

METADOXINE PREVENTS DAMAGE PRODUCED BY ETHANOL AND ACETALDEHYDE IN HEPATOCYTE AND HEPATIC STELLATE CELLS IN CULTURE

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Metadoxine (pyridoxine-pyrrolidone carboxylate) has been reported to improve liver function tests in alcoholic patients. In the present work we have investigated the effect of metadoxine on some parameters of cellular damage in hepatocytes and hepatic stellate cells in culture treated with ethanol and acetaldehyde. HepG2 and CFSC-2G cells were treated with 50 mM ethanol or 175 μ M acetaldehyde as initial concentration in the presence or absence of 10 μ g ml⁻¹ of metadoxine. Twenty-four hours later reduced and oxidized glutathione content, lipid peroxidation damage, collagen secretion and IL-6, IL-8 and TNF- α secretion were determined. Our results suggest that metadoxine prevents glutathione depletion and the increase in lipid peroxidation damage caused by ethanol and acetaldehyde in HepG2 cells. In hepatic stellate cells, metadoxine prevents the increase in collagen and attenuated TNF- α secretion caused by acetaldehyde. Thus, metadoxine could be useful in preventing the damage produced in early stages of alcoholic liver disease as it prevents the redox imbalance of the hepatocytes and prevents TNF- α induction, one of the earliest events in hepatic damage.

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KEY WORDS: metadoxine, ethanol, acetaldehyde, hepatocytes, hepatic stellate cells.

INTRODUCTION

Metadoxine is the ion-pair between pyridoxine and pyrrolidone carboxylate. Both substances have been used in the treatment of alcoholic liver disease. Pyridoxine is metabolized in the liver and released to systemic circulation principally as pyridoxal-P. It has been reported to reverse the reduction in the apoenzyme for tryptophan pyrrolase caused by chronic ethanol intake in rats, an effect suggested to be due to NAD⁺ and NADP⁺ regeneration [1]. Pyrrolidone carboxylate is a cyclic lactam of glutamic acid first reported as an intermediate in the γ -glutamyl cycle, a metabolic pathway that accounts for the synthesis and degradation of glutathione [2]. The novelty of metadoxine is that it supplies both substances with high efficiency in equimolar amounts. Long-term alcohol exposure enhances the reduced glutathione depletion, the decrease of glutathione reductase and the increase of

glutathione transferase activities caused by age in rats. These effects are significantly reduced by metadoxine supplementation [3]. Pretreatment with metadoxine one hour before acute ethanol administration in rats has shown significant protection against reduced glutathione depletion and decreased activity of glutathione reductase [4]. Pretreatment with the drug before ethanol administration also inhibited free fatty acid and fatty acid ethyl esters accumulation on different organs. These effects were concomitant with the decreased ethanol blood levels found in alcohol-intoxicated rats pretreated with metadoxine [5].

Several studies concerning metadoxine's effect on hepatic fibrosis have been conducted. After 6 weeks of CCl₄ treatment rats developed fibrosis and inflammation of the liver, while those treated with CCl₄ and metadoxine had less severe lesions. There were striking increases in fibronectin and pro-alpha 2(I) collagen mRNA contents in the livers of CCl₄-treated animals and these enhancements were less evident in the metadoxine-treated rats [6]. Serum levels of prolyl hydroxylase have been reported to be similar to controls and in rats chronically treated with CCl₄ and metadoxine, in

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contrast with the ones treated only with CCl_4 which have significantly higher levels of the enzyme [7].

Clinical trials on chronic alcoholic patients using metadoxine have been made. In patients admitted to hospital for acute alcohol intake, metadoxine showed a significant improvement of the values of GGT, GPT, blood ammonia, blood alcohol and neuropsychic and behavioral parameters such as agitation, tremor, asterixis, sopor and depression compared with sedative and multivitamin drugs [8]. When it comes to evaluating its effect on fatty liver, after a three month treatment compared with placebo, metadoxine accelerates the normalization of liver function tests such as serum levels of bilirubin, aminotransferases and gammaglutamyl transpeptidase and the ultrasonographic changes [2].

Many alterations of cellular function have been documented due to ethanol itself and to its metabolite acetaldehyde. Ethanol diminishes glutathione content and increases lipid peroxidation damage in HepG2 cells [9]. Acetaldehyde increases collagen secretion in CFSC-2G cells [10, 11]

Since cell cultures constitute a good model for studying specific drug effects eliminating possible distracting factors, we decided in the present investigation to approach metadoxine's effect in preventing the damage produced by ethanol and acetaldehyde treatment on hepatoma and hepatic stellate cell lines. Oxidized and reduced glutathione, lipid peroxidation, collagen and cytokine secretion were evaluated.

MATERIALS AND METHODS

Cell culture

The human hepatocellular carcinoma cell line HepG2 retains many of the hepatocyte functions and was obtained from the American culture collection. HepG2 cells were routinely grown in monolayer culture in Williams E medium supplemented with 8% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin ($100 \text{ units ml}^{-1}$) and streptomycin (100 mg ml^{-1}). Rat hepatic stellate cells (CFSC-2G) were donated by Dr Marcos Rojkind. These cells' phenotype is similar to freshly isolated hepatic stellate cells [11]. These cells were cultured in minimum essential medium (MEM) supplemented with 8% bovine fetal serum (Hyclone, Logan, UT, USA), 1% nonessential amino acids, 2% glutamine, penicillin ($100 \text{ units ml}^{-1}$) and streptomycin (100 mg ml^{-1}).

Cells were grown at 37°C in disposable plastic bottles (Costar, USA), in a humidified atmosphere of 95% air and 5% CO_2 . The medium was replaced twice a week, and cells were harvested and diluted every 7 days at a ratio of 1 : 3.

Viability tests

Trypan blue. HepG2 and CFSC-2G cells were seeded at a density of 30 000 cells per well on a 24-well multichamber (Costar, USA). After 24 h, the culture medium was changed for one supplemented with

different concentrations of metadoxine (10, 25, 50, 100 and $1000 \mu\text{g ml}^{-1}$). Viability was determined after 24 h by measuring the ability of living cells to exclude Trypan blue vital dye at a 0.2% final concentration. Living cells were counted in a hemocytometer.

MTT assay. This assay measures the viability of the cells, based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate MTT into a dark blue formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the viable cell number in a range of the cell lines [12]. 150 000 cells were seeded on each well of a 24-well multichamber (Costar, USA). After 24 h, the culture medium was changed for a fresh one supplemented with different concentrations of methadoxine (10, 25, 50, 100 and $1000 \mu\text{g ml}^{-1}$). 24 h later, 1 ml of solution 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to each well. Cells were incubated for 3 h at 37°C . Finally, 0.04 M HCl in propan-2-ol was added to each well, mixed thoroughly to dissolve insoluble blue formazan crystals and the samples were spectrophotometrically read at 570 nm.

Treatments

Twenty-four hours after seeding, culture media were changed for serum-free ones containing ethanol 50 mM or acetaldehyde 175 μM (initial concentrations) and $10 \mu\text{g ml}^{-1}$ of metadoxine for 24 h. To control ethanol and acetaldehyde evaporation culture media were pre-equilibrated with 95% air/5% CO_2 , then ethanol or acetaldehyde was added and flask were immediately capped tightly. Cells that were only exposed to each toxin, to metadoxine or to serum-free media served as controls.

Initial ethanol concentration in the culture medium corresponds to the blood level of baboons that were chronically fed ethanol [13]. The initial acetaldehyde concentrations added to the cultures were either the same or higher than those in the hepatic venous blood levels observed in these animals; however, it is known that acetaldehyde concentrations in the liver are higher than those observed in the blood and, therefore, the concentration used in the cell culture media can be expected to be close to the intracellular hepatic acetaldehyde levels [14]. Moreover, in the HepG2 cell line, initial acetaldehyde concentration diminished during treatment due to the fact that this cell line presents aldehyde dehydrogenase activity [15].

GSH content assay

GSH content was assayed in HepG2 and CFSC-2G cells. Cells were washed twice with PBS and gently scraped into 0.5 ml of PBS. An aliquot was taken to determine protein. Standard curves were established using known GSH dilutions. Reduced (GSH) and oxidized (GSSG) glutathione were measured by the method of Tietze [16] by glutathione reductase and

NADPH followed by reduction of the colorimetric reagent 5-5'-dithio-bis-(2-nitrobenzoic acid) by GSH.

Lipid peroxidation

Lipid peroxidation was determined in HepG2 and CFSC-2G cells. Lipid peroxidation was assayed by determining the rate of production of thiobarbituric acid-(TBA-) reactive components [17]. For this determination cells (80×10^3 cells cm^{-2}) were seeded in Petri dishes exposed to the mentioned treatments. Cells were then washed with isotonic NaCl solution, scraped and resuspended in 2 ml of PBS. An aliquot was taken for protein assay [16]. The cellular suspension was centrifuged for 10 min at $3000 \text{ rev min}^{-1}$ and the pellet was re-suspended in 1 ml of PBS. Two ml of a solution containing 15% trichloroacetic acid, 0.25 N hydrochloric acid and 0.5% TBA were added to the suspension. Samples were heated for 25 min in a boiling water bath, cooled and centrifuged for 10 min at $3000 \text{ rev min}^{-1}$ in a clinical centrifuge. The absorbance of the supernatant was determined at a wavelength of 534 nm. An extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ was used to calculate the concentration of malondialdehyde per mg of protein. Protein contents were determined by the method described by Lowry *et al.* [18] with bovine serum albumin as standard. Results were expressed as malondialdehyde (MDA) equivalents, as it has been demonstrated that more than 80% of TBA reactive products are MDA [19].

Collagen secretion determination

Collagen secretion was determined on hepatic stellate cell line CFSC-2G. Culture medium hydroxyproline was measured in each experimental condition by the Woessner method [20]. Samples were hydrolyzed by adding HCl to a final concentration of 6 N. The samples were vacuum-sealed in small Pyrex test tubes and hydrolyzed for 14 h at 110°C . The tubes were then opened, and the contents were decanted into flasks. The solution was evaporated and water was added several times until a neutral dilution was obtained (2 ml final volume). Hydroxyproline oxidation was initiated by adding 100 μl chloramine T to each flask. The flask contents were mixed by shaking a few times and allowed to stand for 20 min at room temperature. The chloramine T was then destroyed by adding 100 μl perchloric acid to each flask. The contents were mixed and allowed to stand for 5 min. Finally, 100 μl of *p*-dimethylaminobenzaldehyde solution were added and shaken. The tubes were placed in a 60°C water bath for 20 min, then cooled in tap water for 5 min. The absorbance of the solution was determined spectrophotometrically at 557 nm. The hydroxyproline values were determined directly from a standard curve.

Immunoassay quantitative determination of IL-6, IL-8 and TNF- α

TNF- α and IL-6 were determined in CFSC-2G cells, IL-6 and IL-8 secretion by HepG2 cells were also

determined. TNF- α , IL-6 and IL-8 were determined with commercial immunoassay (R&D Systems) kits for TNF- α , IL-6 and IL-8. A monoclonal antibody specific for TNF- α , IL-6 and IL-8 has been pre-coated onto a microtiter plate. Standards and samples were pipetted into the wells, where TNF- α , IL-6 or IL-8 was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF- α , IL-6 or IL-8 was added to the wells. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The color development was stopped and the intensity of the color measured at 450 nm spectrophotometrically. All results were expressed in pg per million cells.

Data analysis

Data are reported as mean \pm SD. The SPSS package version 8 was used to run the analysis. Comparison among groups were done by means of ANOVA. Tukey's method was used for multiple comparisons. A $P < 0.05$ was considered as statistically significant.

RESULTS

Viability Assays

The viability of HepG2 and CFSC-2G cells exposed to concentrations $\leq 100 \mu\text{g ml}^{-1}$ of metadoxine was not statistically different to control cells when assessed by trypan blue exclusion (data not shown). When MTT assay was performed to HepG2 cells, the functionality of those exposed to $10 \mu\text{g ml}^{-1}$ of metadoxine was 98% decreasing thereafter to 70% when cells were exposed to metadoxine 25, 50 and $100 \mu\text{g ml}^{-1}$. At concentrations of 1 mg ml^{-1} practically all cells were dead [Fig. 1(a)]. On the other hand, stellate cell functionality measured also by MTT assay showed that the cells exposed to concentrations of $10 \mu\text{g ml}^{-1}$ of metadoxine had 98% of control cells', while exposure to concentrations of 25, 50, 100 and $1000 \mu\text{g ml}^{-1}$ of the drug decreased cell functionality to 91, 80, 77 and 0.9% of control cells' respectively [Fig. 1(b)].

GSH and GSSG determination

HepG2 cells exposed to ethanol had 26% less GSH than controls, and those treated with acetaldehyde reduced their GSH cell contents by 41% compared with control cells. Cells treated with metadoxine prevented this decrease [Fig. 2(a)].

Although oxidized glutathione cell content was higher in HepG2 cells exposed to ethanol or acetaldehyde and metadoxine than in cells treated only with the toxins, no statistically significant differences were found in either cell treatment [Fig. 2(b)]. Concerning stellate cells, no differences were found in reduced or oxidized glutathione cell contents under any treatment condition.

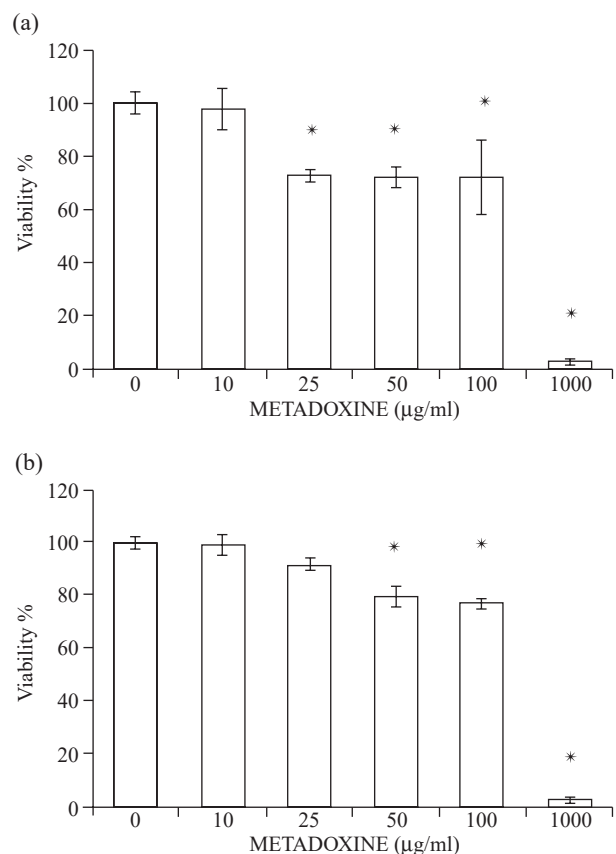


Fig. 1. Viability of HepG2 (a) and hepatic stellate cells CFSC-2G (b) cultured in the presence of metadoxine for 24 h. Viability of cells was determined by MTT assay. Each bar represents the mean \pm SD of at least three independent experiments made in triplicate. *Significantl different from control cells and & ethanol and acetaldehyde treated cells ($P < 0.05$).

Lipid peroxidation

Malondialdehyde production was increased by 23 and 70% in HepG2 cells exposed to ethanol or acetaldehyde respectively compared with control cells'. The increase was prevented by metadoxine administration (Fig. 3). No change in lipid peroxidation damage was observed in hepatic stellate cells.

Collagen secretion

Collagen secretion was 2.3 times higher in stellate cells exposed to acetaldehyde than in controls. Administration of metadoxine at the same time as exposure to acetaldehyde prevented such increase (Fig. 4). No variations in collagen secretion were detected on exposure to ethanol.

TNF- α , IL-6 and IL-8 secretion

TNF- α secretion by stellate cells was increased 16-fold in cells exposed to acetaldehyde compared with controls. When the cells were treated with acetaldehyde and metadoxine, TNF- α secretion was reduced by 63% with respect to the secretion determined in the media of cells exposed only to the acetaldehyde (Fig. 5).

No differences were found in IL-6 and IL-8 secretion to the media of HepG2 cells exposed to the different

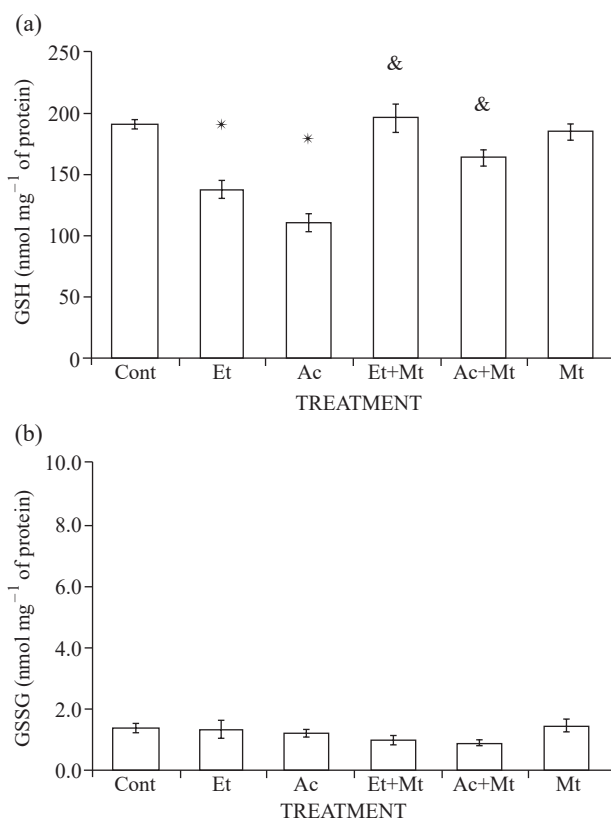


Fig. 2. Reduced and oxidized glutathione content of HepG2 cells treated with 50 mM ethanol (Et), 175 μ M acetaldehyde (Ac), 10 μ g ml⁻¹ metadoxine (Mt), ethanol plus metadoxine (Et + Mt) and acetaldehyde plus metadoxine (Ac + Mt). Cells were exposed to different treatments for 24 h. (a) Reduced (GSH) and (b) oxidized glutathione (GSSG) were determined. Each bar represents the mean \pm SD of at least three independent experiments made in triplicate. *Significantl different from control cells and & ethanol and acetaldehyde treated cells ($P < 0.05$).

treatments with respect to controls. Also, no detectable changes of IL-6 were titered in stellate cells' media (data not shown).

DISCUSSION

Increased deposition of collagen and other extracellular matrix proteins is a feature of many chronic diseases affecting the liver and other organs. In studies of pathogenesis of liver fibrosis particular attention has been paid quite recently to its frequent association with enhanced deranged antioxidant defensive system. Marked oxidative derangement of cell structure and function is known to be able, by various mechanisms, to exert irreversible damage [21]. Ethanol and acetaldehyde modify the redox state of hepatocytes and decrease the intracellular concentration of reduced GSH. Although GSH depletion *per se* may not be sufficien to cause lipid peroxidation it is generally agreed that it may favor the peroxidation produced by other factors; it spares and potentiates. In the present investigation we were able to show that ethanol and acetaldehyde treatment of HepG2

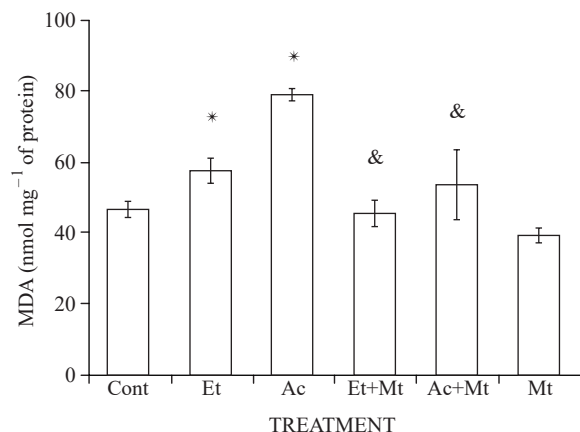


Fig. 3. Lipid peroxidation damage of HepG2 cells treated with 50 mM ethanol (Et), 175 μ M acetaldehyde (Ac), 10 μ g ml⁻¹ metadoxine (Mt), ethanol plus metadoxine (Et + Mt) and acetaldehyde plus metadoxine (Ac + Mt). Cells were exposed to different treatments for 24 h. Lipid peroxidation damage was determined as malondialdehyde (MDA) produced in the presence of thiobarbituric acid. Each bar represents the mean \pm SD of at least three independent experiments made in triplicate. *Significantl different from control cells and & ethanol and acetaldehyde treated cells ($P < 0.05$).

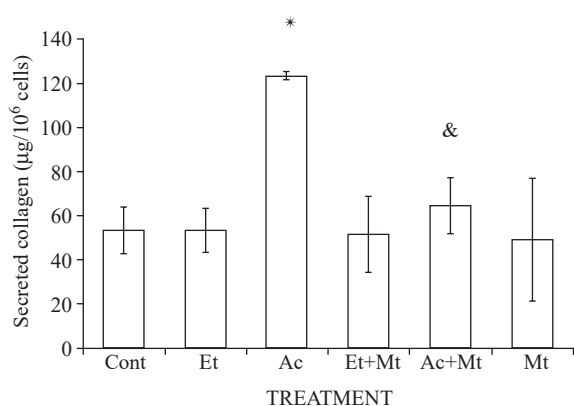


Fig. 4. Collagen secretion of hepatic stellate cells CFSC-2G treated with 50 mM ethanol (Et), 175 μ M acetaldehyde (Ac), 10 μ g ml⁻¹ metadoxine (Mt), ethanol plus metadoxine (Et + Mt) and acetaldehyde plus metadoxine (Ac + Mt). Cells were exposed to different treatments for 24 h and collagen content was determined. Each bar represents the mean \pm SD of at least three independent experiments made in duplicate. *Significantl different from control cells and & acetaldehyde treated cells ($P < 0.05$).

cells led to a significant depletion of reduced GSH and a tendency to increase oxidized GSSG and that metadoxine restored GSH to basal levels. Experimental studies in ethanol fed rats have demonstrated that metadoxine restores hepatic glutathione content and prevents the decrease in hepatic ATP concentration caused by ethanol [22]. Maintenance of normal intracellular redox homeostasis is important for preventing fatty liver and hepatic necrosis caused by ethanol and other toxic substances.

Free radicals have been recognized to induce perturbation of the oxidant/antioxidant balance in the hepatocytes, an effect that may be of relevance in liver fibrogenesis. In a rat model of alcoholic liver disease, the

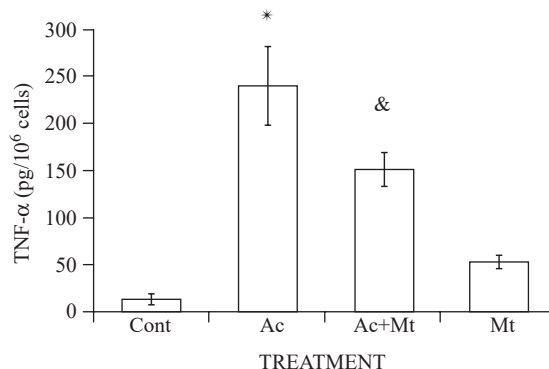


Fig. 5. TNF- α secretion by hepatic stellate cells CFSC-2G treated with 175 μ M acetaldehyde (Ac), 10 μ g ml⁻¹ metadoxine (Mt) and acetaldehyde plus metadoxine (Ac + Mt). Cells were exposed to different treatments for 24 h and TNF- α secretion was determined by ELISA. Each bar represents the mean \pm SD of at least three independent experiments made in duplicate. *Significantl different from control cells and & acetaldehyde treated cells ($P < 0.05$).

importance of oxidative stress was supported by marked accentuation of liver fibrosis by dietary supplementation of iron, a pro-oxidant, and the significant correlation of the liver MDA and 4-hydroxynonenal levels with the hepatic collagen deposition. Particular attention has been given to the possible interference by oxidative stress with gene expression of fibrogenic cytokines, key molecules in the progression of fibrotic tissue degeneration [21]. Recently a cytokine-induced acute inflammatory response has been proposed and increased TNF- α levels have been found in several patients with acute alcoholic hepatitis, particularly in more severe cases. In liver tissue specimens, TNF- α has been detected by immunohistochemical analysis, predominantly in ballooned hepatocytes frequently containing alcohol hyaline, therefore agents with anti-cytokine effects, such as metadoxine, could be of benefit. In the present experiments when we added ethanol or acetaldehyde to stellate cells we observed that acetaldehyde, but not ethanol, increased importantly TNF- α secretion, accompanied by a 2.3-fold increase in collagen secretion. Similar results have been reported by other authors [23, 24]. When acetaldehyde and metadoxine are added together, TNF- α secretion to the culture media is similar to control cells'. TNF- α induction is known to be one of the earliest events in hepatic inflammation triggering a cascade of other cytokines that co-operate to kill hepatocytes, recruit inflammatory cells and initiate a wound healing response that includes fibrogenesis [25]. Metadoxine prevention of the TNF- α increase may in turn avoid other inflammatory and fibrogenic cytokines being secreted by the cells, and as a consequence collagen secretion is not stimulated. The adverse effects restored by metadoxine suggest that the effectiveness reported with the use of this compound when given to patients with early stages of alcoholic liver disease could be attributed to the described restoration of the balance of cellular redox

state and anti-cytokine effects. In conclusion, the present study leads to the appreciation that a substantial decrease in lipid peroxidation damage, collagen and TNF- α secretion and an increase of reduced GSH were achieved with the use of metadoxine in HepG2 and CFSC-2G cell lines. Further studies are to be carried out to elucidate the mechanism by which metadoxine exerts these effects.

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