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EFFECTS OF METADOXINE – A LIVER PROTECTING AGENT ON SHORT-TERM EXTRAHEPATIC CHOLESTASIS

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Metadoxine, consisting of pyridoxine and pyrrolidone carboxylate, has been used as a promising liver protecting agent in alcoholic liver diseases in recent times. It has been shown to be able to restore NADH, GSH, ATP levels and the physiological proportion between saturated and unsaturated fatty acids in many clinical and experimental investigations. Our aim was to investigate the effect of this compound on the redox state in short-term biliary obstruction. Extrahepatic obstruction was induced by 1-hour-ligation of the common bile duct in rats under deep anaesthesia. Group A: sham operated (N = 8), Group B: metadoxine treated sham operated rats (N = 8; 300 mg/bwkg sc.), Group C: rats with common bile duct ligation (N = 8), Group D: rats with extrahepatic cholestasis and metadoxine treatment (N = 8; 300 mg/bwkg sc.). Liver cholestatic injury was proved by routine laboratory parameters (bilirubin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase, alkaline phosphatase). Free SH-group content, hydrogen-donating ability, reducing power property and conjugated diene concentration were measured in sera and homogenates by spectrophotometic method in order to estimate the redox state, as well as free radical-antioxidant balance. Elevated lipid peroxidation and decreased antioxidant capacity of liver homogenates and sera could be observed in the case of ligation. Metadoxine treatment improved antioxidant capacity of the liver and diminished total bilirubin concentration compared to the group without treatment. Metadoxine was able to maintain the cell redox balance in short-term extrahepatic ligation which can be related to its glutathione-saving and proton-donating ability, resulting in the moderation of lipid peroxidation processes.

INTRODUCTION

Cholestasis in liver occurs in various diseases in humans such as late viral hepatitis, bile duct carcinoma, gallstone, primary biliary cirrhosis, sclerosing cholangitis, biliary atresia, alcoholic hepatitis and in relation with certain drugs and physiological conditions such as pregnancy (Eddlestone, 1994). Extrahepatic cholestasis causes known biochemical, pathophysiological and morphological abnormalities produced by interrupted enterohepatic circulation, obstructed hepatic biliary tree, increased biliary pressure, retention of biliary constituents, impairment of hepatocellular transport and induced inflammatory responses as well. The role of free radical reactions has also been mentioned (Scott-Corner & Grogan, 1997; Hagymási, Blázovics, Kocsis, Lugasi & Fehér, 2000).

Metadoxine is the ion-pair between pyridoxine and pyrrolidone carboxylate (or pyroglutamate), the cyclic derivate of glutamic acid. Both substances normally occur in nature, food as well as in the tissues, consequently, there is no risk of untoward toxicity (Drugs of Today, 1998; Moret & Briley, 1988). It has been found to be useful in the prevention and treatment of alcoholic fatty liver or in alcoholic patients with more advanced liver diseases. Experimental studies have demonstrated that it induces an increase in hepatic adenosine triphosphate (ATP) concentration, restores the correct ratio between saturated and unsaturated fatty acids and the hepatic level of reduced glutathione (GSH) and therefore maintains the cell redox balance (Caballeria, Parés, Brú, Mercader, Plaza, Cabelleria, Clemente, Rodrigo, Rodés & the Spanish Group for the Study of Alcoholic Fatty, 1998; Annoni, Khlat, Lampertico, Dell'Oca & Dioguardi, 1988).

The aim of the present study was to evaluate the effects of metadoxine on the redox state of liver consequent to extrahepatic short-term ligation.

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MATERIALS AND METHODS

In vitro natural scavenging capacity of metadoxine was detected by chemiluminometric method with Lumat LB 9051 luminometer, according to the method of Blázovics, Kovács, Lugasi, Hagymási, Bíró & Fehér (1999). Unstable free radicals, originated from H_2O_2 in luminol-microperoxidase system via Fenton type reaction, catalyse the transformation of luminol into amino-phtalic acid. In this reaction monochromatic light is emitted. In the presence of radical scavenging molecules or compounds the emitted light is reduced, expressed as the percentage of the standard light of the H_2O_2/OH˙-luminol-microperoxidase system (RLU% – Relative Light Unit %). Reduced chemilumnescent intensity indicates elevated scavenger capacity.

Extrahepatic cholestatic injury was induced by the ligation of the common bile duct. Male, Wistar albino rats (150–200 g; n = 8) were used. The whole procedure was carried out under Nembuthal anaesthesia (40 mg/kg bw ip.). Group A: sham operated rats, Group B: sham operated animals with metadoxine treatment (300 mg/kg bw sc.) just before the induction of the ligation, Group C: rats with 1-hour-ligation of the common bile duct distal to the entrance of the last lobar duct, Group D: common bile duct ligated rats with metadoxine treatment (300 mg/kg bw sc.).

Routine laboratory parameters were determined in sera in a Hitachi 717 analyser, by standard methods. Liver homogenates were prepared in 0.15 M KCl solution, the protein concentration was assayed by Lowry's method (Lowry, Rosenbrough, Farr & Randall, 1951), using bovine serum albumin as standard. Liver homogenate diene conjugate concentrations were assayed by the AOAC method (1984). Hydrogen-donating ability (HDA) of homogenates and sera was measured according to the method of Hatano, Kagawa, Yasuhara & Okuda (1988), in the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical. DPPH stable radical was found to oxidise cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds and aromatic amines. Reducing power property (RPP) of liver homogenates as well as sera was estimated on the basis of method of Oyaizu (1986), based on the chemical reaction Fe(III)-Fe(II), using ascorbic acid as standard. Increasing absorbance of the sample indicated increasing reducing power and was expressed as mmol/LeqAS. Free SH-group concentration (SH) was determined in homogenates and sera by the method of Sedlak and Lindsay (1986).

1,1-diphenyl-2-picrylhydrazyl, 5,5'-dithiobis-2-nitrobenzoic acid, microperoxidase, luminol were obtained from Sigma (USA), metadoxine was a gift of Kéri Pharma (Hungary), and all other reagents were purchased from Reanal (Hungary).

Results were assessed by one-way analysis of variance (ANOVA) and represent mean ± SD, P values < 0.05 were considered significant.

RESULTS

The *in vitro* scavenger capacity of metadoxine was determined by a chemiluminometric method in H_2O_2/OH˙-microperoxidase-luminol system. The emitted light was diminished in concentration dependent manner (Fig. 1). The chemiluminescent light intensity was reduced in stored sample as well (Fig. 2).

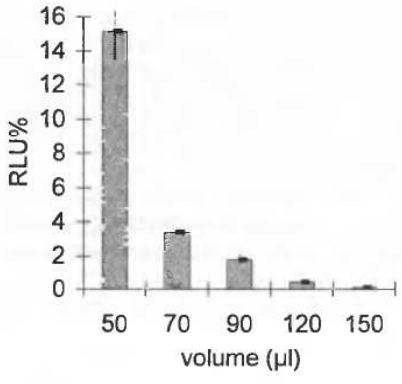

Fig. 1. Concentration dependence of *in vitro* natural scavenging capacity of metadoxine aqueous solution (0.6 mg/ml)

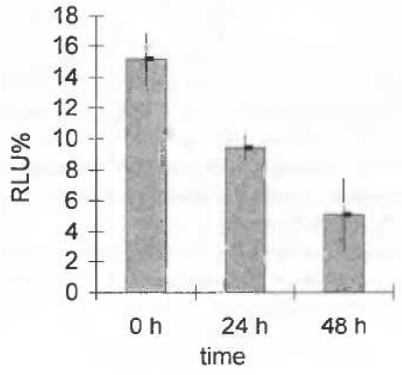

Fig. 2. The *in vitro* scavenging capacity of stored metadoxine aqueous solution (0.6 mg/ml)

Table 1. Routine and biochemical laboratory parameters in sera

	ALP (U/l)	AST (U/l)	ALT (U/l)	GGT (U/l)
Group A	623.8 ± 44.13^{d}	$137.5 \pm 7.17^{b,c,d}$	$50.0 \pm 7.18^{c,d}$	1.60 ± 0.40
Group B	526.8 ± 36.77^{d}	$218.0 \pm 22.26^{a,c,d}$	$50.6 \pm 5.82^{c,d}$	1.75 ± 0.48
Group C	697.6 ± 56.70	$448.8 \pm 30.12^{a,b}$	$122.6 \pm 7.46^{a,b}$	2.00 ± 0.38
Group D	$747.5 \pm 27.36^{a,b}$	$417.8 \pm 48.43^{a,b}$	$129.6 \pm 17.34^{a,b}$	1.80 ± 0.37

	Tbi (μmol/l)	SH (mmol/l)	RPP (mmol/LeqAS)
Group A	1.4 ± 0.24^{c}	0.21 ± 0.02	1.34 ± 0.06^{c}
Group B	1.3 ± 0.25^{c}	0.22 ± 0.01	1.27 ± 0.09^{c}
Group C	$10.7 \pm 2.27^{a,b,d}$	0.20 ± 0.02	$1.11 \pm 0.07^{a,b,d}$
Group D	2.2 ± 0.40^{c}	0.23 ± 0.01	1.35 ± 0.10^{c}

[a]sign. vs. Group A; [b]sign. vs. Group B; [c]sign. vs. Group C, [d]sign. vs. Group D
Group A: sham operated rats; Group B: sham operated rats with metadoxine treatment; Group C: rats with 1-hour bile duct ligation; Group D: ligated rats with metadoxine treatment
Tbi: total bilirubin concentration; ALP: alkaline phosphatase activity; AST: aspartate-aminotransferase activity; ALT: alanine-aminotransferase activity; GGT: gamma-glutamyltransferase acitivity; SH: free SH-group concentration; RPP: reducing power property

Table 2: The redox parameters of liver

	DC (Δabs_{232nm})	SH (mmol/l)	HDA (%)	RPP (mmol/LeqAS)
Group A	0.25 ± 0.01^{c}	$0.36 \pm 0.01^{b,c,d}$	51.11 ± 1.96^{c}	$1.11 \pm 0.11^{c,d}$
Group B	$0.24 \pm 0.001^{c,d}$	0.27 ± 0.02^{a}	53.59 ± 1.80^{c}	1.23 ± 0.12
Group C	$0.30 \pm 0.03^{a,b}$	0.29 ± 0.02^{a}	$37.80 \pm 2.06^{a,b,d}$	0.94 ± 0.09^{a}
Group D	0.27 ± 0.02^{b}	0.29 ± 0.02^{a}	50.37 ± 2.70^{c}	0.95 ± 0.08^{a}

[a]sign. vs. Group A; [b]sign. vs. Group B; [c]sign. vs. Group C, [d]sign. vs. Group D
Group A: sham operated rats; Group B: sham operated rats with metadoxine treatment; Group C: rats with 1-hour bile duct ligation; Group D: ligated rats with metadoxine treatment
DC: conjugated diene concentration; SH: free SH-group concentration; HDA: hydrogen-donating ability; RPP: reducing power property

Significant elevation of serum total bilirubin (Tbi) concentration, aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and non-significant rise of alkaline-phosphatase (ALP) as well as gamma-glutamyl-transferase (GGT) activity indicated cholestatic injury in Group C in experimental investigation. Metadoxine could decrease Tbi concentration in ligated rats, but the activities of liver enzymes were unchanged compared to the Group C (Table 1).

Lipid peroxidation (elevated conjugated diene concentration), decreased non-enzymatic antioxidant activity of liver homogenates (decreased SH, HDA, RPP) and of sera (reduced RPP) could be observed in ligated rats. Metadoxine treatment increased HDA in liver homogenates as well as RPP and SH in sera. Decrease of diene conjugates also proved the improvement of free radical-antioxidant balance in short-term ligated rats with metadoxine treatment (Table 2).

DISCUSSION

Biliary obstruction causes two mechanical problems: interrupts the enterohepatic circulation (concerning cholesterol, phospholipids, folic acid, steroids, vitamin B12, IgA) and elevates pressure in bile ducts which impairs the excretion of bile consists of water, electrolytes, bile salts, lipids and conjugated bilirubin (Scott-Corner & Grogan, 1997). Bile acids can damage hepatocytes when they exceed a critical concentration via cytolysis and/or apoptosis (Stiehl, 1999). Antioxidant bilirubin can be toxic to cells at higher concentrations because it influences the normal redox state and has immunmodulatory activity (Asad, Singh, Ahmad & Hadi, 1999; Lamb, Quinlan, Mumby, Evans & Gutteridge, 1999). When the liver cells are damaged, they can not provide enough energy in the form of ATP for the export pumps and biliary secretion, including bile acid and bilirubin export

pump, is furthermore impaired (Stiehl, 1999). Metadoxine may decrease these effects due to an increase of newly synthesised ATP via two simultaneous mechanisms. Pyrrolidine carboxylate accelerates the uptake of glycine into the purine biosynthesis, increasing AMP concentration. On the other hand pyridoxine facilitates the phosphorylation of ADP to ATP (Baldacci, Catalani, Bartoli & Mura, 1982). Metadoxine was able to reduce bilirubin concentration presumable via acceleration of ATP synthesis.

Also reactive free radicals are supposed to be involved in the pathogenesis of extrahepatic cholestasis. Abnormalities may be caused by increased production of superoxide radical through the activation of xanthine oxidase (Mun, Kwak & Kwon, 1996) and by the secondary generation of inflammatory mediators (eicosanoids, free radicals) as a consequence of cytokine release (Zhou, Chao, Levine & Olson, 1992). There are data regarding decreased antioxidant defence system and protective effect of N-acetylcysteine and silymarin (Mun *et al.*, 1996; Hagymási *et al.*, 2000). Chemiluminometric examination proved the concentration dependence *in vitro* scavenging capacity of metadoxine. Increasing scavenging capacity was observed in stored samples, which may be the consequence of the dissociation of the ion pair. Lipid peroxidation process and decreased antioxidant capacity were observed in extrahepatic cholestatic rats. Our results suggested a shift of the prooxidant/antioxidant balance in concordance with our earlier results (Hagymási *et al.*, 2000). Metadoxine was able to augment the activity of the non-enzymatic antioxidant system. It increased free SH-group concentration and reducing power property moderately in sera, and hydrogen-donating ability significantly in liver homogenates. In earlier investigations metadoxine was proven to be effective in maintaining reduced glutathione level and therefore the cell redox balance. These effects may be related to its normalizing action on GSH reductase and GSH transferase activity (Calabrese, Randazzo, Ragusa & Rizza, 1998).

Other actions of metadoxine should be also mentioned. Pyroglutamate is an intermediate in the γ-glutamyl cycle, which is an amino acid transport system into the cell. It is easily taken up by the central nervous system and facilitates the restoration of its normal functions, deteriorated by alcohol intoxication. It can be hydrolysed by the oxoprolinase and glutamic acid becomes available which has an essential role in the maintaining the nitrogen balance, elimination of ammonia and the biosynthesis of aspartate, an essential element in the urea cycle (Orlowski & Meister, 1970; Textbook of Biochemsitry and Clinical Correlation, 1992). Pyridoxine (vitamine B6) acts as a coenzyme in many reactions in connection with aminoacids, carbohydrates, sphingolipids and heme metabolism and in the neutralisation of biliary acids via the synthesis of taurine (Merril & Henderson, 1987). In carbon-tetrachloride induced rat fibrosis model, metadoxine slowed the development of fibrosis normalising the level of prolyl hydroxylase, which is involved in collagen synthesis, and reduced the increase of pro-alpha2 (I) collagen mRNA content in the liver observed in CCl_4-treated animals (Annoni, Contu, Tronci, Caputo & Aeosio, 1992; Arosio, Santambrogio, Gagliano & Annoni, 1933).

Pyrroline type N-oxide molecules, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), 2,2,2-trimethyl-1-pyrroline-N-oxide (M-3PO), 3,3,5,5-tetramethyl-1-pyrroline-N-oxide (M-4PO), are effective spin traps of singlet oxygen, hydroxyl radical and superoxide anion (Bilski, Reszka, Bilska & Chignell, 1996; Frejaville, Karoui, Tuccio, Le-Moigne, Culcasi, Pietri, Lauricella & Tordo, 1995; Nishi, Hagi, Ide, Murakami & Makino, 1992). We presume that in the presence of free radicals the dissociated molecules of metadoxine may form N-oxide type molecules which are similar to the above mentioned spin traps.

Our results indicate that metadoxine may be a useful agent to improve non-enzymatic antioxidant defensive system and redox state in 1-hour bile duct ligated rats. The pharmacodynamic effects of metadoxine include the prevention or delay the hepatic fibrosis towards cirrhosis so its beneficial effect in fibrous degeneration caused by long-term extrahepatic ligation is supposed, but further examinations are needed to confirm it.

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AOAC Official method of analysis (1984)

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