three groups were sham operated and received metadoxine, colchicine or vehicles as indicated before. Blood was collected by cardiac puncture and the liver was rapidly removed. All the samples were kept on ice until analysis. Animals received human care and the study complies 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.

Serum enzyme activities and bilirubin determinations

Plasma was obtained for the determination of γ-glutamyl transpeptidase (γ-GTP) (11), and alanine aminotransferase (ALT) (12) activities, and for bilirubin content (kit from Merck, Mexico City, Mexico).

Assessment of lipid peroxidation

The extent of lipid peroxidation was estimated in liver homogenates by measurement of malondialdehyde (MDA) formation using the thiobarbituric acid method (13). Protein was determined according to Bradford (14) using bovine serum albumin as standard.

Glycogen determination

Small liver pieces (0.5 g) were separated for glycogen determination using the anthrone reagent according to Seifter et al. (15).

Collagen quantification

Collagen concentrations were determined by measuring hydroxyproline-containing liver samples, after digestion with acid (16). The procedure was as follows. Fresh liver samples (100 mg) were placed in ampoules, 2 ml of 6 N HCl was added, and then the samples were sealed and hydrolyzed at 100 °C for 48 h. Next, the samples were evaporated at 50 °C for 24 h and resuspended in 3 ml of sodium acetate-citric buffer, pH 6.0; 0.5 g of activated charcoal was added, the mixture was stirred vigorously, and then it was centrifuged at 5000g for 10 min. The mixture was kept for 20 min at room temperature and the reaction was stopped by the addition of 2 M sodium thiosulfate and 1 N sodium hydroxide. The aqueous layer was transferred into test tubes. The oxidation product from hydroxyproline was converted into a pyrrole by boiling the samples. The pyrrole-containing samples were incubated with Ehrlich's reagent for 30 min, and the absorbance was read at 560 nm. Recovery of known amounts of standards was carried out on similar liver samples to provide quantification.

Histology

Samples were taken from all the animals and fixed with 10% formaldehyde in phosphate-buffered saline for 24 h. Then, they were washed with tap water, dehydrated in alcohol and embedded in paraffin. Sections of 6–7 μm were mounted in glass slides covered with silane. Gomory trichromic stains were performed in each slide.

Statistics

For statistical analysis, ANOVA followed by the Tukey test was used to compare groups (17). In all cases a difference was considered to be significant when $P < 0.05$.

Results

SHAM-operated animals treated with vehicles, colchicine or metadoxine gained around 20 g per week, while BDL animals, regardless of the

pharmacological treatment, did not gain or lose weight during the 4 weeks of treatment. Livers from SHAM rats weighed around 12 g, while livers from biliary-obstructed rats weighed about 20 g; pharmacological treatments were incapable of modifying liver weights.

ALT and γ-GTP plasmatic enzyme activities are depicted in Fig. 1 (upper and lower panels, respectively). Both activities in the SHAM-operated rats remained within the control values despite pharmacological treatment with metadoxine, colchicine or both. BDL for 4 weeks elevated ALT and γ-GTP about three- and five-fold, respectively. Pharmacological treatment with metadoxine or colchicine prevented these elevations partially, but significantly. It seems that metadoxine afforded a better protection on ALT, while colchicine on γ-GTP; however, the difference did not reach statistical significance. It also appears that the simultaneous administration of both compounds protected better against the elevation of γ-GTP, but again the difference was not significant.

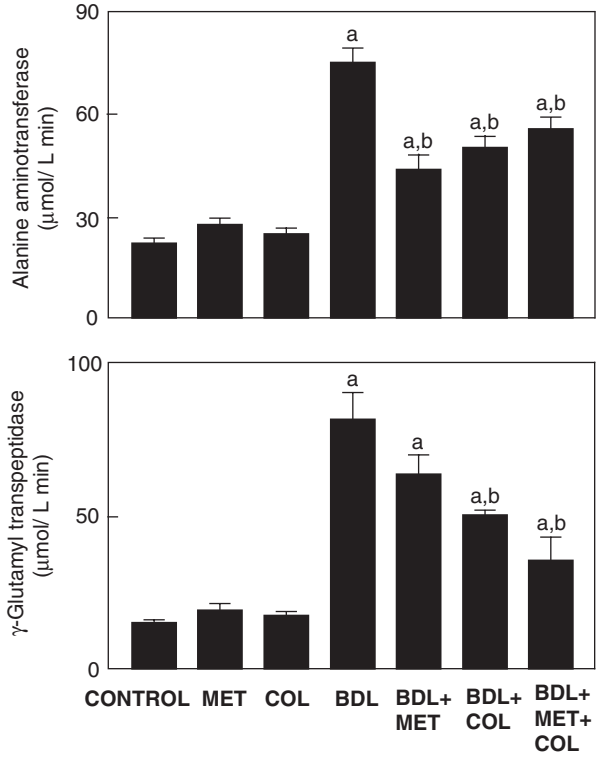

Fig. 1. Enzymatic activities of alanine aminotransferase (ALT; upper panel) and γ-glutamyl transpeptidase (γ-GTP; lower panel), determined in plasma from sham-operated rats treated with vehicle (CONTROL), metadoxine (MET) or colchicine (COL), and bile duct ligated rats (BDL) treated with vehicle, BDL+MET, BDL+COL and BDL+MET+COL. Each bar represents the mean value of experiments performed in duplicate assays with samples from six animals ± SEM. (A) Means different from the CONTROL group, $P < 0.05$. (B) Means different from the BDL group, $P < 0.05$.

BDL in the rat is a model of complete mechanical extrahepatic cholestasis; thus, bilirubins were expected to increase significantly after 4 weeks of BDL. Total bilirubins increased about 40 times by BDL as compared to the SHAM groups treated with the drugs or the vehicle; again, metadoxine, colchicine or both preserved ($P < 0.05$) bilirubin levels partially, but a better protective effect was observed in the animals treated with colchicine and no additive effect was found (Fig. 2).

Lipid peroxidation occurs in this model of liver damage (7); accordingly, in this work, the degree of lipid peroxidation increased nearly three-fold (Fig. 3, upper panel). Interestingly, metadoxine was unable to prevent such an effect, indicating that in this model of cirrhosis, the beneficial properties afforded by this compound are not associated with its antioxidant effects. Colchicine-treated BDL rats showed lower values of lipid peroxidation than nontreated BDL rats; however, the difference was not significant.

Glycogen, the main source of energy in the liver, is a very sensitive factor and thus a reliable marker of liver damage. Fasting decreases glycogen stores; thus care was taken to avoid fasting. Although manipulation may alter glycogen, all animals, including controls, were subjected to the same procedures and the control glycogen values are similar to those reported by several authors. Glycogen was depleted by biliary obstruction for 4 weeks (Fig. 4, lower panel). Colchicine was unable to preserve glycogen stores, but metadoxine efficiently and completely preserved liver glycogen content within the normal values.

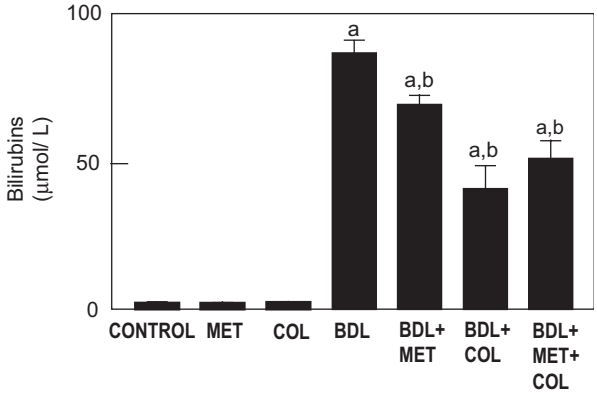

Fig. 2. Total bilirubins determined in plasma from sham-operated rats treated with vehicle (CONTROL), metadoxine (MET) or colchicine (COL), and bile duct ligated rats (BDL) treated with vehicle, BDL+MET, BDL+COL and BDL+MET+COL. Each bar represents the mean value of experiments performed in duplicate assays with samples from six animals ± SEM. (A) Means different from the CONTROL group, $P < 0.05$. (B) Means different from the BDL group, $P < 0.05$.

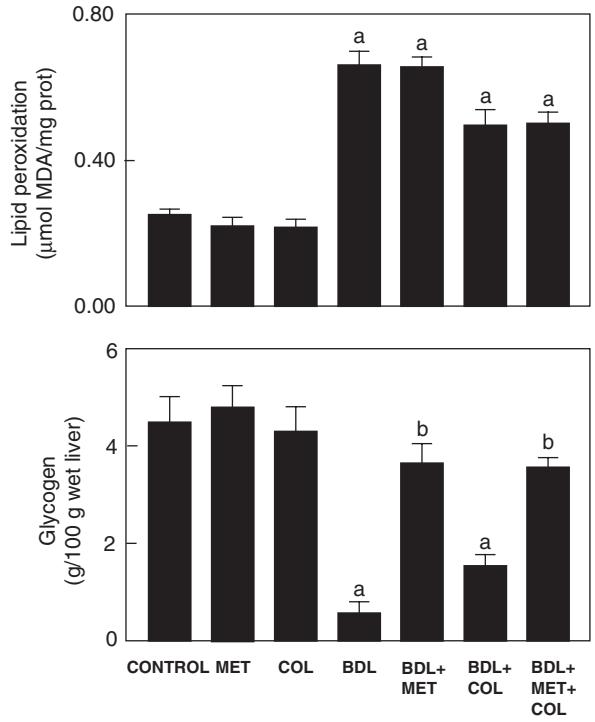

Fig. 3. Lipid peroxidation, expressed as malondialdehyde (MDA) content (upper panel) and glycogen content (lower panel) determined in liver samples from sham-operated rats treated with vehicle (CONTROL), metadoxine (MET) or colchicine (COL), and bile duct ligated rats (BDL) treated with vehicle, BDL+MET, BDL+COL and BDL+MET+COL. Each bar represents the mean value of experiments performed in duplicate assays with samples from six animals ± SEM. (A) Means different from the CONTROL group, $P < 0.05$. (B) Means different from the BDL group, $P < 0.05$.

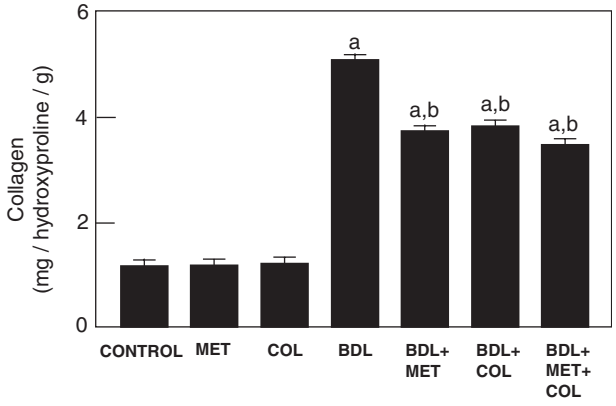

Fig. 4. Liver collagen, expressed as the hepatic hydroxyproline content determined in sham-operated rats treated with vehicle (CONTROL), metadoxine (MET) or colchicine (COL), and bile duct ligated rats (BDL) treated with vehicle, BDL+MET, BDL+COL and BDL+MET+COL. Each bar represents the mean value of experiments performed in duplicate assays with samples from six animals ± SEM. (A) Means different from the CONTROL group, $P < 0.05$. (B) Means different from the BDL group, $P < 0.05$.

Fibrosis, which is the final result of prolonged liver injury and one of the most important features of cirrhosis, was quantified by measuring hydroxyproline, expressed as liver collagen content in Fig. 4. Biliary obstruction during 28 days increased collagen content nearly five-fold; this effect was prevented, partially but significantly, by metadoxine treatment. The antifibrotic effect of the compound used to compare metadoxine properties, colchicine, was identical to that afforded by the former compound. In addition, no further prevention of collagen accumulation was obtained when both compounds were administered simultaneously.

Fibrosis was also evaluated by a histological approach (Fig. 5). Prolonged biliary obstruction was accompanied by a marked increase in collagen deposition around the portal triad, the normal architecture was lost, extended necrotic areas were frequently observed and a marked ductular proliferation was present (Fig. 5B) as compared with the control group (Fig. 5A). Administration of metadoxine to BDL rats resulted in a less severe fibrosis and in a reduction of necrotic areas in the hepatic parenchyma (Fig. 5C). Similarly, colchicine ameliorated the effects produced by BDL (Fig. 5D). Cotreatment of BDL rats with both metadoxine and colchicine did not result in an enhanced antifibrotic or antinecrotic response (Fig. 5E). Metadoxine (Fig. 5F) or colchicine (not shown) treatments to SHAM rats induced no alterations in the liver parenchyma. It is worth noting that the histopathological analysis revealed similar results as the biochemical results with regard to necrosis (Fig. 1, upper panel; ALT is indicator of necrosis) and fibrosis (Fig. 4).

Discussion

In this model of fibrosis and cirrhosis, oxidative stress does not play a causative role (7–9). In addition, metadoxine did not affect lipid peroxidation induced by BDL. Cytochrome P450 is not involved in this model of liver damage (7). Alcohol or any other xenobiotic was not utilized to induce liver damage. Taking this information altogether, it can be suggested that metadoxine possesses important antinecrotic and antifibrotic properties not associated with its well-described effects on alcohol metabolism (1–3) or with its antioxidant activities (1).

Several doses of metadoxine are reported in the literature. For example, Parés et al. (18) administered 80, 160 and 320 mg/kg, daily for 5 weeks to alcohol-treated rats. The first dose resulted in a partial, but significant, effect on ADH activity, the second one provided a full effect and the third one produced no further protection against ethanol intoxication. None of the doses produced

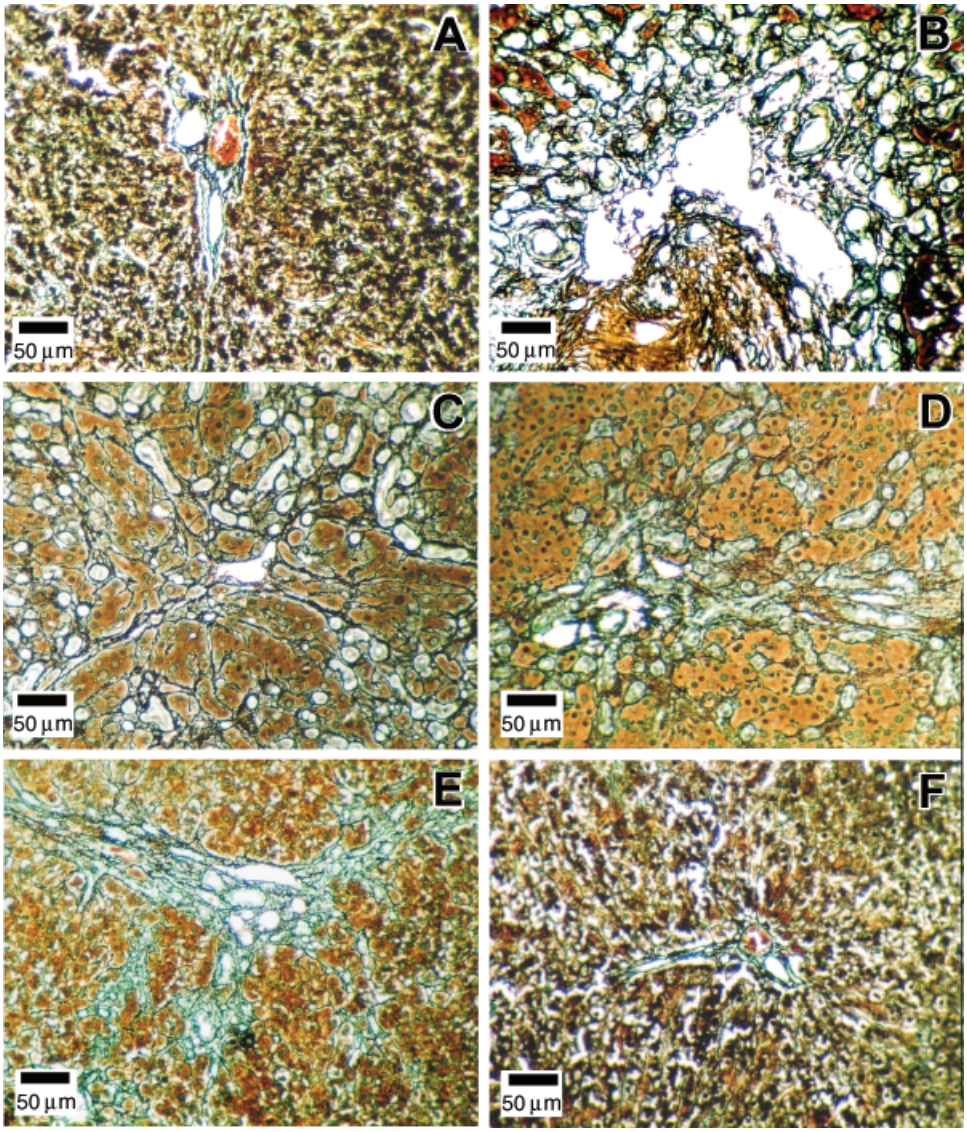

Fig. 5. Gomory's trichromic stains of liver sections from a sham-operated rat treated with vehicle (A), a bile duct ligated (BDL) rat treated with vehicle only (B), a BDL rat treated with metadoxine (C), BDL treated with colchicine (D), BDL treated with both metadoxine and colchicine (E) and a sham rat treated with metadoxine (MET).

any toxic effect. Annoni et al. (5) tested metadoxine at a dose of 200 mg/kg/i.p./day to prevent active fibroplasias in CCl_4-treated rats; this resulted in a significant pharmacological effect and showed no toxicity. Arosio et al. (6) also used 200 mg/kg/i.p., daily with good results. Thus, it seemed reasonable to administer 120 mg/kg of body weight divided into two doses of 60 mg/kg/i.p. Previously we have studied the effect of colchicine at various doses on prolonged bile duct obstruction in the rat (19). We found that the administration of colchicine 10 µg/rat/day for 2 months produced no diarrhea, but 50 µg/rat/day produced diarrhea in all animals treated. Colchicine at a dose of 200 µg/rat/day produced, in addition to severe diarrhea, a 40% mortality after 2 months of treatment. Thus, in that work (19) and in others (10), including the present one, we have chosen a dose of 10 µg/rat/day.

Annoni et al. (5, 6) were the first to demonstrate an antifibrotic effect of metadoxine by utilizing the chronic CCl_4 intoxication model. The beneficial results obtained in that model may be explained by the antioxidant properties of metadoxine or by interferences of CYP2E1 in the bioactivation of CCl_4, which have no relevance in the BDL model utilized in this study. However, they found a very interesting result – a significant reduction in serum levels of immunoreactive prolyl hydroxylase by metadoxine. This observation suggests less procollagen hydroxylation, an

essential step in collagen biosynthesis, and constitutes a possible explanation for the antifibrotic effect of the compound reported herein.

Metadoxine has demonstrated antifibrotic properties in the model of CCl_4 chronic intoxication (5, 6), in alcohol-acetaldehyde induced collagen production *in vitro* (20) and in the model of prolonged BDL (present work). The three models share in common significant increases in cytokines production (20, 21). Cytokines are soluble mediators produced by diverse cell types in response to various stimuli, including antigens, adhesion molecules and other cytokines. Although they play important roles in the normal physiology of cells, cytokines are usually discussed in the context of immune response, inflammation, and tissue injury or repair. Several studies have shown that the production of proinflammatory cytokines, particularly TNF-α, increases in conjunction with liver damage produced by ischemia–reperfusion, endotoxin or hepatotoxicants. Neutralization or suppression of TNF-α decreases the extent of hepatic injury induced by chronic BDL (19), acetaminophen (22), acute CCl_4 intoxication (23), ischemia–reperfusion (24) and cadmium-induced hepatotoxicity (25), among others.

TNF-α may induce both apoptosis in hepatocytes involving caspases (26), and necrosis of hepatocytes via a direct mechanism and through the production of nitric oxide by the hepatocytes themselves (27). The biological effect of cytokines, such as TNF-α, depends on the concentration: low concentrations are involved in homeostasis, whereas an increasing local concentration of TNF-α is associated with local inflammatory response and focal hepatic necrosis (28). Gutiérrez-Ruiz et al. (20) reported that metadoxine inhibits the secretion of TNF-α induced by acetaldehyde in hepatocytes and hepatic stellate cells in culture. TNF-α induction is known to be one of the earliest events in hepatic inflammation, triggering a cascade of other cytokines that cooperate to kill hepatocytes, recruit inflammatory cells and initiate a wound-healing response that includes fibrogenesis (29). Metadoxine prevention of the TNF-α increase (20) may in turn prevent other inflammatory and fibrogenic cytokines from being secreted, and as a consequence collagen secretion and necrosis are attenuated and liver function is preserved.

The most accentuated beneficial effect of metadoxine (not shared by colchicine) was its ability to preserve glycogen levels completely, otherwise depleted by BDL. Felicioli et al. (1) demonstrated the ability of metadoxine to preserve hepatic and cerebral ATP levels in ethanol-treated rats. They attributed this property to the combined action of pyridoxine and pyrrolidone carboxylic acid to stimulate the *de novo* synthesis of purines and to restore the enzymatic activities of pyridoxal phosphate-dependent enzymes. However, further experiments are required to determine the mechanism by which metadoxine preserves liver glycogen stores in BDL-induced liver damage.

The present work demonstrates, for the first time, important antinecrotic and antifibrotic properties of metadoxine, similar to those of colchicine, in a model of liver fibrosis that does not involve, as mechanisms of damage, alcohol, metabolism of xenobiotics or oxidative stress. In addition, metadoxine (but not colchicine) was very effective in preserving liver energy stores in the form of glycogen.

Acknowledgments

The authors express their gratitude to Dr. Victor Tsutsumi and Dr. Mineko Shibayama for performing the histopathological analysis, Mr. Alfredo Padilla for preparing the figures, and Mrs. Patricia González, Mr. Ramón Hernández, Mr. Benjamín Salinas, Mr. Mario Moreno and Miss Verónica Martínez for their excellent technical assistance. This study was supported in part by Grant 34394M from Conacyt, Mexico.

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