Antioxidant mechanism of hepatoprotection by ursodeoxycholic acid in experimental alcoholic steatohepatitis

*Lukivskaya O[1], Zavodnik L[1], Knas M[2], Buko V[1]**

[1] Department of Experimental Hepatology, Institute of Biochemistry, National Academy of Sciences, Grodno, Belarus
[2] Department of Pharmacological Biochemistry, Medical University of Białystok, Białystok, Poland

Abstract

Purpose: The aim of this study was to evaluate the role of an antioxidant factor in the hepatoprotective effect of ursodeoxycholic acid (UDCA) in rat alcoholic steatohepatitis.

Material and methods: The effects of UDCA (40 mg/kg, i.g., 30 days) were studied using rats fed on a high-fat diet (52% calories as fat) and administered with ethanol via intragastric intubation (4 g/kg daily, 30 days) .

Results: The livers of ethanol-treated animals were characterized by fatty dystrophy. The relative liver weight and the square of the sudanophylic area as well as the liver triglyceride content and the activity of the serum marker enzymes, aspartate aminotransferase and γ-glutamyltransferase, were significantly increased. Elevated superoxide dismutase activity as well as increased contents of lipid peroxidation products (hydroxyalkenals, malone dialdehyde, etc.) and lucigenin-enhanced microsomal chemiluminescence were observed in the liver of ethanol-treated rats and the liver reduced glutathione content was decreased. An increase in monoenoic fatty acids, a decrease of the n-6 acid family and an enhancement of microsomal membrane viscosity were found in the liver of these animals. An elevation of the total cytochrome P-450 content and the activity of amidopyrine-N-demethylase were shown in liver microsomes of the ethanol-treated group. The UDCA treatment improved the liver morphology, decreased serum marker enzyme activities, liver triglyceride content and normalized all the indices of oxidative stress. UDCA lowered the viscosity of the microsomal membrane, as assessed by both the fluorescence probe techniques and the saturated/unsaturated fatty acid ratio. The microsomal cytochrome P-450 content and amidopyrine-N-demethylase activity were normalized in UDCA-treated rats.

Conclusions: We can conclude that the hepatoprotective effect of UDCA stipulated by its antioxidant properties is indeed the factor enabling UDCA to control metabolic processes by changing the properties of liver membranes and membranous proteins.

Key words: alcoholic steatohepatitis, ursodeoxycholic acid, liver membranes, oxidative stress.

Introduction

Hepatoprotective properties of ursodeoxycholic acid (UDCA) in alcoholic liver injury have been described quite recently [1,2]. Throughout several decades UDCA has been of considerable clinical use to treat cholestatic liver injuries. The mechanism of the UDCA hepatoprotective effect has been interpreted by the fact that, as a hydrophilic bile acid, it displaces toxic bile acids from the membranes, thus protecting the latter from destruction [3]. However, the protective effect of UDCA on liver alcoholic steatohepatitis cannot be explained in the context of the above hypothesis since this pathology does not involve cholestatic phenomena and, as a consequence, membrane infiltration by hydrophobic bile acids. Some authors explained the UDCA hepatoprotective effect in alcoholic liver injury by its beneficial influence on mitochondrial oxidation [4], improvement of plasma membrane physical properties [1] and recovery of liver prostaglandin level [2].

Numerous experimental and clinical findings suggest that free radical mechanisms contribute considerably to ethanol-induced liver injury [5-7]. The hypothesis for the role of oxidative stress in the pathogenesis of alcoholic liver injury has become generally recognized and serves as a basis for applying antioxidants in treatment of this pathology. However, treatment of alcoholic liver injury by antioxidant drugs has not received a

* CORRESPONDING AUTHOR:
Institute of Biochemistry,
National Academy of Sciences,
BLK-50, 230017 Grodno, Belarus
Tel: +375 152 338411; Fax: +375 152 334121
e-mail: buko@biochem.unibel.by (Vyacheslav Buko)

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wide recognition. For example, clinical estimates of vitamin E efficacy in treatment and prevention of alcoholic liver injury have been ambiguous [8]. At the same time, clinical application of another antioxidant, silymarin, in this pathology seems to be promising [9]. Some authors believe the use of metal chelators, neutralizing the prooxidant effect of iron, to hold much promise as a means for treating alcoholic liver injury [7]. The hepatoprotective effects of prostaglandin E2 and polyunsaturated phosphatidylcholine were also partially achieved by antioxidant mechanisms [10,11].

In our laboratory the antioxidant effect of UDCA *in vivo* was first found when using rats with oxidative stress induced by 1-Gy single γ-irradiation [12]. UDCA lowered the generation of reactive oxygen species and the content of lipid peroxidation end-products in the liver. In this context, it was interesting to assess the antioxidant potential of this compound in alcoholic liver steatohepatitis which, as literature data show, is accompanied by activation of free radical generation [6].

Material and methods

Male albino Wistar rats with an initial body weight ranging from 190 to 210 g were used. The rats from all the groups were fed on a high-fat diet (HFD) *ad libitum* for 30 days. The diet contained the following components (in %): casein, 21.0; starch, 41.5; fat (lard and sunflower oil, 1:1 wt/wt), 26.0; cellulose, 6.5; and vitamin and mineral mix, 5.0 [13]. The diet provided 52% of metabolizable energy as fats. For 30 days, two groups of the animals were treated with ethanol via a gastric tube (4 g/kg b.w., daily). The animals from the second ethanol-treated group were administered with a UDCA aqueous suspension (40 mg/kg b.w., daily). Both groups treated with ethanol (the ethanol group) and ethanol combined with UDCA (the ethanol + UDCA group) were simultaneously fed on a HFD throughout the experiment. The control group fed on a HFD (the HFD group) for 30 days received intragastrically isocaloric amount of sucrose. Each group was of 8 animals.

The rats were decapitated under pentobarbital anaesthesia after 12-h starving. Blood serum was obtained by centrifugation at 3000 g. The liver microsomal fraction was isolated by differential centrifugation at 105000 g using a VAC-602 centrifuge (Janetzki, Germany).

For histological studies, liver samples were fixed in Bouin solution. Histological sections were prepared and stained with hematoxylin and eosin. For histochemical assessment of neutral lipids, liver samples were frozen in liquid nitrogen and cryostat sections were stained with Sudan black B. The sudanophilic areas were measured with a BIOSCAN-NT image computer analyzer (Minsk, Belarus).

The activities of the serum marker enzymes, alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT), were measured by using commercially available kits (Lachema, Czech Republic).

Lipids were extracted with a chlorophorm : methanol mixture (2:1, v/v) [14]. Neutral lipids were separated by thin-layer chromatography into classes [15]. Individual spots corresponding to lipid fractions (triglycerides, phospholipids) were scraped and extracted by methanol. Triglycerides were measured by routine methods using commercial kits from Lachema (Brno, Czech Republic) according to manufacturer instructions. Phospholipids were used for gas-chromatographic determination of fatty acid pattern. Phospholipid fatty acids were methylated with 1.75 M sulphuric acid in absolute methanol at 75°C and separated with a 3700 model gas chromatograph (Moscow, Russia) equipped with a flame ionisation detector and a 1.5x3 mm glass column packed with 10% DEGS on Inerton-Super 100/120 mesh. The temperatures were as follows: column, 197°C; injector, 250°C; detector, 250°C. The flow rates were the following: carrier gas (helium), 30 ml/min; hydrogen, 30 ml/min; air, 300 ml/min. Fatty acid peaks were identified by comparison of the retention time with authentic standards (U.S. Biochemical Corporation, USA).

The activity of superoxide dismutase was measured by the modified method with nitrotetrazolium blue [16]. The method was based on competition of superoxide dismutase and nitrotetrazolium blue for the superoxide ion produced due to interaction of NADPH and phenazine metasulphate. The dinitrophenyl hydrazone mixture was separated in polar and non-polar zones [17]. The extract containing dinitrophenyl hydrazones of aldehydes was separated by double TLC development on 20x20 cm silica gel G-60 plates of 0.25 mm thickness. Dichloromethane followed by benzene were used as mobile phases. Non-polar hydrazones were found in three zones: 4-hydroxyalkenals (zone 1), ozazones (zone 2), alkenals, alkanals and ketones (zone 3). They were identified by the corresponding standards. Individual fractions were scraped, extracted by methanol and determined spectrophotometrically at 365 nm.

NADPH-dependent liver microsomal chemiluminescence was conducted by the use of the chemiluminogenic probes, luminol and lucigenin [18]. The incubation mixture, containing 0.1 M phosphate buffer (pH 7.4), 0.1 μM iron sulphate (II), $6x10^{-4}$ mM luminol and lucigenin, 0.1 nM NADPH and microsomal suspension (5 mg protein), was placed in a cell. The reaction was started by addition of microsomal protein. Chemiluminescence was recorded with a BLM-100 bioluminometer (Krasnoyarsk, Russia) for 4 min.

Lipid peroxidation was assessed by the malone dialdehyde (MDA) level as determined by a thiobarbituric acid method [19]. The molar extinction coefficient of $1.56x10^5$ mol^{-1} cm^{-1} was used to calculate MDA concentration. Trichloroacetic acid was added to control samples before NADPH addition.

Reduced free thiols, among which reduced glutathione (GSH) was more abundant, were measured by the modified method of Ellman [20]. The absorption was recorded at 412 nm with a Specol-211 spectrophotometer (Carl Zeiss, Germany).

The content of cytochrome P-450 in the liver microsomal fraction was determined by the method of Omura and Sato [21]. Amydopyrine-N-demethylase activity was measured according to Klinger and Muller [22]. The incubation mixture (1 ml) contained 100 mM Tris-HCl buffer, (pH 7.4) and 5 mM $MgCl_2$, 8 mM amydopyrine and 2 mg microsomal protein. The reaction was started by addition of 3 mM NADPH, and after 5-min incubation at 370°C for 45 min, absorption was measured at 412 nm with a Specol 221 spectrophotometer and compared to control. Formaldehyde was determined applying the calibration curves constructed by the use of standard solutions.

Figure 1. **Lipid accumulation in the liver. Staining with Sudan black; magnification 10 x. A – Control; B – Ethanol; C – Ethanol + UDCA**

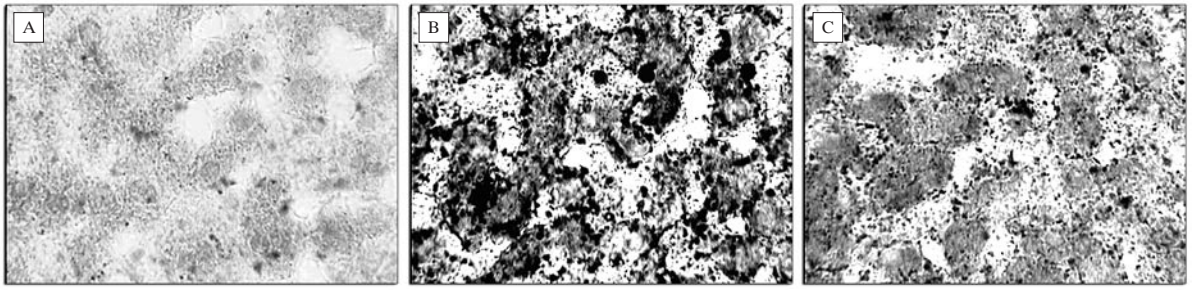

Table 1. **Morphologic characteristics of the liver and serum marker transferases (μmol/mg protein per 1 min) and liver triglyceride content in experimental rats**

Parameter	Control	Ethanol	Ethanol + UDCA
Liver relative weight (g/100 g body weight)	3.12±0.29	4.10±0.26[a]	3.29±0.21
Relative square of sudanophylic area (% to slide square)	0.026±0.0078	0.092±0.040[a]	0.025±0,003[b]
AsAT	1.17±0.088	1.63±0.112[a]	1.03±0.099[b]
AlAT	0.87±0.055	0.98±0.082	0.77±0.070
GGT	2.16±0.38	7.14±0.83[a]	3.89±0.46[ab]
Triglyceride	1618±110	4825±384[a]	1881±57[b]

Values are means ±SEM for eight rats; [a] – $P<0.05$ compared with the control group; [b] – $P<0.05$ compared with the ethanol-treated group

The fluorescence spectra were recorded on a LOMO--SDL-2 spectrofluorimeter (Russia) equipped with a xenon lamp as a fluorescence source. The fluorescence intensities (I) of pyrene monomer and eximers were measured at height of fluorescence band at 373 and 470 nm, respectively. A TRS-2850 standard wolfram lamp was used to calibrate the spectrofluorimeter. The membrane suspensions (5 μl), containing 0.1 ml protein/ml, were titrated with 5 μl portions of pyrene solution in ethanol with intensive stirring. The fluorescence spectra were recorded after 2 min incubation at 250°C with the regular portion of the probe. The maximal volume of pyrene solution added was 50 μl.

The data were processed statistically using two-tailed Student's t-test. The minimal level of significance was set at $p<0.05$.

Results

The long-term treatment of rats fed on the HFD simultaneously with ethanol provoked steatohepatitis which was characterized morphologically by lipid accumulation in hepatocytes and lymphocyte infiltration in the parenchyma. The Sudan black-stained liver preparations showed a diffuse distribution of lipid inclusions, with the lipid vacuoles enlarging to medium sizes, whereas slides from control animals demonstrated very fine, almost indistinguishable, lipid inclusions (*Fig. 1a*, *b*). The morphometric findings suggested an increased relative area of sudanophilic regions on alcohol-treated rat liver preparations (3.5-fold, *Tab. 1*).

The biochemical parameters indicated the presence of alcoholic liver injury in rats treated with ethanol. The activities of the serum marker enzymes, γ-glutamyl transferase and AsAT, were significantly elevated, with AlAT activity remaining essentially unchanged (*Tab. 1*). The content of liver triglycerides was raised 3-fold, which confirms enhanced accumulation of neutral lipids in the liver of the ethanol group. The average liver weight in these animals was also considerably increased.

The administration of UDCA (40 mg/kg, i.g., 56 days) to alcohol-treated animals fed on the HFD essentially normalized the morphologic picture of the liver. Along with the normal liver structure, the animals from this group had some diffuse lymphocyte infiltrations in the parenchyma. However, the sizes and the number of lipid vacuoles were much smaller compared to those in rats treated with ethanol alone (*Fig.1b*). The morphometric evaluation of liver slices stained with Sudan black indicated a pronounced decrease of neutral lipid accumulation in the liver after the application of UDCA (*Tab. 1*).

The UDCA treatment normalized the activity of serum AsAT and significantly decreased that of γ-glutamyl transferase. The content of liver triglycerides reduced virtually to the control values (*Tab. 1*).

The long-term ethanol treatment increased the viscosity of liver microsomal membranes as assessed from the fluorescence intensity ratio of the pyrene monomer to eximer fractions (I372/I470).

In animals with alcoholic steatohepatitis UDCA considerably decreased this parameter (*Tab. 2*). However, the membrane polarity, as shown by the I372/I395 ratio, was unaffected in all the experimental groups.

The increased concentrations of palmitic, palmitoleic and eicosatrienic acids and the diminished level of arachidonic acid were found in liver microsomal phospholipids of animals with alcoholic steatohepatitis (*Tab. 3*). As opposed to the HDF

***Table 2*. The fluorescence intensity of the fluorescent probe pyrene (I_{372}), bound to the liver microsomal membrane, ratio of oscillation band intensities at 372 and 395 nm (I_{372}/ I_{395}), and ratio of the intensities at 372 and 470 nm (I_{372}/ I_{470}) in experimental rats**

Parameter	Control	Ethanol	Ethanol + UDCA
I_{372}	0.84 ± 0.03	1.19 ± 0.05[a]	0.83 ± 0.04[b]
I_{372}/ I_{395}	1.16 ± 0.06	1.15 ± 0.04	1.13 ± 0.04
I_{372}/ I_{470}	4.16 ± 0.12	5.38 ± 0.19[a]	2.11 ± 0.06[ab]

Values are means ± SEM for six rats; [a] – $p<0.05$ compared with the control group; [b] – $p<0.05$ compared with the ethanol-treated group

***Table 3*. Effect of ethanol consumption and UDCA treatment on fatty acid composition (% to total fatty acids) of microsomal phospholipids from the liver of rats pair fed the high-fat diet**

Fatty acid	Control	Ethanol	Ethanol + UDCA
C 16:0	12.7±0.78	14.7±0.56[a]	12.8±0.39[b]
C 16:1 (n-7)	3.5 ±. 27	6.3±0.26[a]	4.3±0.19[b]
C 18:0	20.8±0.75	20.1±0.82	13.8±0.78[ab]
C 18:1 (n-3)	16.8±0.66	18.4±0.71	21.4±0.63[ab]
C 18:2 (n-6)	9.5±0.79	7.6±0.68	13.2±0.73[ab]
C 20:3 (n-3)	0.8±0.07	1.3±0.11[a]	0.4±0.01[ab]
C 20:4 (n-6)	24.3±1.83	17.3±1.56[a]	20.1±2.97
C 20:5 (n-3)	1.1±0.11	1.0±0.12	1.1±0.08
C 22:2 (n-9)	2.5±0.19	2.6±0.23	2.2±0.23
C 22:3 (n-9)	1.4±0.12	1.8±0.17	1.4±0.10
C 22:4 (n-9)	2.4±0.24	2.5±0.25	2.0±0.18
C 22:5 (n-6)	3.0±0.28	2.1±0.22	4.5±0.42[ab]
C 22:6 (n-3)	3.6±0.31	4.0±0.25	3.8±0.33
Saturated/ unsaturated acids	0.51±0.01	0.59±0.02[a]	0.38±0.03[b]

Values are means ± SEM for six rats; [a] – $p<0.05$ compared with the control group; [b] – $p<0.05$ compared with the ethanol-treated group

group, rats administered with ethanol + UDCA had considerably raised percentage of oleic acid, linoleic acid and its derivatives as well as docosapentaenic acid, the levels of stearic acids and group n-9 fatty acids being reduced. The concentrations of palmitic and palmitoleic acids in the ethanol + UDCA group were lower compared to the findings for the group treated with ethanol alone. The ratio of saturated/unsaturated fatty acids, which reflected the viscosity of cell membranes [23], was raised in the ethanol-treated group and decreased after the UDCA administration.

The prooxidant effect of the long-term ethanol administration manifested itself in activation of free radical generation as demonstrated by chemiluminescence intensity of liver microsomes enhanced by luminol and lucigenin, increased superoxide dismutase activity, and lipid peroxidation end-product contents (MDA and polar carbonyls) as well as in a decrease of reduced free thiols (*Tab. 4*). The treatment with ethanol increased nearly two-fold the total cytochrome P-450 content and amidopyrine-N-demethylase activity in liver microsomes.

Our findings show that alongside with the pronounced hepatoprotective effect, the UDCA administration reduced the production of reactive oxygen forms in the liver, the content of lipid peroxidation carbonyl-containing products (alkenals, alkanals, ketones, oxyalkenals and MDA), and the activities of antioxidant defence enzymes (superoxide dismutase) and increased the concentraton of reduced free thiols (*Tab. 4*). Moreover, UDCA normalized liver microsomal cytochrome P-450 level and the activity of amidopyrine-N-demethylase, the end acceptor of the cytochrome P-450-dependent electron transport chain.

***Table 4*. Parameters characterizing oxidative stress and lipid peroxidation in the liver and cytochrome P-450-related indices in liver microsmes of experimental rats**

Parameter	Control	Ethanol	Ethanol + UDCA
NADPH-induced chemiluminescence enhanced by lucigenin, c.p.m./mg microsomal protein per 1 min x 10^6	1.54±0.34	2.92±0.55[a]	1.79±0.31[b]
NADPH-induced chemiluminescence enhanced by luminol, c.p.m./mg microsomal protein per 1 min x 10^3	2.13±0.33	16.65±2.17[a]	2.96±0.49[b]
MDA content, nmol/mg liver protein	0.76±0.049	1.55±0.099[a]	0.64±0.104[b]
SOD activity, μmol/mg cytosolic protein per 1 min	1.72±0.341	2.48±0.358[a]	1.14±0.307[b]
Alkanals, alkenals and ketones content, nmol/mg liver protein	175±21	295±32[a]	204±18[b]
Hydroxyalkenals content, nmol/mg liver protein	99±11	159±16[a]	112±12[b]
Reduced glutathione content, μmol/mg liver protein	5.2±0.31	3.0±0.19[a]	5,5±0.25[b]
Total cytochrome P-450 content, nmol/mg microsomal protein	0.76±0.050	1.550±0.099[a]	0.692±0.104[b]
Amydopyrine-N-demethylase activity, nmol/mg microsomal protein per 1 min	4.21±0.48	8.61±0.73[a]	5.45±0.68[b]

Values are means ± SEM for eight rats; [a] – $p<0.05$ compared with the control group; [b] – $p<0.05$ compared with the ethanol-treated group

Discussion

The pronounced hepatoprotective effect of UDCA in alcoholic liver injury, which we confirmed in the present work, seems to be a multifactor process. In a few studies on this problem, the defence UDCA-governed mechanisms involve improvement of mitochondrial structure and ATP production-related mitochondrial oxidation [4], as well as stabilization of hepatocyte plasma membrane physical properties [1] and recovery of the level of hepatoprotective prostaglandins E [2] in the liver of alcohol-treated rats. Many authors attribute the UDCA defence of liver mitochondria to possible antioxidant properties of this compound [24,25] and stabilization of liver mitochondrial membranes.

The antioxidant effect of UDCA has been studied rather intensively during the last decade. Most frequently, experiments *in vitro* provided a negative answer to this problem. For example, investigators did not find any changes in superoxide anion production by either monocytes of UDCA-treated patients with cholestatic diseases or by cells taken from healthy individuals and preincubated with UDCA [26]. UDCA did not affect the MDA production by macrophage culture and the generation of reactive oxygen forms by rat liver mitochondria, but it prevented a prooxidant effect of hydrophobic bile acids [27,28]. Furthermore, UDCA prevented reduced glutathione depletion in cultivated hepatocytes treated with hydrogen peroxide and cadmium [29]. Thus, the experiments *in vitro* did not confirm with certainty the antioxidant effect of UDCA. In contrast, in studies *in vivo* UDCA acted as a rather effective antioxidant. In rats with alcoholic liver injury, UDCA reduced the content of lipid peroxidation end-products both in the whole liver [30] and in its subcellular fractions, mitochondria [1] and microsomes [31]. UDCA inhibited lipid peroxidation products in the liver of rats with cholestasis provoked by a bile duct ligation [32]. In the liver of rats with γ-irradiation-induced oxidative stress, UDCA normalized the activity of superoxide dismutase, the contents of superoxide radical and lipid peroxidation carbonyl-containing products, diminished chemiluminescence enhanced by luminol and lucigenin and prevented a decrease of reduced glutathione [12]. The distinctions in *in vivo* and *in vitro* results may indicate that the UDCA antioxidant effect may be accomplished via its metabolites.

Moreover, the administration of UDCA normalized the level of liver cytochrome P-450 and the activities of the matched microsomal reactions [31]. Since cytochrome P-450 is the primary source of liver free oxygen radicals in alcohol intoxication, we have every reason to believe that at least one of the mechanisms of the UDCA antioxidant action may be mediated through its effect on the cytochrome P-450 system which, in turn, is involved in bile acid biotransformation [33,34].

The mechanisms of UDCA membranotropic effect, which have been well-studied in cholestatic liver injury [3], do not apply to liver diseases which occur with cholestasis, particularly to alcoholic steatohepatitis. Since in cholestatic diseases the key mechanism of the UDCA therapeutic effect is a replacement of toxic hydrophobic bile acids in membranous structures, alternative mechanisms should be considered for alcoholic liver injuries. We should, however, specify that the UDCA-induced enhanced membrane fluidity may partly be explained in terms of a possible competition with cholesterol for membranous binding sites [35,36]. However, we are inclined to believe that the changes in liver membrane physical properties observed under long-term UDCA administration are related to the increased ratio of unsaturated/saturated fatty acids of the phospholipid microsomal membrane, which in its turn can be accounted for by the antioxidant effect of UDCA preventing membranous fatty acid peroxidation.

The literature data consideration allows us to conclude [37] that hepatoprotective medicinal preparations for treatment of alcoholic liver injuries must at least: a) possess antioxidant properties; b) stabilize cell membranes; c) inhibit microsomal cytochrome P-450-dependent system activity as the primary source of oxygen free radicals in alcohol intoxication [6]; d) have anti-inflammatory properties. From this classification, a substance with the above properties may be considered an ideal hepatoprotector. On the basis of the above data UDCA may be assigned to the category of such "ideal" hepatoprotectors: it possesses antioxidant activity, inhibits the activity of the cytochrome P-450-dependent system of xenobiotic metabolism activated by long-term ethanol consumption; stabilizes physical and chemical properties of the membrane. Moreover, UDCA inhibits production of the principal anti-inflammatory cytokine, TNFα [38]. We can conclude that the hepatoprotective effects of UDCA in alcoholic steatohepatitisis are mediated, at least in part, by its antioxidant actions which are probably linked to an inhibition of cytochrome P-450-related free radical generation. The antioxidant effects of UDCA lead to improvement of liver membrane physical properties and functions which in turn stipulate protection of the liver against the damaging effects of ethanol and oxidative stress accompanying alcoholic liver injury.

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