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Original Research

The Effect of Vitamin C and Glutathione on Ethanol Cytotoxicity and Selected Parameters of Pro- and Antioxidative Processes in Mouse Fibroblasts 3T3-L1

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Abstract

Ethanol metabolites may directly or indirectly induce oxidative stress as a result of disturbed balance between pro- and antioxidative processes. An indirect effect of ethanol on the generation of oxidative stress is associated with the attenuation of intracellular defensive enzymatic antioxidants, e.g. gluthatione peroxidase, catalase, superoxide dismutase as well as water- and/or fat-soluble low-molecular antioxidants (e.g., vitamin C, vitamin E, glutathione, selenium).

The aim of our study was to evaluate the effect of 48h preincubation of mouse embryo fibroblast-like cells (3T3-L1) with vitamin C (VC, 0.06 mM) and glutathione (GSH, 0.05 mM) on their viability after exposure for 4, 8, and 24 h to ethanol (0.3 mM). Additionally, the activity of glutathione peroxidase (GSH-Px) and thioredoxin reductase (TrxR) enzymes as well as the level of thiobarbituric acid reactive substances (TBARS) were assessed in the exposed cells.

In our study, vitamin C and glutathione had no significant effect on cytotoxicity of ethanol. We observed differences in GSH-Px and TrxR activity depending on the duration of exposure to ethanol. The highest GSH-Px activity in cell lysates was measured after 8 and 24h of incubation with ethanol and both antioxidants, or ethanol alone. The highest TrxR activity was observed after 4h of incubation in the presence of ethanol or GSH. There were no effects of VC and GSH on the activity of the antioxidant enzymes in comparison to cells not supplemented with VC and GSH. Similarly, we were unable to show any significant differences in levels of TBARS except incubation for 24h, when concentration of TBARS was highest in cells exposed to ethanol and simultaneously supplemented with the antioxidants.

Keywords: *in vitro* studies, glutathione peroxidase, cytotoxicity, ethanol, TBARS, vitamin C, glutathione

Introduction

In mammals, including humans, ethanol is metabolized via three pathway with the necessary presence of several kinds of enzymes: alcohol (ADH) and aldehyde (ALDH) dehydrogenases with NAD^+ as their co-factor, cytosol and peroximal catalase utilizing H_2O_2 as well as microsomal ethanol oxidation system (MEOS), in which a crucial role is played by cytochrome P450 2E1 (CYP2E1) that requires NADPH and O_2 [1, 2].

Ethanol metabolites may directly or indirectly induce oxidative stress as a result of distorted balance between pro- and antioxidative processes [3, 4]. A direct effect of ethanol on the development of oxidative stress is associated with the generation of free radicals: hydroxyl and

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hydroxylethyl, and reactive oxygen species (ROS), which react with proteins and lipids and induce their peroxidation [3, 5]. The major target of ethanol-induced oxidative stress is mitochondrial DNA, whose damage leads to impaired function of mitochondria and inhibited synthesis of proteins encoded by mitochondrial DNA [6].

Ethanol metabolism may also occur in cerebral cells, heart and lungs. In some tissues (brain, heart, lungs), the process of oxidation due to the absence of ADH involves mainly CYP2E1 [5]. This enzyme converts ethanol into acetaldehyde and forms 1-hydroxyethyl radicals and lipid radicals [7, 8].

An indirect effect of ethanol on the generation of oxidative stress is associated with the attenuation of intracellular defensive enzymatic antioxidants: gluthatione peroxidase (GSH-Px), catalase, superoxide dismutase (SOD) and water- or fat-soluble low-molecular antioxidants (e.g., vitamin C, vitamin E, glutathione, selenium) [4, 9, 10]. Numerous data confirm that ethanol is responsible for reduced activity of glutathione peroxidase and CuZn superoxide dismutase as well as for the diminished concentrations of glutathione (GSH), vitamin C, vitamin E and selenium [3, 10]. Studies performed on rats showed that prolonged intake of ethanol reduces the activity of glutathione peroxidase in hepatocytic cytosol and mitochondria by 40 and 30%, respectively, increasing at the same time growth of oxidative-modified proteins in those cells [3]. Low-molecular antioxidants play a particular role in the defense against oxidative stress as they form the foremost line of defense against ROS in cells. In addition, their concentration depends on diet.

Vitamin C is one of the leading antioxidants involved in scavenging free radicals generated during cellular metabolism [11]. The most important function of vitamin C is associated with regeneration of α-tocopherol as it supplies electrons necessary for hydroperoxide reductase [12] that directly reduces α-tocopheroxy radical to α-tocopherol, thus contributing to the protection of cellular membranes [13, 14]. In the ethanol detoxication pathways, vitamin C activates an alternative pathway of ethanol metabolism – the microsomal ethanol oxidation system – and participates in the regeneration of reduced NADPH [15]. *In vivo* studies have revealed significantly decreased concentrations of malondialdehyde, lipid hydroperoxide and conjugate dienes in ethanol-fed animals supplemented with vitamin C as compared with the control group [8]. It was also shown that a diet supplemented with vitamin C protects against fatty liver degeneration or its necrosis induced by chronic ethanol consumption [16].

GSH plays a particular role in the protection of cells against oxidative stress. This results from the fact that GSH is the major non-protein thiol compound and its presence is necessary to maintain the reduction environment within a cell indispensable for the optimal activity of the majority of enzymes and other cellular macromolecules [17]. Numerous data suggest that both acute and chronic exposure to ethanol reduces GSH content in the liver [18, 19]; however, the mechanism of the reduction has not yet been clearly explained. It is likely that it is associated with the increased oxidation of GSH to GSSG, which is removed from hepatocytes. This in turn may also result from a decreased GSH synthesis, or an increased influx of GSH from hepatocytes [7, 20, 21]. There is evidence that a diet supplemented with cysteine precursors reduces early stages the ethanol-induced liver damage in both humans and experimental animals [18].

The results of numerous *in vitro* studies show that supplementation with low-molecular antioxidants may prove to be an essential factor that protects against toxic effects of compounds produced during ethanol metabolism [8].

The aim of this study was to test whether vitamin C or GSH supplementation before ethanol intoxication can change antioxidative status of the exposed cells. The antioxidative status will be assayed by determination of glutathione peroxidase and thioredoxin reductase (TrxR) activity and TBARS concentration as marker of the prooxidative processes.

Materials and Methods

Cell Line

Embrional mouse fibroblasts 3T3-L1 (ECACC cat. no. 86052701) were cultured using DMEM medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), 2 mM L-glutamine (Sigma), and antibiotics: 100 μg/ml streptomycin and 100 U/ml penicillin, in 75 cm^2 culture flasks (Nunc), at 37^0C, 5%CO_2, and 85% humidity. Cells were passaged 2-3 times a week and enzymatically separated by tripsin/EDTA (Sigma) to prepare cell suspension for assessing basic toxicity and performing biochemical tests. The MTT assay [22] was used to assess ethanol cytotoxicity and determine non-toxic concentrations of antioxidants.

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazole bromide (MTT), dimethyl sulfoxide (DMSO), ascorbic acid, GSH, buffered saline and trypan blue were purchased from Sigma, and 96% ethyl alcohol from Chemical Reagents Co. (Poland). Antioxidant and ethanol solutions were prepared directly before performing cytotoxic tests, w/v or v/v in culture medium (DMEM). Assessment of a wide range of concentrations in the first phase of the experiment was followed by the determination of the range of effective concentrations (able to reduce cell viability in the range $\leq 10\% \geq 90\%$). Effective concentration experiments were repeated three times.

The Effect of Antioxidants on Ethanol Cytotoxicity

To determine the effect of vitamin C and GSH on ethanol cytotoxicity, cell suspensions were seeded in 96 well

plates (Nunc) at a density of $9 \cdot 10^4$ per well, and incubated for 24 h at 37^0C, 5%CO_2, and 85% humidity. Then, the medium was removed from over cells, and vitamin C (0.06 mM) or GSH (0.05 mM) were applied. After 48 h incubation, the medium was removed and cells were exposed to ethanol (0.3mM) for 4, 8 and 24h. After medium removal, MTT solution (0.5 mg/ml) was applied. Following a 3 h incubation with MTT, dye solution was removed and DMSO was added to dissolve formazan. Colorimetric measurements of the dye content in the study and control samples were performed using ELISA reader at 545 nm wave length and 620 nm reference filter. Finally, the percentage viability of cells was calculated according to the following formula:

cell viability % = (absorbance treated wells/absorbance control wells) · 100%.

Preparation of Cells for Assessing Antioxidative Potential

Cells at a density of $13 \cdot 10^4$/ml were placed in culture flasks (75 cm^2). Non-toxic concentrations of vitamin C (0.06 mM) and GSH (0.05 mM) were added. Following a 48 h incubation, the medium was exchanged and ethanol (0.3 mM) was added. After incubation for 4, 8 and 24 h, cells were trypsinized and washed twice with phosphate-buffered saline (PBS). After calculating cell viability using trypan blue, cells (about $2 \cdot 10^6$cells/ml PBS) were frozen at -70^0C. Each experiment was repeated three times. Finally, the antioxidative potential of cells was evaluated.

Determination of GSH-Px and TrxR Activities and Concentration of Tiobarbituric Acid Reactive Substances (TBARS)

After defrosting, cells were lysed with potassium-phosphate buffer (5 mM) containing ethylenediamine tetraacetic acid (EDTA) (0.5 mM) and centrifuged. GSH-Px activity was determined in lysate with t-butyl hydroperoxide as substrate, using the method of Paglia and Valentine [23]; TrxR activity with NADPH and 5,5-dithio-bis (2-nitrobenzoic acid). The activity of both enzymes, GSH-Px and TrxR, was determined with micromethods modified for microplate analyses [24].

TBARS levels was analyzed according to Wasowicz et al. procedure [25] and protein concentrations using the Bradford method [26].

Statistical Analysis

The results are presented as the mean of three repeated experiments ± standard deviation. The statistical analysis was performed using ANOVA test ($p < 0.05$).

Results

A wide range of concentrations of the study substances was assessed in the first phase of the investigation and then, effective concentrations which reduced viability of cells in the range of $\leq 10\% \geq 90\%$ were determined (results not presented). Concentrations of vitamin C or GSH, non-toxic for 3T3-L1 cells, were selected for further studies. For ethanol, concentration that reduced cellular viability by 10% as compared to control was applied.

The experiments showed that a 48 h incubation of cells 3T3-L1 with vitamin C or GSH had no impact on the decrease in cytotoxicity of ethanol at the concentration of 0.3 mM after 4, 8 and 24 h of exposure. A 4 h incubation with ethanol resulted in a slight increase in cellular viability versus cells cultured with the supplement of antioxidants and the control cells (about 5%). However, no significant differences in viability of cells exposed to ethanol alone or ethanol and antioxidants were observed. Figs. 1 and 2 show the rate of changes of GSH-Px activity in cells cultured on medium containing GSH or vitamin C and exposed to ethanol. GSH-Px activity in cultures exposed to ethanol did not differ from that observed in the control ones. Preincubation with GSH and exposure to ethanol for 4 h decreased selenoenzyme activity as compared to the control culture (Fig. 1). In the culture supplemented with vitamin C and exposed to ethanol for 4 h, enzyme activity was also significantly lower than in the relevant control culture (Fig. 2). The most pronounced GSH-Px activity was observed after a 4 h incubation with ethanol in GSH-supplemented culture (Fig. 1).

An increased Trx-R activity was revealed in cultures supplemented with GSH or ethanol. The highest Trx-R activity was observed after a 4 h incubation with ethanol in GSH-supplemented culture ($p < 0.05$) (Fig. 3). A simultaneous incubation of cells in the presence of ethanol and GSH did not change TrxR activity as compared with control cultures. Nor did cultures with vitamin C show significant changes in time intervals applied in the study (Fig. 4).

An 8 h incubation with ethanol in vitamin C-supplemented culture caused a significant decrease in TBARS concentration in relation to the control (Fig. 5), whereas after a 24 h incubation with ethanol in GSH-supplemented culture, a significant increase in TBARS concentration was observed (Fig. 6).

Discussion

Ethanol consumption and the increase of oxidative stress that is asociated with metabolism of ethanol leads to development of a variety of illnesses, including liver diseases and cancer [27]. Therefore, the effective therapies have been searched for years. One of the most interesting ideas is the use of antioxidant therapy. Although many reports show that dietary supplementation with antioxidants can protect cells from oxidative stress induced by ethanol, the idea of the use of antioxidant therapy is still not fully applied [28].

The aim of our study was to examine whether a 48 h preincubation of 3T3-L1 cells with antioxidants in non-toxic concentrations may have some impact on the decrease in ethanol cytotoxicity. Two low-molecular antioxidants, vitamin C and GSH, were selected for the study as they are the first "defence line" involved in scavenging oxidative free radicals generated in the course of cellular metabolism [11]. The role of non-enzymatic antioxidants is of particular importance, as their concentrations depend to a great extent on the applied diet.

In our study, we applied 3T3-L1 cell line which is commonly used to assess basic cytotoxicity of chemical substances. The use of cell cultures gives a better control of experimental conditions. On the other hand, the mechanism of ROS action formed during metabolism of ethanol in cell cultures may differ from *in vitro* studies. Besides, the type of cells used in a particular study and their ability to generate ROS in response to ethanol exposure may play a certain role [29]. The latter conjecture is confirmed by some literature findings, e.g. studies on epithelial cells of the stomach and endothelial cells of the bovine aorta showed that GSH prevented ethanol-induced damages only in epithelial cells [29]. It is not clear whether ROS are generated in 3T3-L1 cells during ethanol metabolism. It is also likely that concentration of ethanol used in our study was too low to observe oxidative injury induced by ethanol because we obtained a relatively low increase in cellular viability as compared to control after four hours of incubation.

Some authors have suggested that ethanol can interact with cell membranes in cells with very low ethanol metabolism activity. The observation of Garriga et al. that ethanol inhibited skeletal muscle proliferation and delayed its differentiation suggests that the effect of ethanol was likely connected to the action of its molecules on cell structures but not with the action of ethanol metabolism products [30].

In the present study, we used vitamin C or GSH in not toxic concentrations to cells 3T3-L1 as well as ethanol in concentrations able to reduce cellular viability by 10% as compared with controls. We failed, however, to evidence the effect of antioxidants in the applied concentrations on the viability of fibroblasts 3T3-L1. The activity of both enzymes, GSH-Px and TrxR, was most pronounced in cultures supple-

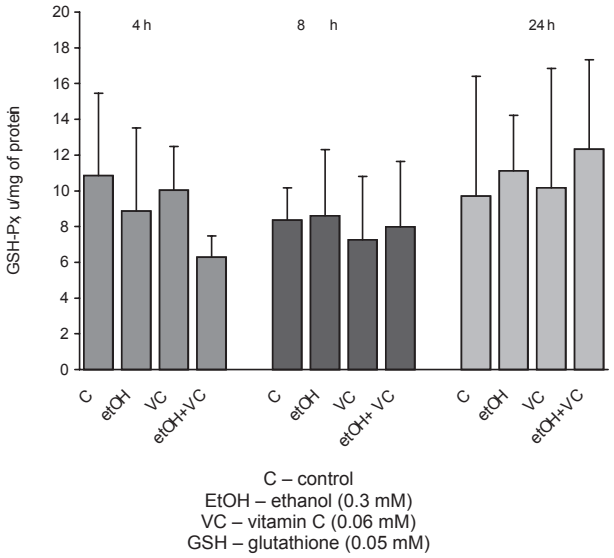

Fig. 2. GSH-Px activity in 3T3-L1 cells preincubated with VC (0.06 mM) and exposed to ethanol (0.3 mM) for 4, 8 and 24 hours. Each column shows the mean ± SD of three separate experiments.

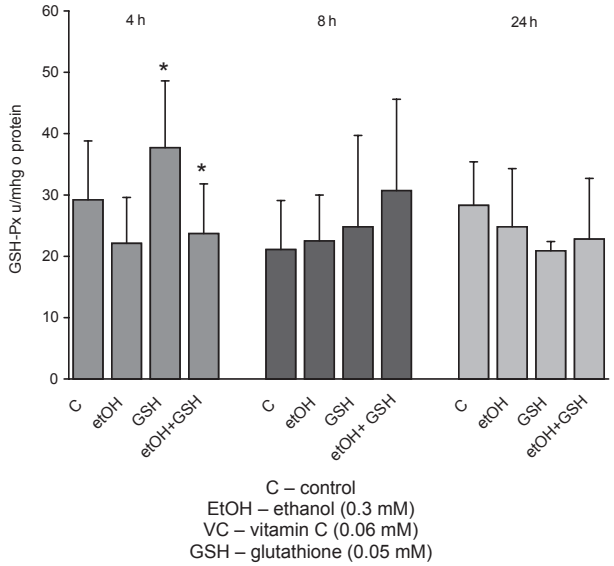

Fig. 1. GSH-Px activity in 3T3-L1 cells preincubated with GSH (0.05 mM) and exposed to ethanol (0.3 mM) for 4, 8 and 24 hours. Each column shows the mean ± SD of three separate experiments. *- $p<0.05$, significant differences compared with untreated control.

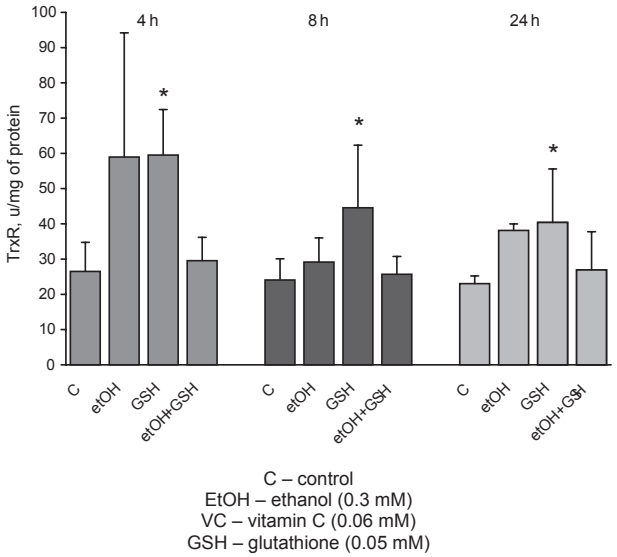

Fig. 3. TrxR activity in 3T3-L1 cells preincubated with GSH (0.05 mM) and exposed to ethanol (0.3 mM) for 4, 8 and 24 hours. Each column shows the mean ± SD of three separate experiments. *- $p<0.05$, significant differences compared with untreated control.

mented simultaneously with ethanol and antioxidants or with ethanol alone, but it was not feasible to show the protective effect of antioxidants on ethanol toxicity. The differences in TBARS concentrations were mostly observed in a 24 h incubation; an evident increase in TBARS concentration was observed in cultures supplemented simultaneously with ethanol and GSH or ethanol alone and vitamin C. An increase in lipid peroxidation after concomitant supplementation with ethanol and vitamin C was also observed in other *in vitro* and *in vivo* studies [31, 32].

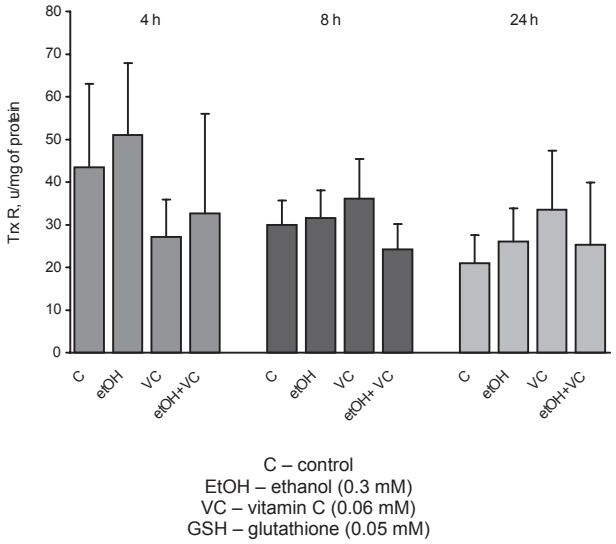

Fig. 4. TrxR activity in 3T3-L1 cells preincubated with VC (0.06 mM) and exposed to ethanol (0.3 mM) for 4, 8 and 24 hours. Each column shows the mean ± SD of three separate experiments.

The results of numerous studies provide evidence that vitamin C may act as pro- or antioxidant, depending on its concentration and the concentrations of other oxidants or antioxidants [33]. The prooxidative effect of vitamin C is probably associated with the ability of this compound to reduce ions of some metals (e.g., Fe^{3+} to Fe^{2+} or Cu^{2+} to Cu^{+}). The reduced ions reduce oxygen to O_2^- and H_2O_2. In the Fenton reaction, both O_2^- and H_2O_2 may generate hydroxyl radical, the most active radical among ROS, which initiates the process of lipid peroxidation [31, 33, 34]. It is likely that in the presence of vitamin C, generation of stable hydroxyethyl radical is also enhanced. This radical reacts with non-saturated fatty acids for a much longer time and in a more effective way than other radicals [32].

It should be noted that the literature data on the effect of vitamin C and GSH on the reduction of ethanol cytotoxicity are ambiguous [18, 35]. Some of them support an observation that vitamin C contributes to a more extensive removal of ethanol and acetic aldehyde from tissues and decreases the concentration of total cholesterol in the liver and serum triglyceride in experimental animals fed vitamin C-supplemented diet [35]. I*n vivo* studies suggest a similar effect of vitamin C [36]. Studies performed on the line of human brain astrocyte cells showed that preincubation of cells with vitamin C not only protected against the toxic effect of this compound, but also decreased the concentration of thermal shock proteins as compared with non-preincubated cells [36]. Neither the effect of ascorbate on activity of alcohol and aldehyde dehydrogenases nor toxic properties of ethanol and its metabolites was confirmed by other literature data.

The fact that standard cellular media do not contain vitamin C because of the instability demonstrated by this compound in an aqueous environment may be of some

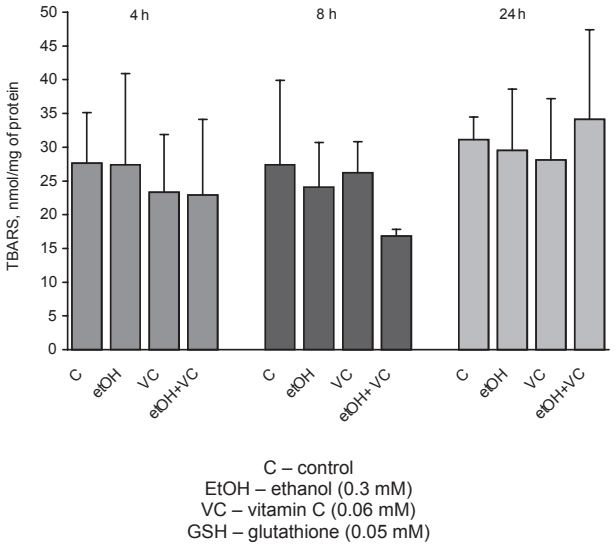

Fig. 5. The TBARS concentrations in 3T3-L1 cells preincubated with VC (0.06 mM) **a**nd exposed to ethanol for 4, 8 and 24 hours. Each column shows the mean ± SD of three separate experiments.

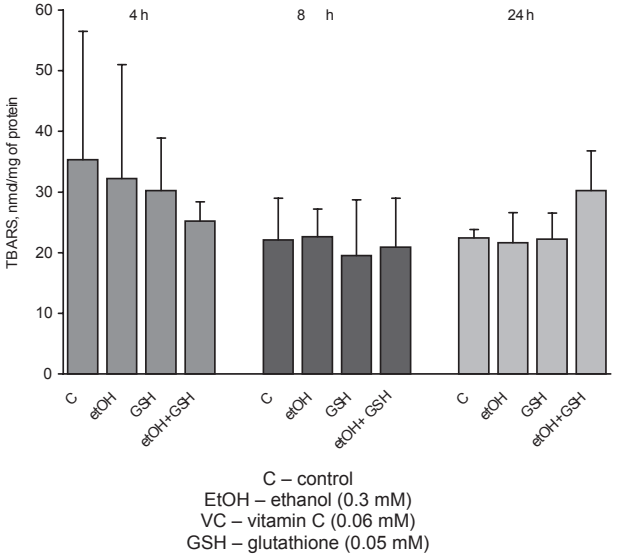

Fig. 6. The TBARS concentrations in 3T3-L1 cells preincubated with GSH (0.05 mM) and exposed to ethanol for 4, 8 and 24 hours. Each column shows the mean ± SD of three separate experiments.

importance in *in vitro* cultures. Cell lines cultured in such conditions are characterized by vitamin C deficiency, whereas in all mammalian cells, both vitamin C and GSH usually occur in millimole concentrations [37]. Studies on cell lines characterized in *in vitro* cultures by vitamin C deficiency have revealed that the supplementation of cellular medium with vitamin C contributed to the increased intracellular concentration of this compound. Its highest concentration was observed after 6 h of incubation. At the same time, an increase in total GSH in relation to GSH/GSSH was also observed, but in case of GSH those changes persisted only for 24 h [38]. It is most likely that vitamin C added to medium protected GSH from oxidation caused by free radicals [38].

Although the idea of using antioxidants as a substance that prevents ethanol toxicity emerged many years ago, it has not yet been fully utilized, mostly because of methodological dilemmas [28]. Recent studies have shown that the stimulation of intrinsic antioxidant production through activation of the antioxidant response element on genes can be a promising therapy in treatment of ethanol-induced liver diseases (e.g. overexpression of SOD with gene therapy would likely prevent the progression of alcohol-induced liver injury) [39]. However, this finding requires further studies in order to ensure that antioxidative gene therapy is safe for humans.

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