

Zinc inhibits HepG2 cell apoptosis induced by acetaldehyde and fatty acid ethyl esters

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Abstract: The vast majority of ingested ethanol is oxidized to acetaldehyde by the hepatocytes of the liver. Oxidation of ethanol by hepatocytes results in many metabolic changes leading to hepatocyte destruction. As zinc is known as a potent antioxidant and an inhibitor of cell apoptosis, the aim of this study was to investigate whether zinc supplementation could inhibit acetaldehyde-induced HepG2 apoptosis, and whether this inhibition was connected with attenuation of oxidative stress and modulation of FasR/FasL system expression. We also examined whether zinc could inhibit fatty acid ethyl esters (FAEEs)-induced apoptosis of HepG2 cells. The results indicated that zinc supplementation significantly inhibited acetaldehyde-induced HepG2 cell apoptosis (measured by cytochrome c release from mitochondria and caspase-3 activation) by attenuation of reactive oxygen species (ROS) production, increase in the cellular level of GSH, inhibition of acetaldehyde-induced FasL over-expression and caspase-8 activation. Moreover, zinc increased cell survival after treatment of HepG2 cells with FAEEs, inhibiting apoptosis of cells via the mitochondrial pathway and caspase-9 activation. Zinc supplementation did not influence acetaldehyde-induced overproduction of TGF- β 1. These results indicate that zinc can inhibit acetaldehyde-induced hepatocyte apoptosis by several independent mechanisms, among others by an indirect antioxidative effect and probably by inhibition of caspase-8 and caspase-9 activation.

Key words: zinc, acetaldehyde, apoptosis, HepG2 cells, reactive oxygen species (ROS); Fas (CD95), FasL, fatty acid ethyl esters (FAEEs), caspases

INTRODUCTION

Alcohol consumption produces a variety of metabolic and pathological alterations in the liver, both in alcoholic patients and in experimental animals [1]. Ethanol induces apoptosis of hepatocytes via two different pathways, by opening mitochondrial permeability transition pores and by upregulating the expression of Fas receptor (FasR, CD95)/Fas ligand (FasL). Overproduction of reactive oxygen species (ROS) by mitochondria, driven by acetaldehyde, metabolite of ethanol triggers both mechanisms [2, 3]. In ethanol-induced damage of the liver, fatty acid ethyl esters (FAEEs), products of non-oxidative metabolism of ethanol, are also involved. The predominant species synthesized by the liver are ethyl linoleate, ethyl palmitate, ethyl oleate, and ethyl stearate [4]. Recently, induction of apoptosis and perturbation of cell cycle by FAEEs in HepG2 cells have been described [5].

Zinc deficiency is common in alcoholic liver disease (ALD) due to reduction by alcohol of the pancreatic exocrine function and reduction of synthesis of ligands, such as picolinic acid, in the liver [6]. Recently, it has also been shown that dietary zinc supplementation prevents alcoholic liver injury through attenuation of oxidative stress. Zinc supplementation particularly prevented decrease in the glutathione level in the liver, suppressed ethanol-induced cytochrome P 4502E1 activity, and increased the activity of alcohol dehydrogenase (ADH) in the liver [7, 8].

Zinc has been shown to exert an antioxidant effect via induction of metallothioneins [9]. Moreover, zinc inhibits cell apoptosis via Bax and Bak activation and cytochrome c release [10] and also influences caspase activation [11]. The influence of zinc on the latter apoptosis pathway via FasR/FasL is poorly understood. The data in the literature are contradictory. In some papers, authors claim that zinc decreases FasR (CD95) expression in human lymphocytes [12], whereas other authors state that zinc inhibits only apoptosis induced via the mitochondrial pathway, not influencing the death-receptor-initiated pathway [10].

The aim of the present study was to investigate the influence of *in vitro* zinc supplementation on acetaldehyde-induced reduction of HepG2 cell viability and apoptosis via mitochondrial and FasR-mediated cell death. To assess the mechanism of the antiapoptotic activity of zinc, we measured the influence of zinc on ROS production, FasR and FasL expression, caspase-3 and caspase-8 activation, and cytochrome c release from mitochondria. To assess the role of zinc supplementation in the inhibition of FAEE-induced HepG2 cell death, we estimated the influence of zinc on cell viability and caspase-3 activation in FAEE-treated HepG2 cells.

MATERIALS AND METHODS

Cell cultures. The human hepatoma HepG2 cell line obtained from American Type Culture Collection (Manassas, VA, USA) was cultured in Eagle's Medium (MEM), supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 1% nonessential amino acids

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(NEAA), 1.5 g/L sodium bicarbonate, and 1% Antibiotic-Antimycotic, pH 7.4. Cells were seeded on tissue culture plates (Falcon, Bedford, MA, USA) and incubated at 37°C in a humidified atmosphere with 5% CO₂. HepG2 cells were subcultured twice a week by trypsinization in 0.25% trypsin-EDTA solution after washing with Ca-Mg-free saline. Culture media and antibiotics were purchased from Gibco (Grand Island, New York, USA), 0.25% trypsin-EDTA, FCS and NEAA were obtained from Sigma-Aldrich (Steinheim, Germany). In some experiments, Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich) was used.

Treatment of HepG2 cells with acetaldehyde, and zinc chloride. HepG2 cells were grown on 96-well plastic plates (Nunc, Roskilde, Denmark), 4×10⁴ cells/well. After 24 h incubation, the medium was replaced with a fresh one with an addition of different acetaldehyde (0-300 μM), or zinc chloride (0-200 μM) concentrations. Acetaldehyde was purchased from Merck (Darmstadt, Germany) and stored as 1 M stock solution. Zinc chloride was obtained from Sigma-Aldrich. The cells, treated with acetaldehyde, or zinc chloride, were maintained in a humidified atmosphere with 5% of CO₂, at 37°C for 24 h. The toxicity of the substances used was determined by MTT assay, as described earlier [13, 14]. All the experiments were conducted in triplicate. For further experiments, two concentrations of acetaldehyde (175 and 75 μM) and one 30 μM concentration of zinc chloride were chosen. HepG2 cells were preincubated with 30 μM zinc chloride followed by removal of the medium containing residual zinc prior to challenge with acetaldehyde. Such a model allowed evaluation of the possible protection against acetaldehyde by pretreatment with zinc. Acetaldehyde was chosen for the experiments because HepG2 cells have been described as having low activity of alcohol dehydrogenase (ADH) [15]. Cells were incubated with zinc chloride for 24 h at 37°C, after which the medium was changed to a new one, with an addition of the selected concentrations of acetaldehyde. The multiwell plates which contained HepG2 cells treated with acetaldehyde were placed on a rack inside a plastic container with a tight-fitting lid. A water bath containing 100 μM acetaldehyde concentration was placed at the bottom of each container. After 24h of incubation at 37°C, cell viability was measured by the MTT method.

Apoptosis measurement. Cell apoptosis was measured by cytochrome c release from mitochondria using Cytochrome c ELISA kit (Bender MedSystem Diagnostic GmbH, Vienna, Austria) according to the manufacturer's instruction. After incubation as described above, cells were centrifuged, washed in PBS, resuspended in Lysis Buffer, and incubated for 1 h at room temperature. After centrifugation (1,000 × g for 15 min), the concentration of cytochrome c was measured in cytosol. Samples were transferred into wells coated with a monoclonal antibody specific for cytochrome c, washed, and incubated with a biotinylated second antibody. After the incubation and washing, a streptavidine-HRP complex was added. After another washing, a substrate solution was added to the wells. Colour development was stopped with sulphuric acid and the intensity of the colour was measured at 450 nm (correction at 610 nm). The limit of cytochrome C detection was 0.08 ng/ml.

After incubation with acetaldehyde, with or without zinc supplementation, and washing with Ca-Mg-free saline, cells

were scraped with a rubber policeman and transferred into Eppendorf tubes with Lysis Buffer (25 μl of cold Lysis Buffer per 1×10⁶ cells). The cell lysate was incubated on ice for 10 min and then centrifuged at 10,000×g for 1 min.

Caspase-3 and caspase-8 activity was determined using a Colorimetric Assay kit (R&D Systems Inc., Minneapolis, MN, USA). The enzymatic reaction was carried out on 96-well flat bottom microplates. 50 μl of cell lysates (derived from 2×10⁶ cells) were tested in duplicate experiments by measuring the proteolytic cleavage of the specific colorimetric substrate DEVD-pNA. Recombinant caspase-3 or caspase-8 (R & D Systems Inc., Minneapolis, MN, USA) were used as a positive control.

sFas, sFasL and TGF-β1 assays. HepG2 cells were cultured in duplicate on 24-well plastic plates (Nunc) at a density of 4×10⁵ cells/ml/well with or without 30 μM ZnCl₂. After 24 h incubation at 37°C, the culture medium was replaced with a fresh one containing the selected acetaldehyde concentrations. After incubation for 24 h at 37°C, 5% CO₂, cell culture supernatants were collected, centrifuged and frozen immediately at -80°C for a further sFas and sFasL or TGF-β1 assay. Culture supernatants were stored for no longer than 3 weeks.

Human sFas and sFasL and TGF-β1 present in supernatants from HepG2 cells were measured by the ELISA method using kits from Bender MedSystem Diagnostic GmbH (Vienna, Austria). The kits contained a specific monoclonal antibody immobilized on a 96-well microtiter plate that bound sFas or sFasL or TGF-β1 in the aliquot and a second enzyme-conjugated specific polyclonal antibody. Following several washings in order to remove unbound substances and antibodies, a substrate solution was added to the wells. Colour development was stopped by sulphuric acid and the intensity of colour was measured by a microtiter plate reader (E-max, Molecular Devices Co, Menlo Park, CA, USA) at 450 nm (correction at 550 nm or 540 nm). The detection limit was sFas >20 pg/ml and sFasL >100 pg/ml and TGF-β1 >1.9 pg/ml. Intra-assay variations were less than 6%.

Glutathione colorimetric assay. HepG2 cells were grown on 6-well plastic plates (4×10⁵ cells/ml, 5 ml/well) in the absence or presence of 30 μM ZnCl₂ for 24 h, as above. The medium was then replaced with a fresh medium with acetaldehyde (final concentration of 175 or 75 μM) for 24 h at 37°C. Control wells were incubated without acetaldehyde. After washing with Ca-Mg-free saline, cells were scraped with a rubber policeman and immediately transferred into Eppendorf tubes with 500 μl of ice-cold 5% metaphosphoric acid (MPA) (Sigma Aldrich). The samples on ice were sonicated with the Sonicator XL 2020 (Farmingdale, New York, USA). After centrifugation (3,000×g/10 min, 4°C) the supernatants were collected and kept for 1 hour at 0-4°C for GSH assay. A Bioxytech GSH-400 kit (OXIS Research, Portland, OR, USA) was used for measurements of the glutathione level in HepG2 cells. The method was based on the two-step chemical reactions. After lysis of cells and centrifugation, the supernatants were treated with R1 reagent. This first step led to the formation of substitution products (thioethers) between the reagent included in the kit and all mercaptans present in the sample. The second step was mediated by 30% NaOH, which specifically transformed the substitution product obtained with GSH into a chromophoric thione with

a maximal absorbance wavelength of 400 nm. Absorbances were measured using the UV-Visible Spectrophotometer (Varian, Optical Spectroscopy Instruments, Switzerland). The glutathione concentration, expressed in nanomoles, was calculated from a standard curve (range 20-100 nm). The detection limit was 5 nanomoles.

Catalase (CAT) activity assay. To measure catalase activity, HepG2 were grown on 6-well plastic plates (4×10^5 cells/ml, 5 ml/well) in the absence or presence of $30 \mu\text{M ZnCl}_2$ for 24 h, as above. The medium was then replaced with HBSS (control), HBSS with acetaldehyde (final concentration of 175 or 75 μM) for 60 min at 37°C . After washing with Ca-Mg-free saline, cells were scraped with a rubber policeman and immediately transferred into Eppendorf tubes, on ice, with 1 ml of 0.05 M potassium phosphate buffer, pH 7.0, and sonicated using a Sonicator XL 2020. After low-speed centrifugation, the supernatants were used immediately for catalase activity measurement.

The assay was performed according to the method described by Pifferi et al. [16] and modified by Nowak [17]. Briefly, the reaction mixture prepared in the Eppendorf tube consisted of 500 μl of 0.05 M phosphate buffer, pH 7.0, 300 μl of distilled water, 50 μl of 1.1 mM H_2O_2 in distilled water and 50 μl of HepG2 cell homogenate (or 50 μl of distilled water as blank). After 5 min of incubation at 25°C , 100 μl of 50% trichloroacetic acid (TCA, Sigma-Aldrich) was added to each tube and the tubes centrifuged ($1,000 \times g$ for 5 min). Next, 10 μl of titanium (IV) reagent [17] was added to each tube and 200 μl of supernatant was transferred into wells on a 96-well microplate. The absorbance was read at 405 nm in a microplate reader. The results were expressed as CAT activity in U/ 10^6 cells/min after comparison with the standard curve, prepared by plotting the absorbance (OD) at 405 nm (ordinate) as a function of standard CAT (Sigma-Aldrich) concentration (abscissa) between 0-33 U/ml. One unit of CAT decomposed 1.0 μmole of H_2O_2 per min at pH 7.0 and at 25°C .

Measurement of superoxide anion (O_2^-) production by the cytochrome c reduction assay [18]. HepG2 cells were grown on 96-well plastic plates (4×10^4 cells/well) with or without $30 \mu\text{M ZnCl}_2$. After 24 h incubation at 37°C , the cultures were washed twice with HBSS and an assay for the superoxide anion was performed. Briefly, HBSS (207.5 μl), 12.5 μl of cytochrome C solution in HBSS (final concentration of 75 μM), 5 μl of either SOD solution (final concentration of 60 U/ml) or 5 μl of distilled water, and 25 μl acetaldehyde solution in HBSS (final concentration of 175 or 75 μM) was added to each well on a 96-well plate. Control wells were also used where cells were incubated without acetaldehyde. The microplate was incubated at 37°C for 60 min and transferred to the microplate reader. The absorbance values at 550 nm (the differences in OD between samples with and without SOD) were converted to nanomoles of O_2^- based on the extinction coefficient of cytochrome C: $\Delta E_{550} = 21 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The results were expressed as nanomoles of O_2^- per 1×10^6 cells per 60 min. All chemicals were purchased from Sigma Aldrich.

Measurement of hydrogen peroxide (H_2O_2) production [19]. HepG2 cells grown on 96-well plastic plates (4×10^4 cells/well) with or without $30 \mu\text{M ZnCl}_2$. After 24 h incubation

at 37°C , the cultures were washed twice with Hanks' Balanced Salt Solution (HBSS), and the medium replaced with HBSS (100 μl /well) with an addition of acetaldehyde (final concentration of 175 or 75 μM) for 60 min at 37°C . In control wells, cells were incubated without acetaldehyde. Those wells were washed twice with HBSS and a measurement of intracellular hydrogen peroxide was performed. The assay was based on horseradish-dependent peroxidation (HRPO) of phenol red by H_2O_2 leading to the formation of a compound that exhibited absorbance at 600 nm. Briefly, the cells were covered with 100 μl /well of the assay solution, which was prepared on the day of the experiment and consisted of HBSS, phenol red (Sigma, final concentration of 0.56 mM), HRPO (Serva, Heidelberg, Germany, final concentration of 20 U/ml). Additionally, 10 μl /well of 1N NaOH was added. After 3 min of incubation, the plate was read at 600 nm in the microplate reader. The results were expressed as nanomoles H_2O_2 per 10^6 cells per 60 min based on the phenol red extinction coefficient ($\Delta E_{600} = 19.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

Treatment of HepG2 cells with different ethyl palmitate (EP), ethyl stearate (ES), or ethyl oleate (EO) concentrations. Fatty acid ethyl esters (FAEEs) obtained from Sigma-Aldrich (Steinheim, Germany) were dissolved in DMSO (EP, EO) or in isopropanol (ES) in 200 mM stock solution and subsequently bound to albumin according to Chang and Borensztajn's method [20]. HepG2 cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark), 4×10^4 cells/well. After 24 h incubation, the medium was replaced with a fresh one with an addition of 1 mM DETAPAC (zinc chelator) or $30 \mu\text{M}$ zinc chloride. Each medium also had an addition of FAEE in an albumin binding form at various concentrations (ranging from 10 μM - 5 mM). Control samples contained no FAEE but contained the same amount of DMSO or isopropanol (final concentration 2.4%). After 24 h incubation, the toxicity of used FAEEs was determined by MTT assay.

Preparation of HepG2 cells for the caspase-3 activity assay after incubation with FAEEs. HepG2 cells were treated with FAEEs and zinc as described above. Each sample was prepared in triplicate; with or without caspase-8 or -9 inhibitor at a final concentration of 20 μM (R & D Systems Inc., Minneapolis, MN, USA). After 20 h incubation and washing with Ca-Mg-free saline, cells were scraped with a rubber policeman, transferred into Eppendorf tubes with Lysis Buffer (25 μl of cold Lysis Buffer per 1×10^6 cells). The cell lysate was incubated on ice for 10 min and then centrifuged at $10,000 \times g$ for 1 min. Caspase-3 activity was examined as described above.

Statistical analysis. Values are expressed as mean \pm SD. The significance of differences was determined with the use of the analysis of variance (Statistica computer package). A number of statistical tests were used; these include two-way ANOVA test with *post-hoc* Tukey's test and Wilcoxon paired test for comparisons inside groups. P values < 0.05 were considered to be significant.

RESULTS

The influence of acetaldehyde, and zinc chloride on HepG2 cell viability. As can be seen from Fig. 1, acetaldehyde (A), and zinc chloride (B) decreased cell viability in a time-and-dose dependent manner. For further experiments, two acetaldehyde concentrations (175 μM and 75 μM), and one (30 μM) zinc chloride concentration were chosen. Zinc supplementation significantly increased the viability of HepG2 cells treated with different acetaldehyde concentrations (Fig. 1C). When acetaldehyde-induced HepG2 cell apoptosis was measured, zinc supplementation significantly reduced the amounts of cytochrome released from mitochondria (Fig. 2A) and significantly inhibited the activity of caspase-3 (Fig. 2B), which indicated that decrease in cell viability was, at least partially, caused by induction of the mitochondrial pathway of apoptosis.

The influence of zinc supplementation on reactive oxygen species production, GSH level, and catalase activity in HepG2 cells. It is well established that acetaldehyde-induced cell apoptosis is caused, at least partially, by oxidative stress. Therefore, to assess the mechanism in zinc-mediated HepG2 protection, we measured zinc influence on the superoxide anion extracellular level induced in HepG2 cells by acetaldehyde treatment. As can be seen from Fig. 3A,

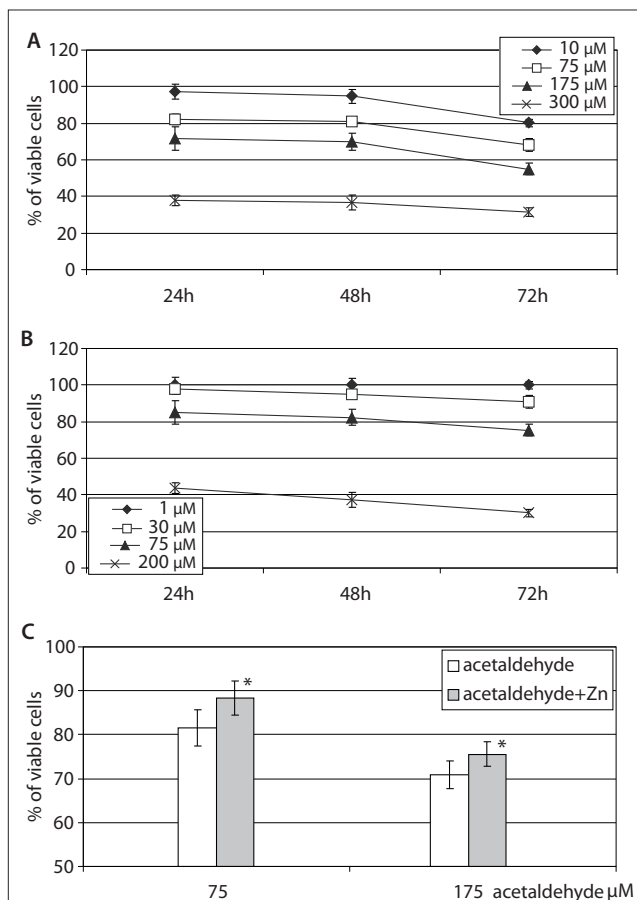


Figure 1 Dose-dependent and time-dependent cytotoxicity of acetaldehyde (A) and zinc chloride (B) for HepG2 cells, measured by the MTT method. The influence of 30 μM zinc chloride on the viability of cells treated with acetaldehyde (C). Results are expressed graphically as a percentage of the control (untreated) cells viability. Values are means \pm SD of results from five experiments. * Significantly different in comparison to control without zinc chloride, $p \leq 0.05$.

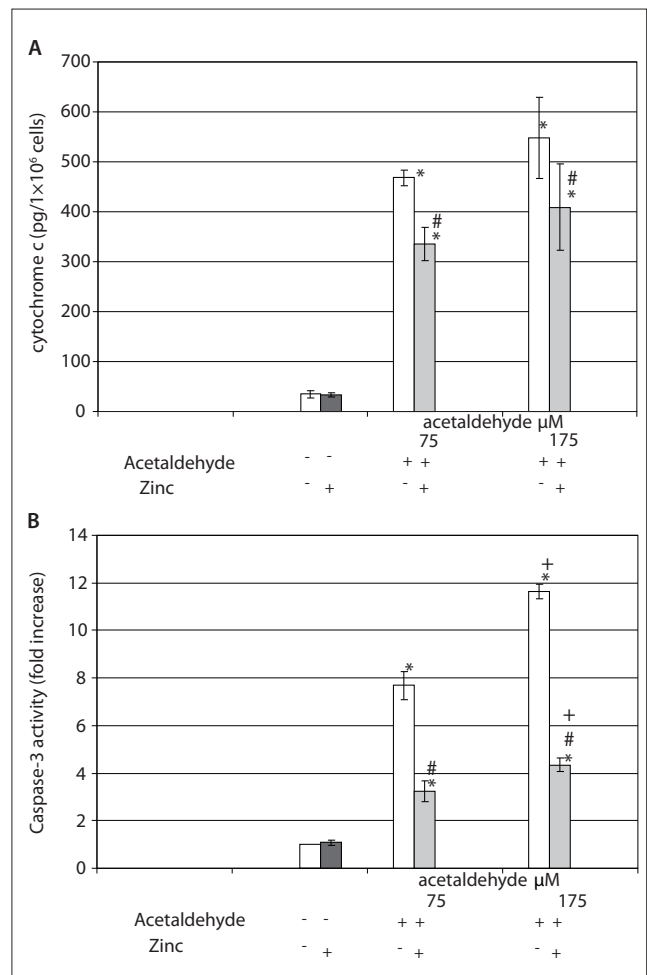


Figure 2 Level of apoptosis (measured as cytochrome c release $\text{pg}/1 \times 10^6$ cells) after treatment of HepG2 cells with acetaldehyde. Cells were preincubated with zinc for 24 h. After treatment, the medium was changed and different acetaldehyde concentrations added. Apoptosis was assayed after 24 h incubation. (B) Caspase-3 activation in HepG2 cells treated with acetaldehyde. Cells were preincubated with zinc for 24 h. After treatment, the medium was changed and different acetaldehyde concentrations added. Caspase-3 activation was assayed after 24 h incubation. Results are expressed as mean \pm SD of three independent experiments.

* Significantly different from respective control (cells not treated or treated only with zinc), $p \leq 0.001$.
Significantly different in comparison to acetaldehyde control, $p \leq 0.005$.
+ Significantly different in comparison to cells treated with 75 μM acetaldehyde, $p \leq 0.05$. Zinc significantly changed acetaldehyde effect, $p \leq 0.01$ (two-way ANOVA).

zinc supplementation significantly decreased superoxide anion production, especially when high doses of acetaldehyde were used. Zinc supplementation also inhibited acetaldehyde-induced hydrogen peroxide production in HepG2 cells, although its effect was rather weak (Fig. 3B). The presence of oxidative stress in acetaldehyde-treated HepG2 cells was also confirmed by a significant drop in the intracellular GSH level. When zinc was added to HepG2 cell cultures, the GSH level was significantly higher (Fig. 4A). In order to detect whether the decrease in the hydrogen peroxide level was caused by the positive influence of zinc on catalase activity, we compared its level in HepG2 cells treated with acetaldehyde, in the presence or absence of zinc (Fig. 4B). Generally, catalase activity was lower in HepG2 cells treated with acetaldehyde, and zinc supplementation tended to inhibit its activity. One exception was the slight increase in catalase activity after zinc supplementation of cells treated with 75 μM acetaldehyde. It seems likely that the decreased level of hydrogen peroxide was

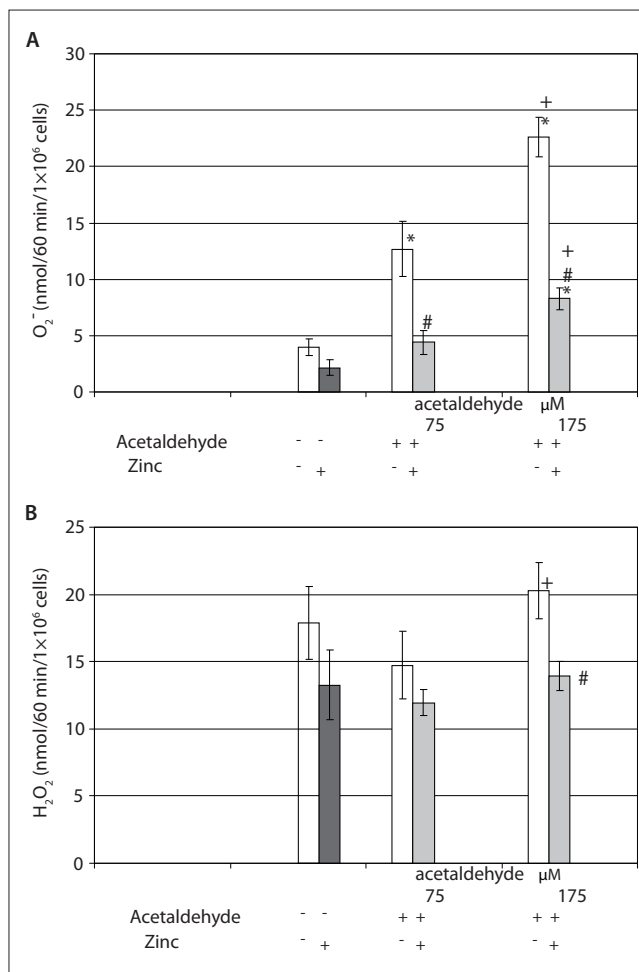


Figure 3 Influence of zinc chloride supplementation on superoxide anion (nmol/60 min/ 1×10^6 cells) release from HepG2 cells treated with acetaldehyde. (B) Influence of zinc chloride on hydrogen peroxide (nmol/60 min/ 1×10^6 cells) release from HepG2 cells treated with acetaldehyde. Results are expressed as mean \pm SD of four independent experiments.

* Significantly different from respective control (cells not treated or treated with zinc), $p \leq 0.01$.

Significantly different from acetaldehyde control, $p \leq 0.001$.

+ Significantly different compared to cells treated with 75 μM acetaldehyde, $p \leq 0.05$. Zinc significantly changed acetaldehyde effect, $p \leq 0.01$ (two-way ANOVA).

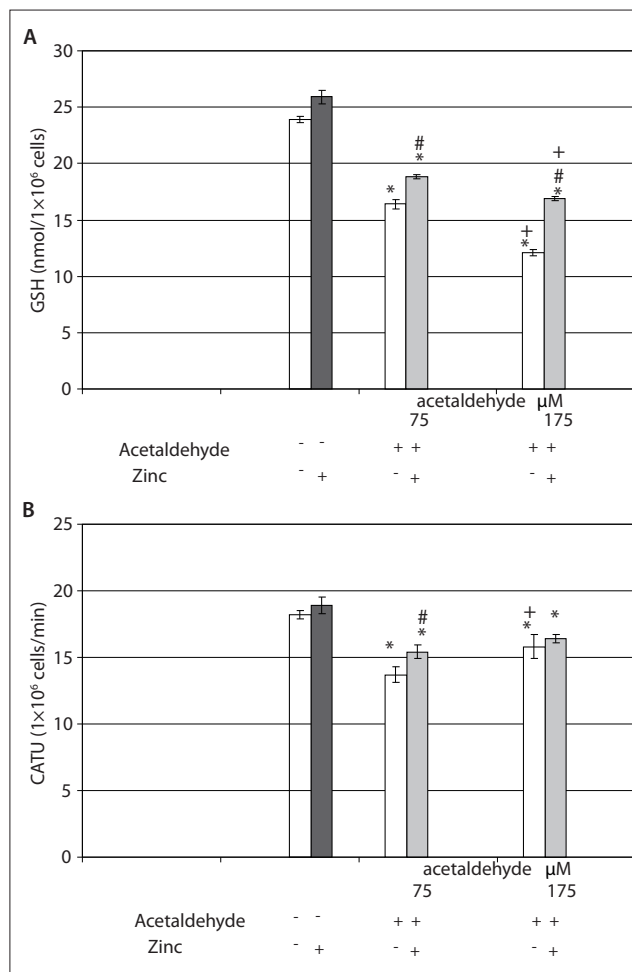


Figure 4 GSH (nmol/ 1×10^6 cells) level in HepG2 cells treated with acetaldehyde. Cells were preincubated with zinc for 24 h. After treatment, the medium was changed and different acetaldehyde concentrations added. GSH level was assayed after 24 h incubation. (B) CAT level (U/ 1×10^6 cells/min) in HepG2 cells treated with acetaldehyde in absence or presence of 30 μM zinc chloride. Results are expressed as mean \pm SD of three independent experiments.

* Significantly different from respective control (cells not treated or treated with zinc), $p \leq 0.01$.

Significantly different in comparison to acetaldehyde control, $p \leq 0.001$.

+ Significantly different in comparison to cells treated with 75 μM acetaldehyde, $p \leq 0.05$. Zinc significantly changed acetaldehyde effect, $p \leq 0.002$ (two-way ANOVA).

rather the consequence of an indirect antioxidative effect of zinc than its influence on catalase activity.

The influence of zinc supplementation on acetaldehyde-induced FasR-mediated apoptosis of HepG2 cells and caspase-8 activation and TGF- β production. In order to check whether acetaldehyde-induced apoptosis involves the FasR/FasL system, we examined zinc influence on sFasR and sFasL levels in a cell culture medium. In our experiments, ethanol induced sFas release from HepG2 cells, while zinc supplementation inhibited this process. Acetaldehyde seemed to modulate sFas release: while the 175 μM concentration significantly increased sFas concentration in cell culture supernatants, the 75 μM concentration significantly reduced it in comparison to untreated cells. Moreover, zinc supplementation did not change this modulation (Fig. 5A). Acetaldehyde modulated sFasL release, but in contrast to sFas, the low acetaldehyde concentration significantly enhanced sFasL release, whereas the high 175 μM concentration inhibited its release. Zinc supplementation tended to inhibit sFasL release, but the inhibition was significant only with high

doses of acetaldehyde (Fig. 5B). When the level of caspase-8 activity was examined, ethanol acetaldehyde significantly enhanced, while zinc supplementation inhibited caspase-8 activation (Fig. 6A).

TGF- β -induced apoptosis of hepatocytes is well documented. As can be seen from Fig. 6B, HepG2 cells can produce significant amounts of TGF- β after acetaldehyde treatment, but zinc supplementation does not influence the production of TGF- β .

The influence of zinc on FAEE-induced apoptosis of HepG2 cells. The recently detected higher levels of FAEE synthesis in HepG2 cells treated with ethanol prompted us to investigate whether zinc could also inhibit FAEE-induced apoptosis of HepG2 cells. Three ethyl esters, palmitate (EP), oleate (EO) and stearate (ES) were dissolved in DMSO or isopropanol and bound to albumin, as described in Materials and Methods. HepG2 cells treated with 5 mM concentrations of all the ethyl esters of fatty acids used had a significantly

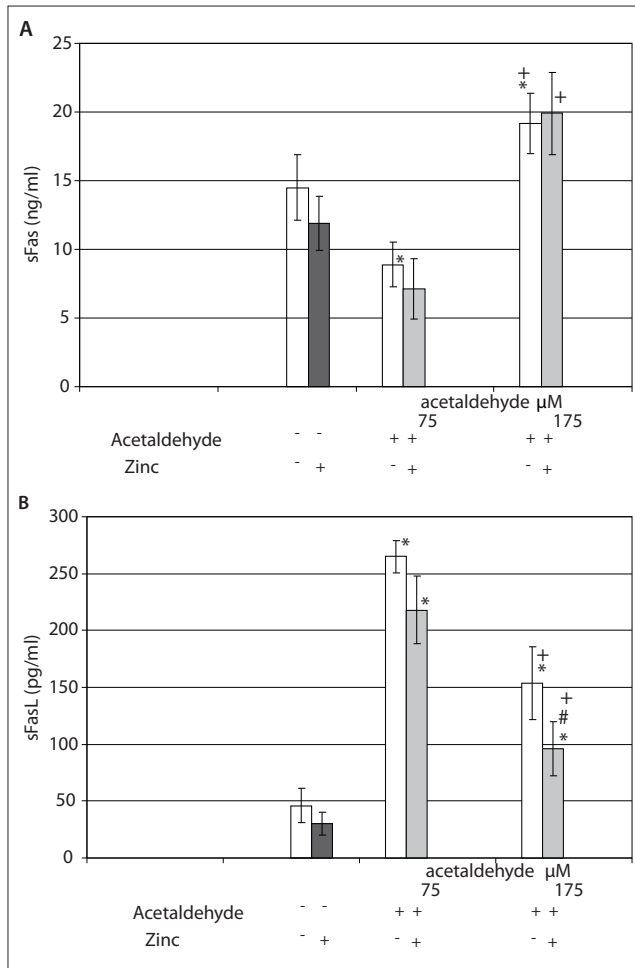


Figure 5 sFas (ng/ml) release from HepG2 cells treated with acetaldehyde. Cells were preincubated with zinc for 24 h. After treatment, the medium was changed and different acetaldehyde concentrations added. sFas level in cell culture medium was assayed after 24 h incubation. (B) The influence of zinc chloride supplementation on sFasL (pg/ml) release from HepG2 cells after treatment with acetaldehyde. Cells were preincubated with zinc for 24 h. After the treatment, the medium was changed and different acetaldehyde concentrations were added. sFasL level in cell culture medium was assayed after 24 h of incubation. Results are expressed as mean ± SD of three independent experiments.

* Significantly different from respective control (cells not treated or treated with zinc), p≤0.001.
Significantly different from acetaldehyde control, p≤0.001. Zinc significantly changed acetaldehyde effect, p≤0.02 (two-way ANOVA).

lower cell viability. The viability of cells decreased to 83% of that in control cells (data not shown). This effect was partially reversed by zinc supplementation. Moreover, FAEEs, in the presence of DETAPAC as zinc chelator, induced apoptotic HepG2 cell death, as indicated by caspase-3 activation, via the mitochondrial pathway, as was shown by using the specific inhibitor of caspase-9. Zinc supplementation significantly inhibited FAEEs-induced caspase-3 activation (Fig. 7).

DISCUSSION

The plasma and hepatic levels of zinc have been found to be reduced in alcoholic liver disease (ALD) [21]. Moreover, in the animal model it has been detected that zinc treatment exerts an inhibitory action on alcohol-induced liver damage and activation of inflammation. Zinc-mediated liver protection is complex. It is partially due to zinc-induced decrease in

intestinal permeability to bacterial toxins and its influence on ammonia metabolism [22, 23], but its main effect is through a direct influence of zinc on hepatocyte metabolism (induction of metallothioneins). Recently, it has been shown that dietary zinc supplementation prevents alcoholic liver injury through attenuation of oxidative stress. Zinc supplementation particularly prevented the decrease in the glutathione level in the liver, suppressed ethanol-induced cytochrome P4502E1 activity, and increased the activity of alcohol dehydrogenase (ADH) in the liver [7, 8]. Zinc has also been described to inhibit ethanol-induced hepatocyte apoptosis *in vitro* [24], but the mechanisms of its action have yet to be studied.

To assess the mechanisms of zinc-mediated hepatoprotection, we examined the *in vitro* influence of zinc supplementation on acetaldehyde-induced HepG2 cell apoptosis. For this, we chose acetaldehyde as ethanol oxidation metabolite which is known to be involved in hepatotoxicity of ethanol. In our study, we used a HepG2 cell line sensitive to acetaldehyde treatment, and in which acetaldehyde induced cell apoptosis,

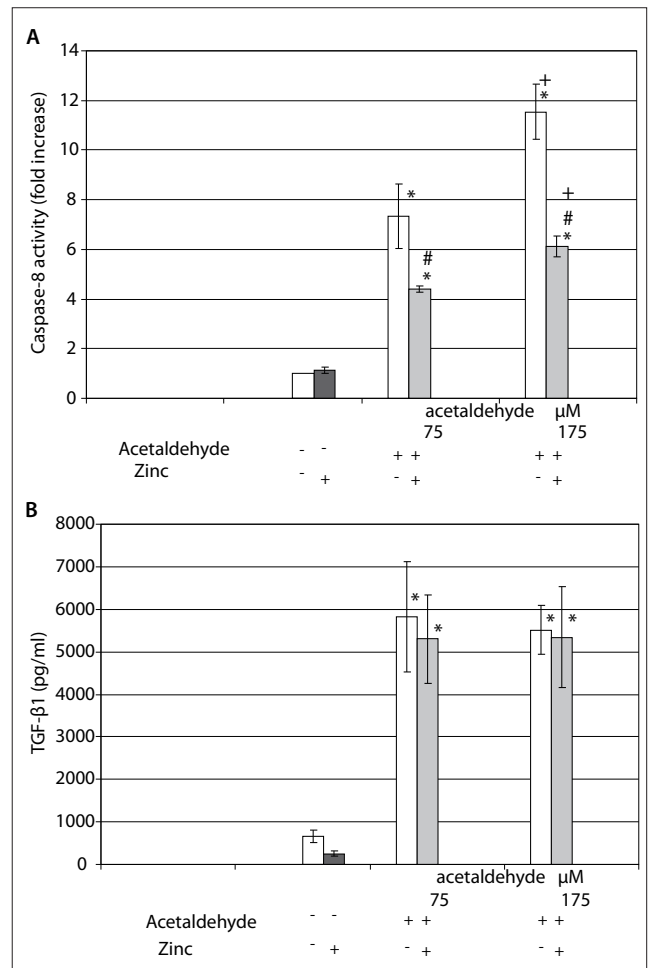


Figure 6 (A) Caspase-8 activity in HepG2 cells treated with acetaldehyde. Cells were preincubated with zinc for 24 h. After treatment, the medium was changed and different acetaldehyde concentrations added. Caspase-8 activity was assayed after 24 h incubation. (B) TGF-β1 (pg/ml) production by HepG2 cells treated with acetaldehyde in the absence or presence of 30 μM ZnCl₂. Results are expressed as mean ± SD of four independent experiments.

* Significantly different from respective control (cells not treated or treated with zinc), p≤0.01.
Significantly different from acetaldehyde control, p≤0.003.
+ Significantly different in comparison to cells treated with 75 μM acetaldehyde, p≤0.05.

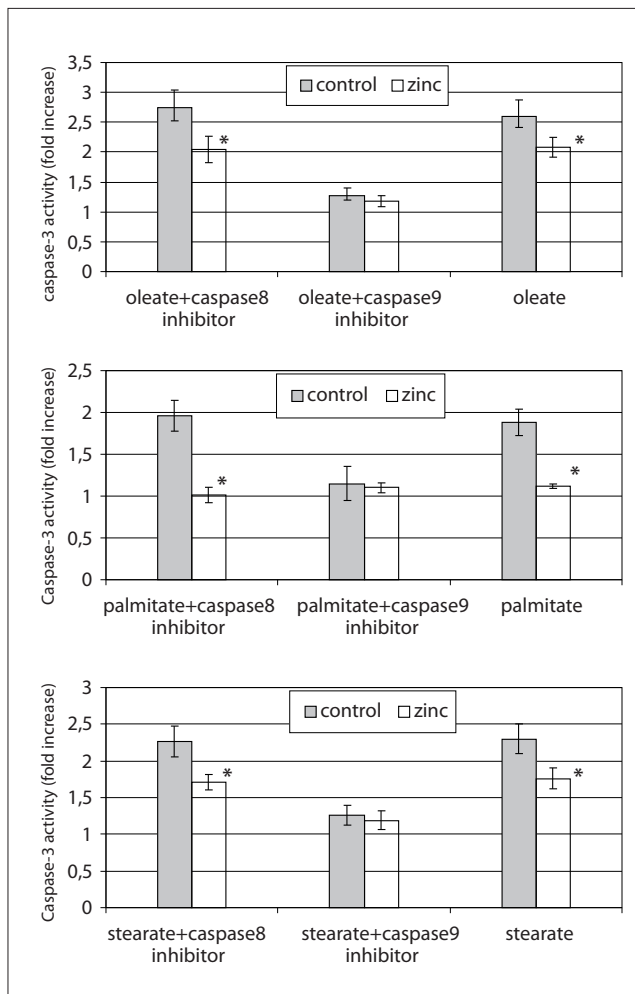


Figure 7 Influence of zinc chloride supplementation on caspase-3 activation after treatment of cells with FAEEs. Results are expressed as mean \pm SD of four independent experiments.
 * Significantly different from control (cells not treated with zinc), $p < 0.01$.

and confirmed that acetaldehyde treatment can induce the loss of viability, cytochrome release from mitochondria of HepG2 cells and activate caspase-3.

There are two pathways that lead to cell apoptosis:

- 1) positive induction by ligand binding to a plasma membrane receptor;
- 2) negative induction by loss of the suppressor activity.

The former is mediated through death domain-containing transmembrane receptors such as Fas (CD95). The activation of Fas by Fas ligand (FasL) induces a signal complex that recruits caspase 8 [25]. Induction of apoptosis by loss of suppressor activity involves mitochondria. Altered permeability of the mitochondrial outer membrane is essential for initiation of apoptosis through this pathway due to the release of cytochrome c into cytosol. In the cytosol, cytochrome c triggers activation of caspase-9 [26]. In the HepG2 recombinant cells [27] constitutively expressing ADH, ethanol oxidation was required for ethanol-induced reduction in cell viability. On the other hand, it has been detected that apoptosis of HepG2 cells induced by a low concentration of ethanol is associated with Fas receptor (FasR) activation and subsequent caspase-8 activation [28]. The role of acetaldehyde in ethanol-induced apoptosis via FasR/FasL, however, is not known. In our study, we confirmed that in HepG2 cell apoptosis both pathways were

involved. Cells treated with acetaldehyde released cytochrome c into cytosol and activated caspase-3, which indicates that the mitochondrial pathway was involved. Moreover, acetaldehyde modulated sFas and sFasL release to the culture medium. Acetaldehyde also increased caspase-8 activation.

We also detected that zinc inhibited acetaldehyde-induced apoptosis of HepG2 cells. Several papers indicate that zinc could inhibit apoptosis of different kinds of cells. Blood leukocytes can be rescued from apoptosis with physiological concentrations of zinc salts (5-25 μ M) [29]. In our experiments we used slightly higher zinc concentrations which were non-toxic for HepG2 cells and protected the cells against acetaldehyde-induced apoptosis. The mechanism of this protection is not well-understood, but it has been described that zinc can inhibit the mitochondrial pathway of apoptosis by inhibition of Bax and Bak activation, cytochrome release from mitochondria, and all the subsequent steps of cell apoptosis, such as caspase-3 activation and Ca^{2+}/Mg^{2+} DNA nuclease activity [11, 30].

In our experiments, zinc was able to inhibit acetaldehyde-induced cell death, we therefore suspected that it affected proapoptotic mechanisms that occur in the cells downstream of ADH. As oxidative stress during acetaldehyde metabolism is known to be a major cause of hepatocyte death, we examined zinc influence on ROS generation. Not surprisingly, we detected that 30 μ M zinc inhibited superoxide anion and hydrogen peroxide release from HepG2 cells after acetaldehyde treatment. In our study we observed a significant increase in the intracellular GSH level after zinc supplementation, and suppose this to be the consequence of antioxidative activity of zinc rather than direct zinc influence on the GSH level.

The ability of zinc to retard oxidative processes has been recognized for many years. In general, the mechanism of antioxidation can be divided according to acute or chronic effects. Chronic effects involve induction of antioxidants, such as metallothioneins. Acute effects involve the protection of protein sulfhydryls and reduction of hydroxyl radical formation from hydrogen peroxide through the antagonism of iron and copper as transition metals. For example, zinc reduces superoxide anion production induced by PMA (phorbol-12myristate-13acetate) in leukocytes [31].

In our study, we detected that acetaldehyde modulated Fas/FasL system by increasing the release of soluble Fas as well as sFasL. *In vitro* experiments by other authors have revealed that zinc preferentially interfered with Fas/FasL system-induced cell apoptosis by inhibiting expression of FasL [11]. We also detected in our experiments that zinc supplementation significantly inhibited acetaldehyde-induced sFasL release, and that zinc inhibition of sFasL resulted in an inhibition of caspase-8 activation.

It has already been found that in HepG2 cells which express a low level of ADH activity, after ethanol treatment, the products of nonoxidative metabolism, such as FAEEs, cause apoptosis via the intrinsic pathway. This apoptosis was mediated by release of mitochondrial cytochrome c release, and activation of caspase-9 and caspase-3 [15]. Moreover, the treatment of HepG2 cells with FAEEs also induced apoptosis in those cells [5]. We confirmed in our experiments that FAEEs such as ethyl oleate, ethyl palmitate or ethyl stearate induced apoptotic death of HepG2 cells involving caspase-9 and caspase-3 activation. Not surprisingly, we also detected that zinc supplementation inhibited FAEe-induced cell death, probably by inhibiting the activation of caspases.

SUMMARY

In acetaldehyde-induced HepG2 cell apoptosis both apoptosis pathways were involved, i.e. through mitochondria and through the Fas/FasL system. Moreover, after acetaldehyde treatment, HepG2 cells produced TGF- β which might also be involved in autocrine stimulation of apoptosis. Zinc supplementation inhibited HepG2 cell apoptosis by inhibiting release of superoxide anion and hydrogen peroxide and by attenuating oxidative stress, which was indicated by increased levels of cellular GSH. Moreover, zinc inhibited acetaldehyde-induced sFasL over-expression and caspase-8 activation. Zinc also increased cell survival after treatment of HepG2 cells with FAEEs, inhibiting apoptosis of cells via the mitochondrial pathway and caspase 9 activation. Zinc supplementation did not influence acetaldehyde-induced TGF- β 1 overproduction. These results indicate that zinc could inhibit acetaldehyde-induced hepatocyte apoptosis by several independent mechanisms, among others by indirect antioxidative effect, modulating FasL expression, and probably by inhibition of caspases activation.

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