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Reproductive Toxicology

Zinc attenuates ethanol-induced Sertoli cell toxicity and apoptosis through caspase-3 mediated pathways

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ABSTRACT

Ethanol enhances apoptosis in testicular germ cells. Zinc reduces ethanol-induced apoptosis of somatic cells through inhibition of caspase-mediated pathways. Little is known about the effects of ethanol on Sertoli cells and the effects of Zinc on ethanol-induced testicular injury. The hypothesis tested was that ethanol enhances apoptosis of Sertoli cells through up-regulation of caspase-3 and Zinc inhibits ethanol-induced effects. Cultured Sertoli cells (TM4) were exposed to ethanol (160 mM), Zinc (8 μM) and Zinc prior to ethanol for duration of 24 or 48 h and their effects on TM4 cell viability was then investigated by MTT assay. Caspase-3 mRNA expression was also investigated using real-time RT-PCR. Cell viability decreased and caspase-3 mRNA expresstion increased in cells exposed to ethanol, while exposure to Zinc showed opposite effects. Pretreatment with Zinc recovered ethanol-induced anti-proliferative effects and over-expression of caspase-3. Zinc reduced ethanol-induced Sertoli cell toxicity and apoptosis via caspase-3 mediated pathways.

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1. Introduction

Humans are exposed to varied environmental agents potentially hazardous to the reproductive system. Male reproduction, which its dysfunction is known to contribute to more than half of all cases of global childlessness [1], is highly responsive and sensitive to a variety of chemical and physical agents [2]. Chronic stress, obesity, urogenital trauma and inflammation, smoking and alcohol abuse are associated with decreased male fertility [3,4]. Alcohol has been shown to have a deleterious effect at all levels of male reproductive system, including the hypothalamus, anterior pituitary gland and the testes [5]. Chronic alcohol intake in experimental animals has been associated with marked degenerative changes such as germ cell damage, testicular atrophy and reduction in seminiferous tubular size [6–10]. Moreover, spermatogenic failure, hypogonadism, gonadal dysfunction and infertility by triggering testicular germ cell apoptosis have been reported in humans with a history of chronic alcohol abuse [11]. However, the mechanism(s) of ethanol-induced infertility remain poorly understood [12]. Ethanol exposure also adversely affects the secretory function of testicular Sertoli cells [7], and disrupts Sertoli cell-mediated blood-testes barrier [13]. Sertoli cells play a supportive and crucial role in spermatogenesis by providing physical support, nutrients and survival signals, controlling germ cell proliferation and apoptosis which is necessary for successful spermatogenesis [14–17]. Ethanol is very toxic for Sertoli cells, making them to be the primary cellular target of ethanol toxicity with marked Sertoli cell vacuolization being a morphological marker of ethanol-induced testicular injury [7,9,18–20].

Alcohol consumption induces apoptotic cell death in different cell lines and various organs including the brain, liver, thymus and testis [14,21–23]. Within the testes, apoptosis occurs under physiologic conditions and provides to avoid overproduction of normal cells and maturation of abnormal cells. Under pathologic conditions, such as an exposure to specific environmental toxins, a greater portion of testicular cells degenerate through the apoptosis, leading to spermatogenic failure and infertility [24]. The process of

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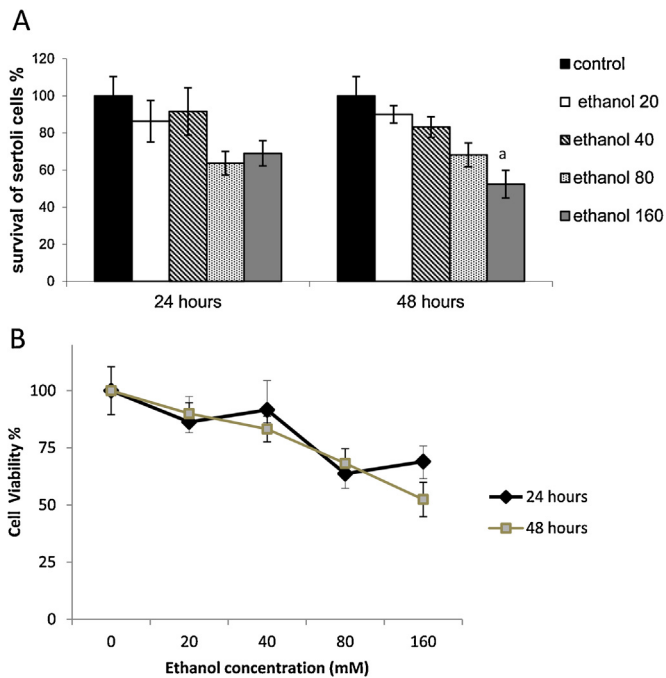
Fig. 1. The effect of ethanol on TM4 cell viability. (A) Comparison of the effects of different concentrations of ethanol on TM4 cell viability following 24 or 48 h exposure periods. [a] Represents p = 0.005 compared to the control group (one-way ANOVA). All results are presented as mean ± S.E.M derived from two determinations in duplicate cultures. (B) Comparison of data between 24 and 48 h exposure periods revealed no significant changes. As concentration of ethanol increased, cellular viability decreased and eventually reached to approximately 50% of control level at concentration of 160 mM following 48 h exposure period.

Fig. 2. The effect of Zinc on TM4 cell viability. (A) Comparison of the effects of different concentrations of Zinc on TM4 cell viability following 24 or 48 h exposure periods. At concentration of 8 μM of Zinc, cell viability and proliferation increased compared to control and higher concentrations of Zinc. [a] Represents p < 0.01 compared to the control and the 64 μM Zinc-treated groups. [b] Represents p < 0.01 compared to the control. [c] Represents p < 0.05 compared to the 64 μM Zinc-treated group (one-way ANOVA). All results are presented as mean ± S.E.M derived from two determinations in duplicate cultures. Comparison of data between 24 and 48 h exposure periods revealed no significant changes. (B) As concentration of Zinc increased cell viability decreased and eventually showed a trend around the control level.

apoptosis is triggered and regulated by the expression of several proteins. Caspase, a highly specific class of cysteine proteases, is expressed in many mammalian cells and known to mediate a crucial stage of the apoptotic process [25,26]. Of particular interest is caspase-3, the most widely studied member of the caspase family, which is, at least partially, responsible for the degradation of DNA and cleavage of cellular proteins, leading to apoptosis [27–30]. Previous *in vitro* and *in vivo* studies have shown involvement of several proteins and pathways in ethanol-induced apoptosis of testicular cells, including Fas system, active caspases and bax-dependent signaling pathways [14,22,23]. Based on the results, ethanol appears to induce apoptosis in Leydig cells via bax-dependent caspase-3 activation [22].

It has been reported that ethanol-induced testicular damage is mainly mediated by DNA damage, oxidative stress and androgen suppression [7,14,23,31]. Oxidative stress is caused by imbalanced between the production of reactive oxygen species (ROS) and the production of antioxidant system that is responsible for their neutralization and removal. Increased oxidative stress is a well-accepted mechanism of alcohol induced tissue injury, particularly in the liver, heart, central nervous system and testis [3,5,32,33]. In the testis, oxidative stress affects reproductive activity, damages DNA structure and induces apoptosis, leading to fertility disturbances or embryo development disorders [3,34,35]. Zinc as an antioxidant agent, reduced ethanol-induced apoptosis of somatic cells in different non-sexual organs [36,37]. Zinc plays important roles in various biological activities but its role in spermatogenesis is not well known. Zinc deficiency has been documented in alcoholic liver disease, skin, esophagus, gastric and kidney damages, in both patient and animal [36–40]. It has been shown that Zinc deprivation induces apoptosis in diverse cell lines and potentiates death receptor-mediated apoptosis [41,42]. In this respect, testicular apoptosis after dietary zinc deficiency has also been reported
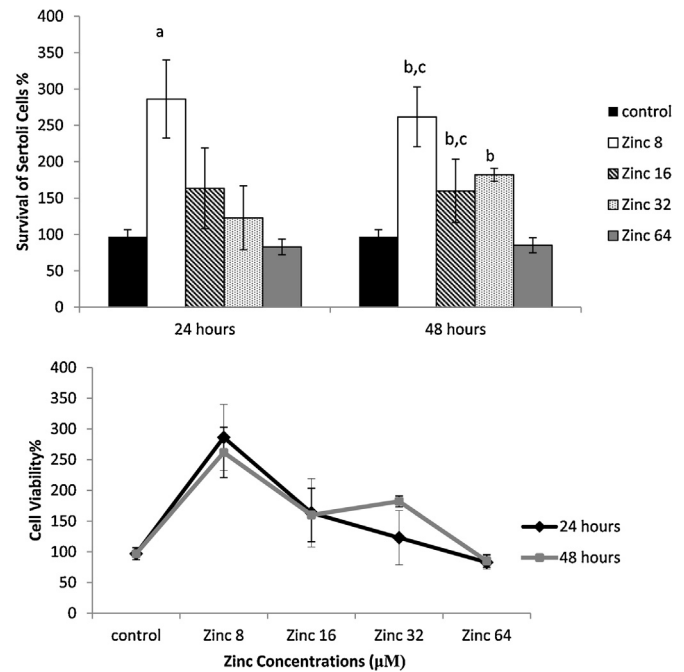
[43]. Moreover, Zinc inhibits ethanol-induced hepatic apoptosis in both cell culture and mice subjected to a long-term ethanol exposure [36,37], however its protective effects on ethanol-induced testicular injury is poorly understood.

Despite of toxic effects of ethanol and protective effects of zinc on multiple cells and organs, there is little available information regarding their effects on testis and testicular somatic cells. In the present study, the effect of different concentration of ethanol and zinc (single and co-administration) on the cell viability and apoptosis (by measuring caspase-3 gene expression) in cells of the TM4 cell line, derived from mouse Sertoli cells, were investigated to determine whether an enhancement of Sertoli cell death occurs as a result of ethanol exposure and whether zinc can inhibit ethanol-induced effects.

2. Material and methods

2.1. Cell line and culture conditions

The Mus Musculus Sertoli cell line, TM4 (purchased from Pasteur Institute, Tehran, Iran) was used in all experiments. The TM4 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12/hepes media (Atocel, Austria) supplemented with 2.5% fetal bovine serum (Atocel, Austria), 5% Horse serum (Atocel, Austria), penicillin (50 units/ml) and streptomycin (50 units/ml), in 25 cm^2 tissue flasks maintained at 37 °C in a humidified atmosphere with 95% O_2 and 5% CO_2 in a cell incubator. The medium was routinely aspirated and replaced with fresh medium every 24–48 h. When the cultured cells achieved 70–80% confluence, the cells were transferred after short term (maximum, 1–2 min) trypsinization with 1% trypsin and subcultured twice per week. Cell viability was
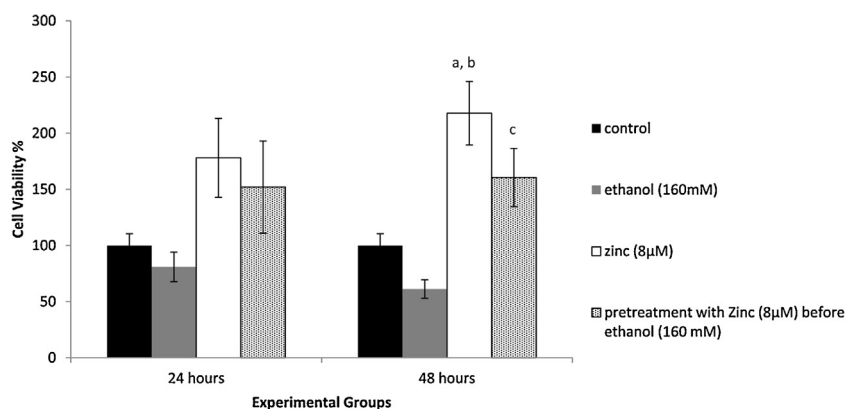
Fig. 3. Comparison of the effects of different treatments on TM4 cell viability. The experimental groups were assigned to treatment conditions that included ethanol (160 mM), Zinc (8 μM), pretreatment and control (un-treated) groups, following 24 or 48 h exposure periods. In the pretreatment group, the cells were pretreated with Zinc (8 μM, for 48 h), before ethanol (160 mM) exposure. Results are presented as mean ± S.E.M derived from two determinations in duplicate cultures. [a] Represents $p < 0.001$ compared to the control group. [b] Represents $p < 0.001$ compared to the ethanol group. [c] Represents $p < 0.05$ compared to the ethanol group. Comparison of data between 24 and 48 h exposure periods revealed no significant changes.

determined by the trypan blue staining followed by hemocytometry and 10^5 cells were seeded in 5 ml fresh medium in 25 cm^2 flasks. All chemicals and reagents purchased are of molecular biology grade.

2.2. Cell viability (MTT assay)

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit (Cayman Company, USA) according to the manufacturer's protocol. In order to determine the effect of ethanol and Zinc, cells were seeded to 96-well culture plate at a density of about 5×10^3 cells/well, and. Although the Sertoli cells have high adhesion properties, but to prevent the possible effects of unknown laboratory errors a stabilization period of 24 h has been assigned. After this stabilization period, cells were treated with ethanol (ethanol for molecular biology, Fluka, Sigma/Aldrich, Steinheim, Germany) at concentrations of 0, 20, 40, 80 and 160 mM, for 24 or 48 h. To determine the effect of Zinc, TM4 cells were treated with 0, 8, 16, 32 and 64 μM Zinc sulfate (Sigma, USA) for 24 or 48 h in a final volume of 100 μl. At the beginning of treatments, the medium was aspirated from each well and replaced with 100 l ethanol or Zinc containing medium, at described concentrations (4 wells each and control groups of 10 wells). In control wells, the medium was aspirated from each well and replaced only with a free medium. After 24 h of the last treatment, a 10 μl sample of the MTT labeling reagent was added to each well, and the cells were then incubated in the CO_2 incubator. After an incubation period of 3–4 h, 100 μl of Crystal Dissolving Solution was added to each well. The absorbance and optical density (O.D.) were then measured with a micro plate reader at 545–630 nm. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100. After primary analysis, a concentration of 160 mM for ethanol and 8 μM for Zinc was considered as effective concentrations. Accordingly, in another set of experiments, TM4 Sertoli cells were cultured and investigated in four main groups including: Ethanol (160 mM), Zinc (8 μM), pretreatment and control groups in two subgroups including: 24 and 48 h exposure periods. In each of duplicate cultures, cells were seeded to 96-well culture plate at a density of 5×10^3 cells/well (4 wells each group/exposure period, total number of 24 wells and control group of 10 wells). Therefore cells for all groups and subgroups were seeded to a single culture plate, seeded at the same time, with the same density. After a stabilization period of 24 h, in 48 h exposure subgroups, the medium was aspirated from each well and replaced with a Zinc containing
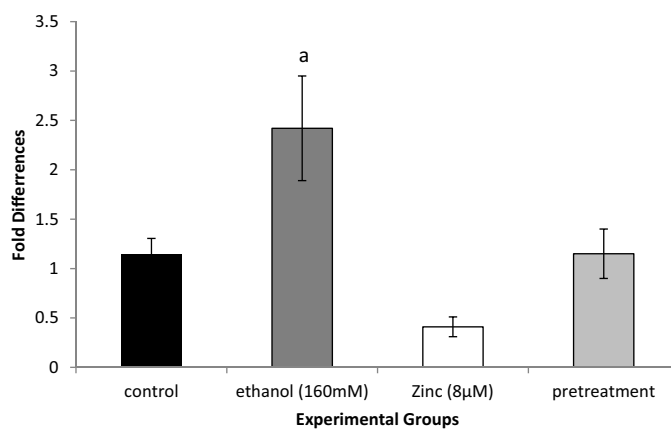

Fig. 4. Quantitative real-time PCR analysis of caspase 3 mRNA level in different treatment groups. The experimental groups were assigned to treatment conditions that included ethanol (160 mM), Zinc (8 μM), pretreatment and control (un-treated) groups. In the pretreatment group, the cells were pretreated with Zinc (8 μM, for 48 h), before ethanol (160 mM) exposure. Caspase 3 mRNA expression was normalized to β-actin in each sample. Vertical bars indicate the mean fold differences ± SEM for three independent experiments in each treatment group. [a] Represents $p < 0.05$ compared to the control and Zinc groups.
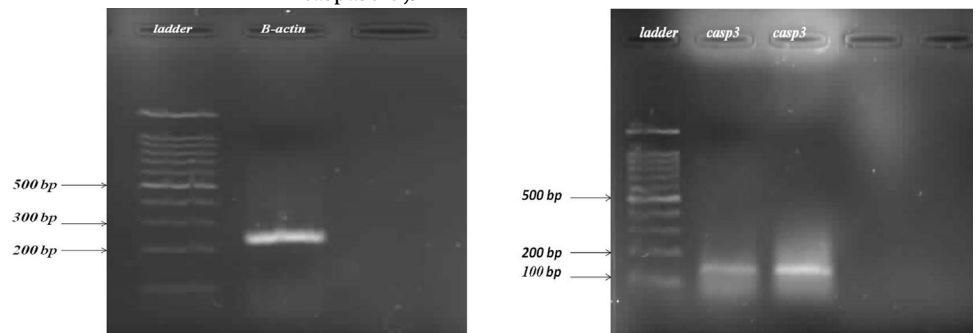
medium for pretreatment wells and a free fresh medium for all other wells, for following 48 h. The medium was changed to the fresh related medium in the midpoint of culture. The day after that, the medium was aspirated from each well and replaced with an ethanol containing medium for wells of pretreatment and ethanol groups, with a Zinc containing medium for Zinc group and with a free medium for control group, again for following 48 h. For wells of 24 h exposure subgroups, the same protocol of 48 h exposure period was applied except that the pretreatment started one day later and extended for 48 h and ethanol and Zinc exposure was lasted only for 24 h. Therefore, pretreatment duration of Zinc for both 24 and 48 h subgroups was same and only treatment exposure time was different. MTT assay was then performed to investigate cell viability of different groups (for all wells, together simultaneously).

As the best effect of ethanol and Zinc was observed at the above-mentioned concentrations, these concentrations, at 48 h exposure period, were applied for the following experiments.

2.3. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

In each set of triplicate cultures, 105 TM4 cells/well were seeded in 6 well plates for 24 h, followed by a treatment according to the

the procedure at 72 °C for 5 min. After amplification, the resulting products were separated on 2% agarose gel and stained with safe stain. The stained gel was visualized under ultraviolet light and its band width was measured by 100 bp DNA Ladder. The expected sizes of the PCR products were 250 bp (for β-actin) and 136 bp (for caspase-3).

RT-PCR for β-actin (left, 250 bp) and Caspase-3 (right, 136 bp) of TM4 cells.

experimental main groups: ethanol (160 mM), Zinc (8 μM), pretreatment and control (one well each). Following a stabilization period of 24 h, the well of pretreatment group was exposed to Zinc and the other wells were exposed to free medium for following 48 h. The medium was changed to the fresh related medium in the midpoint of culture. The day after that, the medium was aspirated from each well and replaced with an ethanol containing medium for wells of pretreatment and ethanol groups, with a Zinc containing medium for Zinc group and with free medium for control group. After 48 h of last treatment, the cells were trypsinized and cell pellets were used for RNA extraction with RNX-Plus (CinnaGen, Iran) according to the manufacturer's instructions. Briefly, 1 ml of RNX plus was added to a tube containing 10^6 homogenized cells, and the mixture was incubated at room temperature for 5 min. Chloroform was added to the solution and centrifuged at 12,000 rpm for 15 min. The upper aqueous phase was then transferred to a new tube and an equal volume of isopropanol was added. The mixture was then centrifuged at 12,000 rpm for 15 min and the resulting pellet was then washed in 70% ethanol and dissolved in DEPC-treated water. RNA concentration and purity were determined by measuring the ratio of optical density at 260 nm to that at 280 nm and 1% agarose gel electrophoresis. Extracted total RNA was then used to generate single-stranded cDNA with 2-step RT-PCR kit (Vivantis, Malaysia) by the following reaction (according to the protocol provided by the manufacturer): 10 μg of RNA of each treatment was mixed with 1 μl of random Hexamers primer (50 ng/μl), 1 μl dNTPs mix (10 mM) and up to 10 μl of nuclease-free water. This mixture was added to a cDNA synthesis mix that included 2 μl of buffer M-MuLV (10X), 100 unit M-MuLV reverse transcriptase and up to 10 μl of water and was followed by incubation of the mixture at 42 °C for 60 min, and then for 5 min at 85 °C. The synthesized cDNA was directly used as template for RT-PCR by the following reaction: 5 μl of cDNA template was amplified by RT-PCR in 25 μl of total reaction volume containing 1 μl of each specific primer β-actin (housekeeping gene) and Caspase-3 (target genes) primer sequences (takapozist, Iran). Primer sequences for β-actin were 5′-TAGGCGGACTGTTACTGAGC-3′ (sense) and 5′-GCTCCAACCAACTGCTGTC-3′ (anti-sense) and for Caspase-3 were 5′-GCAGCTTTGTGTGTGTGATTC-3′ (sense) and 5′-AGTTTCGGCTTTCCAGTCAG-3′ (anti-sense). RT-PCR procedure (2-steps RT-PCR, Vivantis, Malaysia) was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions (briefly): initial denaturation at 94 °C for 2 min, followed by 30 amplification cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 40 s and extension at 72 °C for 50 s, with an additional extension step at the end of

2.4. Real time RT-PCR assay

The expression of caspase-3 gene was analyzed by real-time RT-PCR, using the mentioned specific primers and Bioneer AccuPower qPCR master Mix (South Korea) in a total volume of 25 μl according to the manufacturer's instructions. mRNA expression of Caspase-3 gene was analyzed with quantitative real-time RT-PCR using iQ5 Real-Time PCR Detection System (Bio-Rad, CA, USA). A no template control was used to test the potential contamination and primer dimer formation. The reactions were prepared in a 96-well optical plate for 10 min at 95 °C followed by 40 cycles performed with 20 s at 95 °C and 45 s at 60 °C. A melting curve analysis was conducted to confirm the specificity of the amplification reactions. Each sample was replicated three times and the threshold cycle (Ct) values were the corresponding mean. The relative expression of each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, where Ct is the threshold cycle (analysis of relative gene expression data using real-time quantitative PCR and the $2^{-(\text{DeltaDeltaCt})}$ method). Relative expression levels of mRNA were normalized to β-actin and analyzed for statistical significance using one-way analysis of variance (SPSS 16). Results were expressed as mean fold differences ± SEM for three independent experiments in each treatment group.

2.5. Statistical analysis

All data were presented as mean ± standard error for at least three separate experiments for each treatment. Statistical significance of differences between mean values was analyzed by one way ANOVA followed by Tukey's HSD post-hoc test using SPSS 16 statistical analysis software (SPSS Inc., Chicago, IL). The level of significant difference was set at $P < 0.05$. The fold differences of gene expression normalized to control were presented graphically in the form of histograms, using Microsoft Excel computer program.

3. Results

3.1. Effects of ethanol on Sertoli cell viability

In order to investigate the effect of ethanol on Sertoli cell viability and proliferation, cultured TM4 cells were exposed to ethanol at final concentrations of 0 (cells exposed to an ethanol free media, as control group), 20, 40, 80 and 160 mM, for 24 and 48 h and MTT assay was then carried out. In both 24 and 48 h exposure periods, cell viability decreased as concentration of ethanol increased in

comparison to control group (Fig. 1). After 48 h exposure period, cell viability significantly decreased from $100 \pm 12\%$ in control group to $54.6 \pm 4\%$ in cells exposed to 160 mM ethanol (P < 0.05). Ethanol decreased viability and inhibited proliferation of TM4 cells in a concentration-dependent manner with IC50 values of 160 mM for 24 h and 230 mM for 48 h exposure. Comparison of data between 24 and 48 h exposure periods revealed no significant changes (Fig. 1).

3.2. Effects of Zinc on Sertoli cell viability

To investigate Zinc-induced changes in Sertoli cell viability and proliferation, TM4 cells were incubated with Zinc at final concentrations of 0 (cells exposed to Zinc free media, as control group), 8, 16, 32 and 64 μM for 24 and 48 h and then MTT assay was carried out. In both 24 and 48 h exposure periods, cell viability increased in comparison to control in lower concentrations of Zinc (Fig. 2). The cellular viability in control group was %98 ± 10, which in cells exposed to 8 μM Zinc increased significantly to %286 ± 53 and %261 ± 41 following 24 and 48 h exposure periods, respectively (P ≤ 0.001). At higher concentrations of Zinc, cell viability and proliferation decreased as concentration of Zinc increased, in comparison to cells exposed to 8 μM Zinc, and at the highest concentration of Zinc (64 μM) cellular viability showed a trend almost around the control level (Fig. 2). Comparison of data between 24 and 48 h exposure periods revealed again no significant changes (Fig. 2).

3.3. Effects of pretreatment with Zinc on ethanol-induced changes in Sertoli cell viability

Following above mentioned primary experiments and analysis, a concentration of 160 mM for ethanol and 8 μM for Zinc was considered as effective concentrations. In order to investigate the effect of pretreatment of Zinc on ethanol-induced cell viability suppression, in another set of experiments, cultured TM4 cells were exposed to 8 μM Zinc for 48 h before addition of ethanol and cellular viability was then assessed and compared with groups exposed to ethanol and Zinc alone. As shown in Fig. 3, exposure to ethanol inhibited cell proliferation, whereas cellular viability and proliferation increased in Zinc group and in the group received Zinc prior to ethanol, compared to the control and ethanol groups. Pretreatment with Zinc inhibited ethanol-induced cell proliferation suppression (Fig. 3). Comparison of data between 24 and 48 h exposure periods revealed again no significant changes.

3.4. mRNA expression of caspase-3 in Sertoli cells exposed to ethanol, Zinc and Zinc prior to ethanol

Quantitative real-time RT-PCR analysis of the mRNA levels of caspase-3 was performed, in order to determine the relative levels of expression of this gene, in the experimental groups. The experimental groups were assigned to treatment conditions that included ethanol (160 mM), Zinc (8 μM), pretreatment (cells were pretreated with Zinc before ethanol exposure) and control (untreated) groups. Expression of β-actin gene was tested for internal control of the mRNA levels. Expression of caspase-3 gene was normalized to the endogenous control to acquire the relative threshold cycle (ΔCt) and related to the ΔCt of un-treated condition to find the relative expression level ($2^{-\Delta\Delta Ct}$) of treated groups. Caspase-3 mRNA levels in the un-treated cells were set at 1.00.

Caspase-3 mRNA levels was significantly increased to 2.42 ± 0.53 by treatment with ethanol (P ≤ 0.03, Fig. 4). On the contrary, caspase-3 mRNA levels were decreased to 0.41 ± 0.16 by treatment with Zinc, but these differences were not found to be significant. As shown in Fig. 4, mRNA level of caspase-3 in the pretreatment group decreased to around control level

and showed approximately same gene expression level with un-treated conditions.

4. Discussion

In the present study cell viability and proliferation as well as caspase-3 gene expression have been investigated in TM4 Sertoli cells, exposed to ethanol, Zinc or Zinc prior to ethanol, for 24 or 48 h. The main findings were that cell viability and proliferation decreased in cells exposed to ethanol while increased in cells exposed to Zinc, in a concentration-dependent manner. Pretreatment with Zinc prior to ethanol exposure decreased ethanol-induced anti-proliferative effects. There were not significant differences in Sertoli cell viability between 24 and 48 h exposure periods in experimental groups. Moreover, caspase-3 mRNA levels increased in ethanol-treated cells while decreased in Zinc-treated cells, compared to untreated group. Pretreatment with Zinc recovered ethanol-induced elevation of caspase-3 mRNA expression levels to approximately control levels. These findings suggested that ethanol shows cytotoxic and apoptotic effects in TM4 Sertoli cells, whereas Zinc shows anti-apoptotic effects and attenuates ethanol-induced cellular toxicity.

It is widely accepted that ethanol is a gonadal toxin. A growing body of evidence in humans and experimental animals indicates that ethanol abuse leads to testicular atrophy and male infertility [5,6,32,44]. It is well known that ethanol exposure disrupts the HPG axis, adversely affects the secretory function of Sertoli cells, and produces a certain degree of testicular dysfunction. Furthermore, increases in germ cell apoptosis were observed after testicular exposure to ethanol in human, laboratory animals and cell cultures [14,22,23]. Apoptosis has been indicated as an important mechanism by which ethanol caused tissue injury and enhanced toxicity of testicular germ cells [23]. In spite of intensive studies for ethanol-induced injury or apoptosis in germ cells, little is known about the effects of ethanol on isolated Sertoli cells. Since Sertoli cells have a supporting influence on spermatogenesis and do not turn over, the degradation of the Sertoli cells might be a major factor responsible for the loss of germ cells.

In the present study evaluation of cell viability via MTT assay confirmed that ethanol decreases Sertoli cell survival and exhibits an anti-proliferation effect, in a concentration- dependent manner. This finding is in agreement with the other studies which showed ethanol at concentrations comparable to those used in this study, exerts a cytotoxic effects on isolated Leydig [22], Sertoli [7,23] and germ cells [14,23]. Moreover, the ethanol concentrations used in this study at 80 and 160 mM, correspond to 360 and 720 mg/dl. In in vivo animal study in rat, the serum ethanol concentrations were reached to about 200, 300 and 500 mg/dl 3 h after subcutaneous injection of 2.5, 3 and 5 g/kg ethanol, respectively [45]. As previously reported, the concentrations used in this study seem as clinically heavy drunken condition [22,45]. National Institute on Alcohol Abuse and Alcoholism (2004) has designated a blood alcohol concentration level of 80 mg/dl or higher as an indicator of heavy drinking [46]. Previous study has been reported a toxic threshold for alcohol in the range of 180–200 mg/dl [45]. Accordingly, maintaining blood ethanol concentrations at or above 200 mg/dl for four consecutive hours was the minimum condition for triggering degenerative response. Some of the previous studies have also revealed a time-dependent effect of ethanol on reproductive activity [47–50]. According to our results no significant differences were found between 24 and 48 h exposure periods in the experimental groups. Most of the above mentioned studies are in vivo, in which hypothalamic pituitary gonadal (HPG) axis activity is performed to regulate gonadal function. Duration dependent impairment of testicular function in alcohol abusers or in experi-

mental animals has been reported to might be due to impaired HPG axis [47,48,51] which is absent in cell culture *in vitro* studies.

It is well known that caspase family concentration is critical in determining the susceptibility of cells to apoptotic stimuli. Caspase-3, in particular, is a key player in the execution of apoptosis and has many cellular targets that, when activated, produce the features of apoptosis. The present data have also revealed ethanol-induced up-regulation and increased expression of caspase-3 mRNA in Sertoli cells, which has already been reported in other cells and tissues exposed to ethanol [14,22,52–54]. Decreased cell viability and over-expression of caspase-3 mRNA in Sertoli cells exposed to ethanol may be a reflection of ethanol-induced testicular injury resulting in enhanced Sertoli cell apoptosis, which may be partly involved in infertility associated with alcohol abuse.

It is well known that ethanol produces oxidative stress within the testes [33,55,56]. Excessive ROS production beyond critical levels overwhelmed the antioxidant defense strategies of spermatozoa in seminal plasma and resulted in increased oxidative stress. Zinc, a cytoprotectant and anti-oxidant agent, coordinates intracellular signaling pathways, stimulates cell proliferation and suppresses apoptosis [41]. The hepatoprotective effect of Zinc against ethanol induced-hepatocyte injury has been well documented and seems to be related to its antioxidant properties. Accordingly, it has been reported that Zinc can inhibit ethanol-induced hepatocyte apoptosis by several independent mechanisms: by an indirect antioxidative effect and probably by inhibition of caspase activation and death receptor-mediated pathways [36,37]. Our data is the first report that Zinc, inhibited ethanol induced testicular injury. Ethanol exerted anti-proliferation and cytotoxic effects on Sertoli cells as well as induced apoptosis, at least in part, through over-expression of caspase-3. However, all these parameters exhibited, inhibited or recovered when the ethanol exposure was pretreated with Zinc.

At concentrations of 8 and 16 μM, Zinc elevated cell viability and proliferation throughout the 24 and 48 h exposure periods maximally up to 2–3 fold the control level. In addition, caspase-3 mRNA expression was below the control level in cells exposed to Zinc. These data suggest that Zinc not only suppressed ethanol-induced anti-proliferation and apoptotic effects, but also inhibited duration-dependent and spontaneously-induced apoptosis, confirming the previous evidences that Zinc regulates intracellular signaling pathways and reduces apoptosis and changes in Zinc levels are sufficient to alter susceptibility to apoptosis [38,41,57]. Irrespective to this, Zinc at higher concentrations (32 and 64 μM) did not affect significantly cellular viability of TM4 cells, suggesting that its protective and anti-apoptotic effects occurs at lower concentration which is in agreement with previous study showing that antioxidant agents at low concentration can inhibit or delay oxidative damage [58]. Zinc itself at higher concentrations (100–200 μM) induces apoptosis which has been reported previously in different cells and tissues [15,59]. The role of oxidative stress and antioxidants in male fertility has been previously discussed. Accordingly, the type, dose and duration of antioxidant therapy differ substantially and should probably be adjusted to the level of oxidative stress [3]. The efficacy of Zinc supply seems to be strictly related to the dose and length of the treatment. After Zinc physiological supplementation, recovery has been observed in elderly, in cancer, in infections as well as in patients with cell disease, since it decreased oxidative stress [38,60–61]. However, long treatment or high doses of Zinc may provoke a Zinc accumulation with subsequent damage, causing toxic effects [62]. Therefore the cellular level of Zinc must be controlled within a suitable range [62,63]. Nevertheless, in the case of oxidative stress, doses of antioxidants should be higher than usual daily dose. Determination of the most preferred active doses of antioxidants like Zinc requires further research.

Despite of toxic effects of ethanol and protective effects of Zinc on Sertoli cells reported in the current study, it is not possible to define with any certainty that protective effects of Zinc is generalized or is a cell type-specific phenomenon. Investigating the protective effects of Zinc on cultured primary Sertoli cells and non-Sertoli cells will be informative to show whether Zinc is specifically protects Sertoli cells, or will any cultured cell line behave similarly under these conditions.

In summary, decreased cell viability and enhanced apoptosis through over-expression of caspase-3 mRNA occurred in Sertoli cells exposed to ethanol. Pretreatment of Zinc prior to ethanol exposure attenuated ethanol-induced Sertoli cell toxicity and apoptosis through inhibition of caspase-3 mediated pathways. Since Sertoli cells are the key supportive cells for spermatogenesis and they are very receptive to modification, a better understanding of the role of Sertoli cells in ethanol-induced testicular injury would make it feasible to identify novel mechanisms of male infertility in alcoholism and lead to more effective therapeutic approaches.

Conflict of interest

The authors have no conflicts of interest to declare regarding the study described in this article and the preparation of the article.

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