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Short Communication

Ethanol promotes apoptosis in rat ovarian granulosa cells via the Bcl-2 family dependent intrinsic apoptotic pathway

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Abstract: Excessive and indulgent alcohol consumption causes tremendous public health issues worldwide. Not only is ethanol associated with a broad spectrum of critical chronic diseases, ethanol is also demonstrated to exert striking reproductive toxicity for both males and females. Epidemiological investigations suggested that ethanol is closely related to fertility decrease in women. Animal studies showed that ethanol intake obstructed ovulation and reduced ovarian weight during gestation. However, cellular mechanism for this inhibitory effect of ethanol on female fertility is yet to be explored. This study recruited rat ovarian granulosa cells, the primary effector of ovary, to investigate the effects of ethanol on cell apoptosis and explore potential mechanism. Ovarian granulosa cells treated with ethanol for three hours manifested observable reduction in cell viability and apparent apoptosis. In the presence of 200 and 300 mmol/l of ethanol, the percentages of apoptotic cells increased to 33% and 36%, respectively. In addition, apoptosis related caspase-3 activity was elevated with increasing concentrations of ethanol, suggesting a dose dependent effect. Furthermore, high concentrations of ethanol significantly disturbed the transcriptional and translational regulation of anti-apoptotic Bcl-2 and pro-apoptotic Bax, which are the two key members of Bcl-2 family tightly involved in intrinsic apoptotic pathway. These results indicated that ethanol promotes apoptosis in rat ovarian granulosa cells, possibly via the intrinsic apoptotic pathway.

Key words: Ethanol; Alcohol; Apoptosis; Ovarian granulosa cell; Female reproductive toxicity.

Introduction

Alcohol consumption is becoming an increasingly popular choice of lifestyle in the modern times. Rapidly developing alcohol industries provide a wide variety of alcoholic beverages accessible to the public. However, serious concerns on alcohol consumption are raised due to the high incidences of over-dosages and rising frequencies of drinking at both social and domestic occasions. In addition, alcohol related occupational risks are prone to rise among laborers with massive and chronic contact of ethanol. It is generally believed that excessive alcohol consumption can cause severe public health issues worldwide (1). Numerous studies reported that alcohol consumption was related to various critical diseases including hypertension, cardiovascular diseases, hepatic diseases, diabetes mellitus, Alzheimer's disease and cancer (2-5). Especially, the effects of ethanol on male reproductive system were intensively investigated in rat, monkey and human (6-8). Ethanol was shown to distinctly disrupt the hypothalamic-pituitary-gonadal taxis, cause significant spermatozoon morphological changes, influence sperm motility, decrease seminal fluid volume and sperm concentration, induce spermatogenic arrest and exert genotoxicity in the male offspring (9-11).

Furthermore, ethanol was also reported to possess pronounced toxicity on female reproductive system. A variety of evidences supported that alcohol consumption was able to cause substantial damages to the fetus. It was documented that maternal alcohol consumption during pregnancy resulted in low birth weight, preterm birth, as well as fetal alcohol spectrum disorders characterized by craniofacial abnormalities, growth defects and central nervous system damage (12,13). Thus, in some countries including the United States and China, the authorities highly recommend abstinence for pregnant women. Apart from birth defects, epidemiological studies also suggested that alcohol consumption was strongly associated with decreased female fertility (14,15). In order to further unravel the details behind the alcohol related fertility issues, preliminary animal studies were performed, demonstrating inhibited ovulation and reduced ovarian weight under the stimulation of ethanol (16,17). However, the cellular mechanisms of ethanol on female fertility remain to be fully elucidated.

Female fertility is closely associated with follicular function, which largely depends on the conditions of ovarian granulosa cells. Being the primary constituent of follicles, granulosa cells afford fundamental endocrinal functions by secreting growth factors, cytokines, steroid hormones, estradiol and progesterone (18,19). In addition, ovarian granulosa cells play crucial regulating roles in the development of female reproductive process, including oocyte maturation and follicles ovulation (19,20). As one of the most important functions of the ovary, ovulation is the primary prerequisite of fertilization. Under normal circumstances, only a few

follicles in the ovary undergo maturation and ovulation, while most follicles not selected to ovulate go through the process of atresia. Follicular atresia is an integral part of the regular ovary function, and about 99% of follicular atresia is primarily triggered by apoptosis of granulosa cells (21). When apoptosis is induced in ovarian granulosa cells by xneobiotics, over-atresia would occur in follicles subsequently, leading to abnormalities in ovary function, and thereby influencing female fertility. Accordingly, exploring the impact of ethanol on ovarian granulosa cells may bring insight into the details of alcohol-induced female fertility. Given that alcohol intake is able to inhibit ovulation, we propose a hypothesis that ethanol may promote apoptosis in ovarian granulosa cells, and consequently undermine ovulation and female fertility.

Thus, the present study was conducted to validate the hypothesis that ethanol may induce apoptosis in ovarian granulosa cells, and if so, to explore the corresponding mechanism of action through following experiments "cell viability analysis, apoptosis evaluation, caspase-3 activity assays, transcriptional and translational investigations of anti-apoptotic Bcl-2 and pro-apoptotic Bax" in rat ovarian granulosa cells exposed to varying concentrations of ethanol.

Materials and Methods

Chemicals and reagents

Pregnant mare's serum gonadotropin (PMSG) was obtained from Second Hormone Factory (Ningbo, China). Dulbecco's modified Eagle's medium (DMEM/F12) and trypsin solution (0.25%) were brought from Hyclone (Logan, UT). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY). Rabbit polyclonal anti-follicle-stimulating hormone receptor antibody and Streptavidin-Biotin complex immunohistochemical staining kit were brought from Boster (Wuhan, China). Cell counting kit-8 (CCK-8) and annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit were obtained from Dojindo Molecular Technologies (Kumamoto, Japan). Caspase-3 activity assay kit and ECL western blotting substrate kit were brought from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit monoclonal anti-Bax (No. ab32503) and anti-Bcl-2 antibody (No. ab32124), and horseradish peroxidase goat anti-rabbit IgG (No. ab6721) were from Abcam China (Shanghai, China). TGX Stain-Free FastCast Acrylamide Kit was from Bio-Rad (Hercules, CA).

Animals and cells

The animal experiment ethics committee of Sichuan University approved this research protocol. Immature female Sprague-Dawley (SD) rats (21-25 days old) were obtained from Dashuo Experimental Animal Co. Ltd. (Chengdu, China). Animals were housed in a controlled environment with a 12/12-h light/dark cycle and were fed with standard rat chow *ad libitum*. Animals were injected subcutaneously with 40 IU of PMSG to stimulate the growth of ovarian granulosa cells. The rats were then sacrificed by cervical dislocation after stimulated for 48 hours. Bilateral ovaries were collected aseptically and the adipose tissues and oviducts were removed. Then the ovaries were punctured with a sterile 26-gauge needle to release cells from follicles into the DMEM/F12 growth medium supplemented with 10% (v/v) FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. In order to obtain granulosa cells, the cell suspensions were filtered through 40-μm nylon meshes allowing granulosa cells but not oocytes to pass through. The acquired granulosa cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 for 72 hours. Immunocytochemistry was used to determine the purity of granulosa cells as follicle-stimulating hormone receptor (FSHR) was only expressed in granulosa cells in the ovary.

Cell viability analysis

Cell viability was determined using CCK-8 assay kit. Briefly, rat ovarian granulosa cells were plated in 96-well plates (2×10^4 cells/well) and treated with ethanol at varying concentrations of 10, 25, 50, 75, 100, 125, 150, 175, 200 and 300 mmol/l for one or three hours. The ethanol concentrations were physiologically relevant based on previous studies (22-25). CCK-8 was added and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 1.5 hours. The absorbance at 450 nm was recorded by Multiskan spectrum microplate spectrophotometer (Thermo Fisher, Waltham, MA). Controls were used for each experiment, and survival was considered 100%.

Apoptosis evaluation

Apoptosis was determined using annexin V-FITC/PI apoptosis detection kit. After treatment with ethanol at different concentrations of 50, 100, 150, 200 and 300 mmol/l for three hours, rat ovarian granulosa cells were harvested and then washed twice with phosphate-buffered saline (PBS) by centrifugation at 200 *g* for three minutes and resuspended in annexin V binding solution to a final concentration of 1×10^6 cells/ml. Afterwards, 5 μL of annexin V-FITC and 5 μL of PI were sequentially added. Cell suspensions were incubated in the dark at room temperature for 15 minutes. Eventually, additional 400 μL of annexin V binding solution was supplemented, and the proportions of apoptotic cells were analyzed by FACSVerse flow cytometer (BD, Franklin Lakes, NJ).

Caspse-3 activity assessment

Caspase-3 activity was determined by enzymatic assay kit. In brief, after treatment with ethanol at concentrations of 50, 100, 150, 200 and 300 mmol/l for three hours, harvested rat ovarian granulosa cells were washed twice with PBS by centrifugation at 4 °C and 600 *g* for five minutes. Then the lysis solution was added to cells on ice for 15 min and the supernatant was collected by centrifugation at 4 °C and 2000g for 15 min. The optical density (OD) of each reaction mixture was measured spectrophotometrically at 405 nm. Caspase-3 activity was calculated based on the standard curve and normalized to respective protein concentration determined by Bradford protein assay.

RT-PCR analysis

After exposure to ethanol at concentrations of 50, 100, 150, 200 and 300 mmol/l for three hours, the me-

dium was removed and total cellular RNA was extracted from rat ovarian granulosa cells using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was quantified by spectrometry at 260/280nm using NanoDrop 2000 (Thermo Fisher, Waltham, MA). The extracted RNA was subjected to reverse transcription using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Primer pairs were selected as previously reported (26). 18S was utilized as an internal control. The RT-PCR reactions were run in the CFX96 System (Bio-Rad, Hercules, CA). The relative expression of each mRNA was calculated and quantified by the $2^{-\Delta\Delta Ct}$ methods. The Ct value of 18S mRNA control did not change significantly under the treatment in this study.

Western blot investigation

After treatment with ethanol at concentrations of 50, 100, 150, 200 and 300 mmol/l for three hours, the total protein from the cells were extracted using the RIPA lysis buffer (containing 1 mmol/l of PMSF). Protein concentrations were measured using the bicinchoninic acid (BCA) protein assays. The proteins were separated by TGX Stain-Free gels (12%) (Bio-Rad) and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% (w/v) dried fat-free milk in TBS containing 0.1% tween-20 (TBST) at room temperature for one hour. Then the membranes were incubated with rabbit monoclonal anti-Bcl-2 or anti-Bax antibody overnight at 4 °C, followed by incubation with horseradish peroxidase goat anti-rabbit IgG. The protein bands were visualized using ECL substrate kit. Optical intensities of immunoreactive protein bands were analyzed using the Image Lab 5.1 software and normalized to total protein content quantified with the stain-free technology (Bio-Rad).

Statistical analysis

All experiments were repeated three times at least. All data were presented as mean ± standard deviation (SD). Differences among groups were tested using either one-way ANOVA (for homogeneous variances) or Kruskal-Wallis test (for heterogeneous variances). Statistical analyses between two groups were conducted using Dunnet t test (for homogeneous variances) or Games-Howell test (for heterogeneous variances). P value<0.05 was considered statistically significant. All statistical analyses were performed using SPSS 20.0 software.

Results

Primary culture of rat ovarian granulosa cells

Under optical observations, obtained cells from the primary culture were anchorage dependent and formed a monolayer. In order to identify the purity of obtained rat ovarian cells, immunocytochemistry was adopted for follicle-stimulating hormone receptor (FSHR) that is specifically expressed in ovarian granulosa cells. Cells with dark brown cytoplasm and dark blue nucleus were considered FSHR-positive ovarian granulosa cells. The results demonstrated that the obtained cells were predominantly FSHR-positive, suggesting that the purity of rat ovarian granulosa cells was high and could be used in the following experiments (Figure 1).

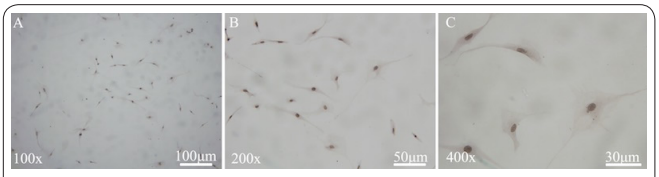

Figure 1. Primary culture of rat ovarian granulosa cells. Immunocytochemistry was adopted for follicle-stimulating hormone receptor (FSHR) that is specifically expressed in ovarian granulosa cells. Cells with dark brown cytoplasm and dark blue nucleus were considered FSHR-positive ovarian granulosa cells. Magnifying resolutions of 100× (A), 200× (B) and 400× (C) were applied. Optical observations demonstrated the purity of rat ovarian granulosa cells was high.

Effect of ethanol on the viability of rat ovarian granulosa cells

In order to investigate the effect of ethanol on the viability of rat ovarian granulosa cells, two sets of independent experiments were performed when cells were treated with increasing concentrations of ethanol for one or three hours. The duration and concentrations of ethanol treatment were reported to be physiologically relevant based on the previous studies (22-25). In addition, the concentrations of ethanol used in these studies were physiologically and clinically relevant to blood levels of binge alcohol drinkers (27). Cell viability was determined independently for each sample using CCK-8 assay. The survival was considered 100% for the control. When the cells were treated for one hour, no significant differences were observed with varying concentrations of ethanol (data not shown). When the cells were stimulated for three hours, the viability was notably reduced with increasing concentrations of ethanol (Figure 2). Compared to the control, cell viability was significantly decreased 20%, 21%, 25%, 34% and 45% when the concentrations of ethanol were at 125

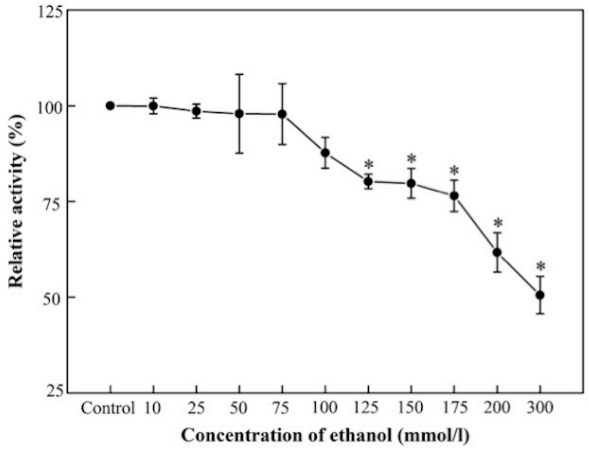

Figure 2. Ethanol decreases the viability of rat ovarian granulosa cells. Rat ovarian granulosa cells were treated with ethanol at varying concentrations of 10, 25, 50, 75, 100, 125, 150, 175, 200 and 300 mmol/l for three hours. Following incubation with CCK-8, absorbances at 450 nm for each sample were recorded. The relative activity for each sample was calculated based on the control. Distinct decreases in cell viability were noted with increasing concentrations of ethanol and significant reductions were observed when ethanol concentrations were above 125 mmol/l. Data is presented as mean ± SD. *Significantly different from control (P<0.05).

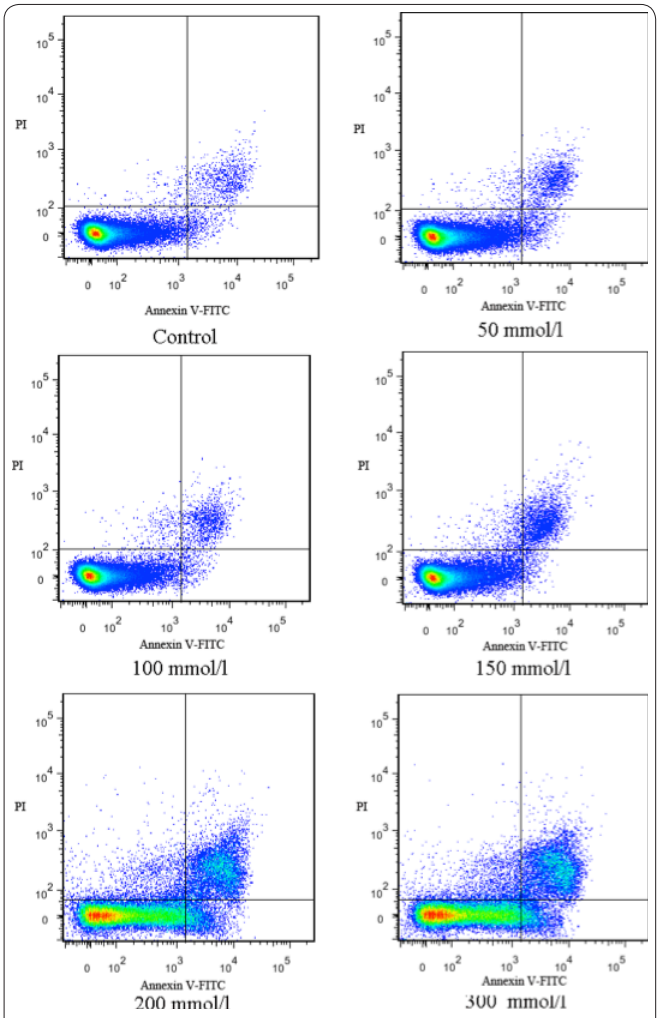

Figure 3. Ethanol promotes apoptosis of rat ovarian granulosa cells. Rat ovarian granulosa cells were treated with ethanol at varying concentrations of 50, 100, 125 200 and 300 mmol/l for three hours followed by annexin V-FITC/PI staining and flow cytometric analysis. Apparent apoptosis were observed when ethanol concentrations were 200 and 300 mmol/l.

mmol/l, 150 mmol/l, 175 mmol/l, 200 mmol/l and 300 mmol/l, respectively. These results suggested that relatively high concentrations of ethanol was able to inhibit the viability of rat ovarian granulosa cells in a concentration dependent manner, and the effect was apparent under the stimulation beyond three hours. Therefore, rat ovarian granulosa cells were treated with ethanol for three hours in the following investigations.

Effect of ethanol on apoptosis of rat ovarian granulosa cells

In order to evaluate the effect of ethanol on the apoptosis of rat ovarian granulosa cells, annexin V-FITC/PI double staining was used to label the cells treated with 50, 100, 150, 200 and 300 mmol/l of ethanol for three hours. The proportion of apoptotic cells was then analyzed by flow cytometry (Figure 3). Normal cells free from the annexin V-FITC or PI stains were represented in the lower left region of each flow cytometric graph. Early apoptotic cells that were solely reacted with annexin V-FITC were shown in the lower right region of each flow cytometric graph. Late apoptotic and necrotic cells were labeled by both annexin V-FITC and PI staining, and was located in the upper right region of each flow cytometric graph. The results from flow cytometry showed that ethanol had little apoptotic effect on rat ovarian granulosa cells at the concentrations of 50 mmol/l, 100 mmol/l and 150 mmol/l. When ethanol concentrations were at 200 mmol/l and 300 mmol/l, apparent early, late and total apoptosis were noted (Table 1). In the presence of 200 mmol/l of ethanol, 33% of all the cells went through apoptosis. Under the stimulation of 300 mmol/l of ethanol, the percentage of apoptotic cells increased to 36%. These results demonstrated that relatively high concentrations of ethanol were able to induce distinct apoptosis of rat ovarian granulosa cells.

Effect of ethanol on caspase-3 enzymatic activity

Caspase-3 is an effector enzyme activated in apoptotic cells via both intrinsic and extrinsic pathways. Thus, the activity of caspase-3 is usually recruited to serve as a molecular marker for apoptosis. In order to determine the effect of ethanol on caspase-3 enzymatic activity, rat ovarian granulosa cells were stimulated with 50, 100, 150, 200 and 300 mmol/l of ethanol for three hours. Caspse-3 activity was assessed using colorimetric assay and normalized to respective protein concentrations (Figure 4). In all ethanol-treated cells, the caspase-3 activity was significantly higher than that in the control. In the presence of 50, 100, 150, 200 and 300 mmol/l of ethanol, the caspase-3 activity increased 1.8-fold, 2.4-fold, 2.5-fold, 2.7-fold and 3-fold, respectively. These results showed that the enzymatic activity of caspase-3 increased with increasing concentrations of ethanol, substantiating the occurrence of apoptosis. Nevertheless, whether the apoptosis was activated through intrinsic or extrinsic pathway required further evaluations.

Effect of ethanol on the transcription of Bcl-2 and Bax

The intrinsic pathway of apoptosis is activated by intracellular signals, and depends on the release of mitochondrial proteins, which are regulated by mammalian Bcl-2 family including Bcl-2 and Bax. To investigate the effect of ethanol on transcription of anti-apoptotic

Table 1. Effects of ethanol on apoptosis of rat ovarian granulosa cells (%).

Concentration	Early apoptosis	Late apoptosis	Total apoptosis
0 mmol/l	0.633±0.013	0.942±0.008	1.575±0.006
50 mmol/l	0.655±0.009	1.240±0.012	1.895±0.010
100 mmol/l	0.768±0.016	1.430±0.007	2.198±0.011
150 mmol/l	1.070±0.013	2.730±0.019	3.800±0.015
200 mmol/l*	18.700±0.561	13.800±0.636	32.500±0.347
300 mmol/l*	20.600±0.685	15.400±0.798	36.000±0.578

* The percentages of early apoptosis, late apoptosis and total apoptosis of rat ovarian granulosa cells in 200 mmol/l and 300 mmol/l groups were all significantly higher than those in the control group (P<0.05). Data are presented as mean ± SD.

Bcl-2 and pro-apoptotic Bax, RT-PCR analysis was performed on cells were treated with 50, 100, 150, 200 and 300 mmol/l of ethanol for three hours. Compared to the control, relatively high concentrations of ethanol significantly reduced Bcl-2 mRNA levels, while increased Bax mRNA levels (Figure 5A and B). In the presence of 150, 200 and 300 mmol/l of ethanol, the Bcl-2 transcription decreased 2-fold, 2.5-fold and 2.9-fold, respectively. In the presence of 150, 200 and 300 mmol/l of ethanol, the Bax transcription increased 3.6-fold, 3.5-fold and 4-fold, respectively. In addition, the Bax/Bcl-2 mRNA ratio rose with the increasing concentrations of ethanol, and was significantly higher in 150, 200 and 300 mmol/l groups than that in the control (Figure 5C).

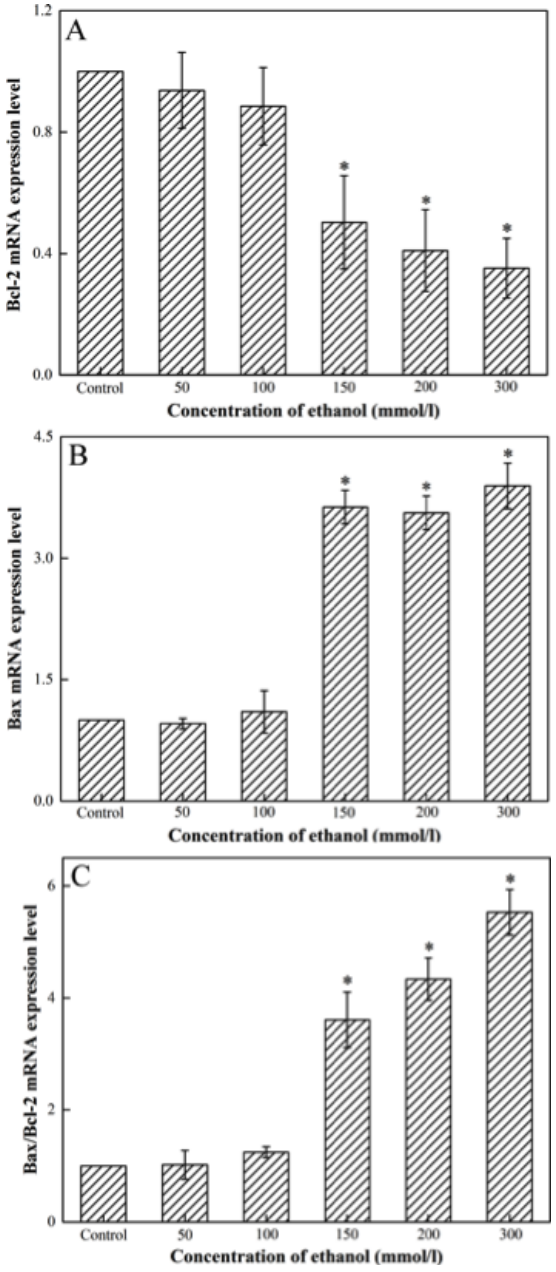

Figure 5. Ethanol suppresses Bcl-2 transcription and stimulates Bax transcription. Rat ovarian granulosa cells were treated with ethanol at varying concentrations of 50, 100, 125 200 and 300 mmol/l for three hours followed by RT-PCR assays. With increasing concentrations of ethanol, Bcl-2 transcription was down regulated (A) and Bax transcription was up regulated (B). Bax/Bcl-2 mRNA ratio increased significantly in the presence of 150, 200 and 300 mmol/l of ethanol (C). Data are presented as mean ± SD. *Significantly different from control (P<0.05).

These results indicated that ethanol was able to induce the down-regulation of anti-apoptotic gene Bcl-2 and the up-regulation of pro-apoptotic gene Bax.

Effect of ethanol on the translation of Bcl-2 and Bax

In order to determine whether the effects of ethanol on Bcl-2 and Bax were also prominent at translation level, western blot analysis was conducted to examine the protein expression levels when rat ovarian granulosa cells were incubated with 50, 100, 150, 200 and 300 mmol/l of ethanol for three hours. The expression levels of Bax and Bcl-2 were quantified using total protein normalization as described previously (28,29). The expression levels of Bcl-2 protein were significantly lower, while the expression levels of Bax protein were higher in the presence of ethanol at concentrations of 150, 200 and 300 mmol/l (Figure 6). Moreover, the Bax/Bcl-2 protein ratio showed a rising trend as the concentration of ethanol increased, illustrating a dose-dependent effect. These results suggested that ethanol induced imbalanced expression of Bax and Bcl-2, as well as apoptosis in rat ovarian granulosa cells were possibly associated with Bcl-2 family dependent intrinsic pathway.

Discussion

Alcohol consumption has been reported to display considerable adverse consequences in female reproductive system, such as decreased fertility. Ovary, as the major female reproductive organ, is considered the target of numerous xneobiotics with female reproductive toxicity (30). Therefore, ovarian granulosa cells are likely to be the target cells of xneobiotics due to the vital roles in regulating ovarian functions. In order to

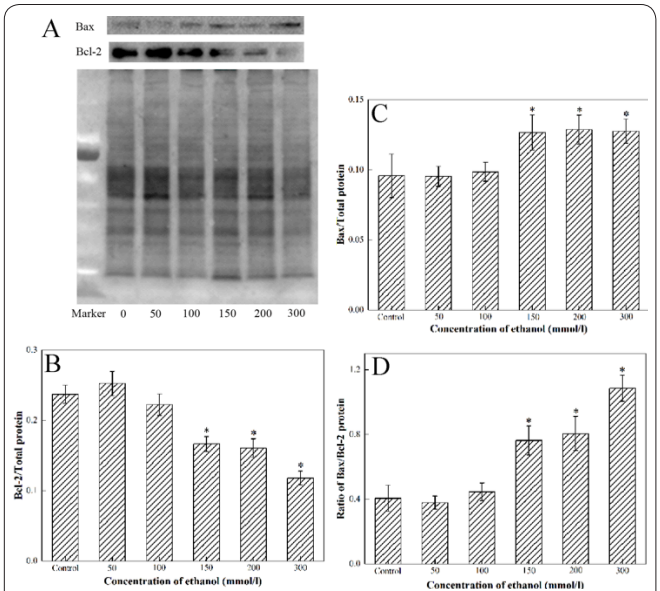

Figure 6. Ethanol reduces Bcl-2 translation and increases Bax translation. Rat ovarian granulosa cells were treated with ethanol at varying concentrations of 50, 100, 125 200 and 300 mmol/l for three hours followed by western blot analysis. Western blot images were shown (A). With increasing concentrations of ethanol, Bcl-2 translation was down regulated (B) and Bax translation was up regulated (C). Bax/Bcl-2 protein ratio was elevated significantly in the presence of 150, 200 and 300 mmol/l of ethanol (D). Data are presented as mean ± SD. *Significantly different from control (P<0.05).

thoroughly comprehend the effect of ethanol on female fertility, the *in vitro* ability of ethanol to induce apoptosis was investigated using rat ovarian granulosa cells.

The development and proliferation of ovarian granulosa cells are essential for ovarian function. Thus, the effect of ethanol on the viability of rat ovarian granulosa cells was first determined. The results in this study showed that viability was not significantly altered when rat ovarian granulosa cells were treated with varying concentrations of ethanol for one hour, possibly due to relatively short exposure duration. After treatment with ethanol for three hours, cell viability significantly decreased in concentrations higher than 100 mmol/l compared to the control, suggesting that ethanol exerted female reproductive toxicity via suppressing the viability of ovarian granulosa cells in a concentration dependent manner. Although the ethanol concentrations over 100 mmol/l might seem to be relatively high for conventional alcohol consumers, these high concentrations were indeed reported to be physiologically relevant under certain circumstances based on the previous studies (22-25).

Whether a follicle will be selected for ovulation or undergo atresia depends on both the survival and death of ovarian granulosa cells (31-33). The apoptotic programmed death of ovarian granulosa cells underlies the initiation and progression of follicular atresia (34). When exposed to xneobiotics, increased apoptosis of ovarian granulosa cells can result in follicular over-atresia and subsequently ovulation failure, which is a possible mechanism for the impairment of reproduction (35). Apoptosis of ovarian granulosa cells is a genetically controlled mechanism that plays a vital role in homeostasis and maintenance of ovaries (31-33,36). Previous studies demonstrated that both Bcl-2 family proteins and caspases, especially caspase-3 were involved in the apoptotic process of ovarian granulosa cells (37). Hence, in this study, the induction of apoptosis by ethanol in rat ovarian granulosa cells was examined through flow cytometric analysis, evaluation on caspase-3 enzymatic activity, and investigations of Bcl-2 and Bax expression at both transcriptional and translational level.

The results from flow cytometry demonstrated that significant portions of rat ovarian granulosa cells underwent apoptosis in the presence of 200 and 300 mmol/l of ethanol, suggesting that high concentrations of ethanol were able to induce programed cell death. While perturbations in cell viability were observed when ethanol concentrations were above 100 mmol/l, it is speculated that the effect of ethanol on rat ovarian granulosa cells was firstly manifested as inhibition of cell proliferation, then followed by induction of apoptosis with the accumulation of ethanol. The occurrence of apoptosis in high concentration of ethanol was also validated with elevated activity of caspase-3. Interestingly, in the presence of 50, 100 and 150 mmol/l of ethanol, little portions of cells were observed to undergo apoptosis, while the activity of casepase-3 increased 1.8-fold, 2.4-fold and 2.5-fold. One possible explanation could be that caspase-3 in this case was turned on at the early prophase stage before the annexin V was expressed and the nucleus was accessible. Nevertheless, the results from this study did demonstrate that ethanol significantly increased the activity of caspase-3 in a concentration dependent manner. Caspase-3 belongs to a cysteine proteases family involved in both the initial and final stages of apoptosis. Casapse-3 is believed to be the final executor responsible for cleaving targeted substrates and apoptosis (32,38). Together with the results from flow cytometric analysis, it was evident that ethanol promoted apoptosis in rat ovarian granulosa cells in a dose dependent fashion.

Two classical activation pathways are involved in the initiation and regulation of apoptosis. The extrinsic pathway is activated by extracellular signals including tumor necrosis factor and Fas ligand. Then, the binding of extrinsic ligands to the receptors on the cell membrane leads to the formation of the death-inducing signaling complex (DISC), followed by the activation of caspases and apoptosis. The intrinsic apoptotic pathway is activated by stress related intracellular signals including mitochondria intermembrane protein cytochrome c, which initiates cascades of signal transduction and eventually caspase-3 activation. Cytochrome c is released from mitochondria through the mitochondrial apoptosis-induced channel (MAC), which is tightly regulated by mammalian Bcl-2 family. Therefore, the expression levels of Bcl-2 family are frequently utilized to indicate an intrinsic apoptotic pathway. The dominant pair of Bcl-2 family is the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax. Since ethanol is speculated to be a stress related signal, investigations were conduced to determine whether ethanol-induced apoptosis was associated with intrinsic pathway. Transcriptional and translational studies showed that Bcl-2 was significantly down regulated and Bax was significantly up regulated when ethanol was at concentrations of 150, 200 and 300 mmol/l, suggesting that Bcl-2 and Bax were involved in ethanol-induced apoptosis in rat ovarian granulosa cells. Since Bax forms heterodimers with Bcl-2 to suppress the anti-apoptotic function, the balance between Bcl-2 and Bax levels presumably determines the cell fate (19,39-40). When Bcl-2 exceeds Bax in the expression level, the cells tend to survive. Thus, the ratio of Bax versus Bcl-2 for both mRNA and protein levels were also assessed in the study. The results showed that ratios of Bax/Bcl-2 expression exhibited an elevating tendency as ethanol concentrations increased and were significantly higher when the cells were stimulated with 150, 200 and 300 mmol/l of ethanol. These results were consistent with previous observed reduction in cell viability and induction of apoptosis, indicating that ethanol acted on rat ovarian granulosa cells possibly through the Bcl-2 family dependent intrinsic apoptotic pathway. It is necessary to mention that stain-free gel was used in this study. Stain-free gel is a recently developed technology on detecting total protein as internal control for Western blot assays (41-43) and has been widely used to normalize the target proteins (44-46). The stain-free gel has been evidenced to show better stability and less viability of protein compared with β-actin, β-tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (47). Moreover, stain-free gel demonstrated superior performance in that they were faster, required fewer steps and consumables, and provided checkpoints throughout the Western blotting process by allowing rapid visualization of gel separation and protein transfer especially before committing

to costly and time-consuming Western blots (41,48). Accordingly, stain-free gel was recruited in this study.

The effect of ethanol on ovarian function and fertility has been widely discussed. Earlier studies reported that ethanol exposure could lower ovarian weight, suppress follicular development, and reduce levels of serum estradiol in female rats (49,50). The adverse actions of ethanol on ovarian steroidogenesis were further investigated (51). Furthermore, chronic alcohol intake was observed to cause estral cyclicity irregularities and impairments of ovarian structural components (52). Ethanol was also found to decrease oocyte quality in mice (53). However, there were few studies concerning the induction of apoptosis by ethanol. An *in vivo* study by reported that the mRNA expression level of Bax was significantly higher than Bcl-2 in mice treated with alcohol, which is consistent with our *in vitro* study (54). Except for the mRNA levels, the proteins levels of Bcl-2 and Bax were also analyzed in this study. In addition, the underlying mechanism was explained to elucidate the apoptotic induction by ethanol in granulosa cells.

In conclusion, the present study demonstrated that high concentrations of ethanol significantly inhibited cell viability and promoted apoptosis in rat ovarian granulosa cells with elevated caspase-3 activity and increased Bax/Bcl-2 ratios at both transcriptional and translational levels, likely to be a contributing factor to alcohol-induced female fertility decrease. Further comprehensive investigations are necessary to discern whether ethanol also initiates the formation of the death-inducing signaling complex and participates in the extrinsic apoptotic pathway.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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