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Effects of Ethanol on Embryonic and Neonatal Rat Testes in Organ Cultures

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ABSTRACT: Ethanol exposure in adult animals and humans has shown to elicit significant inhibitory effects on the function of male reproduction, but consequences of ethanol exposure on the embryonic and early postnatal testis development are not known. The current study investigated the effect of ethanol on embryonic and neonatal testis development using an organ culture technique. In embryonic day 13 (E13) testis organ cultures, ethanol had no effect on the testicular cord formation, the expression of Müllerian-inhibiting substance (MIS) in Sertoli cells or the number of gonocytes. Similarly, in the ethanol-treated embryonic day 18 (E18) testes, both the number of gonocytes and the expression of GATA-4 and MIS were similar to those from the control testes. In contrast, in postnatal day 3 (P3) testes, ethanol at concentrations of 150 and 200 mM significantly decreased the number of gonocytes without affecting the expression of GATA-4 and MIS in Sertoli cells. This effect was shown to be resulting from the enhanced apoptosis of gonocytes. In addition, ethanol abnormally activated retinoic acid receptor alpha (RARα), as indicated by increased nuclear localization of RARα with increasing doses of ethanol treatment. These observations suggest that the effect of ethanol on testis varies at different stages during embryonic and neonatal testis development. Furthermore, germ cells may be the main target for the action of ethanol on the early postnatal testis.

Key words: Gonocytes, Sertoli cells, testis, seminiferous cords, apoptosis.

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Testicular development involves a complex combination of cell differentiation, migration, proliferation, and apoptosis, which occurs in a strict temporal order and anatomical pattern (Capel, 2000). Male and female rat gonads at embryonic day 13 (E13) are morphologically identical and therefore called bipotential or indifferent gonads. These indifferent gonads arise from the urogenital ridge, a region adjacent to the mesonephros that ultimately contributes cell lineages to the adrenal cortex, gonads, and kidney. In the rat, the first morphological change in male gonad is the formation of testicular cords that occurs from E13.5 to E14.5, whereby germ cells are surrounded by Sertoli cells, which are in turn surrounded by peritubular myoid cells (Magre and Jost, 1991). After testicular cord formation, the testis becomes twice the size of ovary. Sertoli cells continue proliferating until 3 weeks after birth (Mittwoch et al, 1969). Gonocytes, on the other hand, proliferate until E17.5, after which they are mitotically quiescent. Then on postnatal day 3 (P3), some of the gonocytes resume mitosis and migrate from the central position in the seminiferous tubules towards the basement membrane (Orth, 1982; McGuinness and Orth, 1992). The resumption of mitosis and migration of gonocytes are required for further development of the gonocytes and their progeny, spermatogonia. Thus, the normal development of gonocytes in the first 3–5 postnatal days is critical to proper spermatogenesis in adults (Orth et al, 1988; de Rooij, 1998).

Numerous studies have indicated that ethanol exposure has profound inhibitory effects on adult testis function in animals and humans. For example, chronic ethanol abuse in males has resulted in decreased testosterone production, reduced sperm output, and testis atrophy (Van Thiel et al, 1980; Adler, 1992; Villalta et al, 1997). Histological examinations of the testes of chronic alcoholics have revealed decreased diameter of the seminiferous tubules, resulting primarily from the loss of germ cells (Van Thiel et al, 1975). In animals, there is evidence that ethanol exposure of adults can increase germ cell apoptosis (Zhu et al, 2000) and cause an adverse effect on the secretory function of Sertoli cells (Zhu et al, 1997).

However, the effect of ethanol on the development of fetal and neonatal testes is not known. In this study, we employed organ culture technique (Cupp et al, 1999; Livera et al, 2000) to investigate the effect of ethanol on the testicular cord formation and the development of germ and Sertoli cells in the embryonic and neonatal rat testes. Testes at three developmental stages were used: embryonic day 13 (E13), when testicular cord formation and

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Sertoli cell differentiation begin; embryonic day 18 (E18), when the gonocytes begin a mitotically quiescent stage; and postnatal day 3 (P3), when gonocytes resume mitosis (Kluin and de Rooij, 1981; McGuinness and Orth, 1992).

To assess the effect of ethanol on Sertoli cells, we used Müllerian-inhibiting substance (MIS) and GATA-4, which are widely used as functional markers for Sertoli cell differentiation (Raymond et al, 2000; Colvin et al, 2001). MIS, a member of the transforming growth factor beta (TGFβ) family, is expressed specifically in the Sertoli cells of the fetal testis, in the mouse beginning at E11.5 and E12.5 until shortly after birth (Munsterberg and Lovell-Badge, 1991). Similarly, GATA-4, a member of the GATA family of zinc finger proteins, has been shown to be expressed normally in the nuclei of Sertoli cells at a high level during embryonic and early postnatal development (Viger et al, 1998; Ketola et al, 1999). Moreover, GATA-4 has recently been characterized to be involved in early gonadal development and sex differentiation in mice (Tevosian et al, 2002).

Materials and Methods

Animals

Pregnant female Sprague-Dawley rats were obtained from Charles River Laboratories (Hollister, CA). Plug date was considered to be embryonic day 0 (E0). Animal experimentation was conducted in accordance with the highest standards of humane animal care as outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Organ Cultures

E13 gonads were dissected out with the mesonephroi and cultured as previously described (Cupp et al, 1999). An indifferent gonad and the neighboring mesonephros were cultured in a drop (2 μL) of medium on a Millicell CM filter (Millipore, Bedford, MA) floating on the surface of CMRL 1066 medium (Life Technologies, Rockville, MD), supplemented with penicillin-streptomycin, insulin (10 μg/mL), and transferrin (10 μg/mL). One gonad with its corresponding mesonephros was cultured in medium containing ethanol, and the other gonad with its corresponding mesonephros from the same fetus were cultured in medium without ethanol as a control. The ethanol concentrations of 50–200 mM were chosen for study because 50–100 mM are usually observed after an excessive intake of alcohol in normal individuals and the concentration of 150 mM is noted after an acute ethanol intake in chronic alcoholics (Szabo et al, 1994). To determine the sex of E13 embryos, polymerase chain reaction analysis for sex-determining region, Y chromosome (Sry) was conducted on genomic DNA isolated from E13 embryos (Cupp et al, 1999). Using 5′-CGGGATCCATGTCAAGCGCCCCATGAATGCATTTATG-3′ and 5′-GCGGAATTCACTTTAGCCCTCCGATGAGGCTGATAT-3′ primers, we obtained a 234-bp fragment if the embryo is a male. Images of organ cultures of E13 testes were obtained using a digital imaging system (Optronics, Goleta, CA).

For E18 and postnatal day 3 (P3) organ cultures, testes were cut into small pieces (halves for E18 and 8 pieces for P3 testes). All the pieces from the same testis were cultured on a Millicell filter floating on 1 mL of culture medium and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 3 days. The culture medium was changed every 24 hours. At the end of culture, the tissues were fixed for 1 hour at room temperature in Bouin solution, embedded in paraffin, and cut into 5-μm sections.

Gonocyte Counting

Changes in the number of gonocytes were analyzed by counting the gonocyte nuclei in sections stained with hematoxylin-eosin. Gonocytes were easily identified by their relatively large, round, and lightly stained nuclei containing 1 to several globular nucleoli (Clermont and Perey, 1957). Counts were made using an ocular grid (area, 56406 μm^2) with a ×20 objective (final magnification, ×200). Gonocytes in 3 grids for E13 testes, 10 fields for E18 testes, and 20 fields for P3 testes were counted from each of the 3 sections from every treatment group per experiment. Sertoli cells in the same areas were also counted to normalize the number of gonocytes against the number of Sertoli cells. Sections for analysis were collected at 20-μm intervals to avoid counting the same cell twice.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Akmal et al, 1996). The tissue sections were deparaffinized, rehydrated, microwaved for 16 min in 10 mM sodium citrate to unmask antigenic sites, treated with 0.3% H_2O_2 to quench endogenous peroxidases, and incubated in 10% rabbit normal serum for 10 minutes at room temperature to block nonspecific binding. For detection of MIS, GATA-4, and retinoic acid receptor alpha (RARα), the sections were incubated with goat or rabbit polyclonal antibodies (MIS: 1:300; GATA-4: 1:150; RARα: 1:150; Santa Cruz Biotechnology, Santa Cruz, CA) in a humidified chamber overnight at 4°C. The next day, sections were washed with phosphate-buffered saline 3 times and treated with 1:300 dilution of biotinylated rabbit antigoat secondary antibody (Vector Laboratories, Burlingame, CA), followed by incubation with peroxidase-conjugated streptavidin and substrate-chromagen mixture containing aminoethyl carbazole (AEC) from Zymed Laboratories (South San Francisco, CA). As negative controls, serial sections were incubated with primary antibody preabsorbed with a 50-fold excess of synthetic immunizing peptide (Santa Cruz Biotechnology). Immunohistochemistry was performed on the testis cross sections from at least 3 embryos or P3 rats.

Proliferation Analysis

5-Bromo-2′-deoxyuridine (BrdU) at 1:100 dilution (Zymed Laboratories) was added to the culture medium 3 hours prior to the completion of the culture. BrdU immunohistochemistry on tissue sections was performed following the protocols provided by the supplier (Zymed Laboratories). Briefly, randomly chosen sections was treated with 0.3% H_2O_2 in methanol to quench endogenous peroxidases and immersed in 1N HCl for 30 min to de-

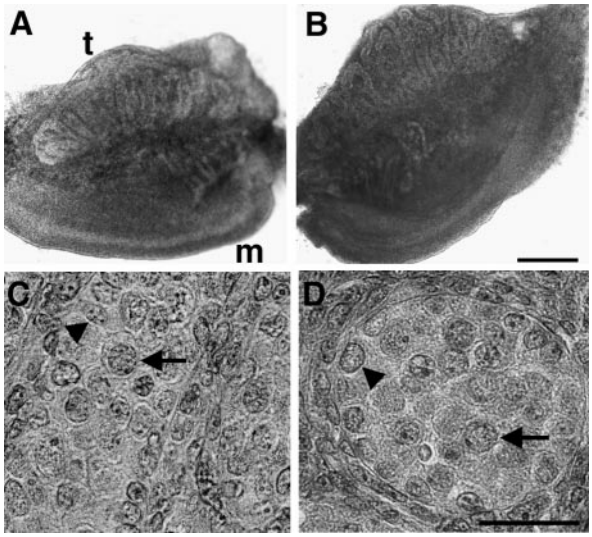

Figure 1. Effect of ethanol on formation of testicular cords in embryonic day 13 (E13) testis organ cultures. E13 gonads plus mesonephros were cultured in the absence **(A, C)** or presence of 200 mM ethanol **(B, D)**. Sections from the E13 testes were stained with hematoxylin and eosin. t indicates testis; m, mesonephros; arrows, gonocytes; arrowheads, Sertoli cells. Bar in B = 500 μm and bar in D = 25 μm.

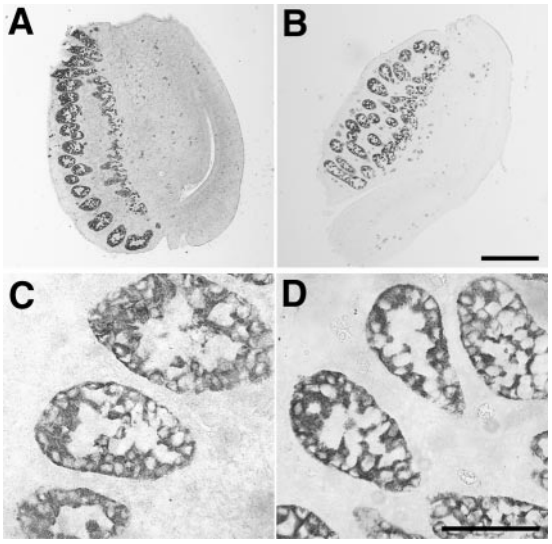

Figure 2. Müllerian-inhibiting substance (MIS) expression identifies Sertoli cells within the seminiferous cords. Embryonic day 13 gonads plus mesonephros were cultured in the absence **(A, C)** or presence of 200 mM ethanol **(B, D)**. Testicular sections were immunostained with an antibody against MIS. Bar in B = 500 μm and bar in D = 50 μm.

nature the genomic DNA. After rinsing, the sections were treated with blocking solution and incubated for 1 hour with biotin-conjugated anti-BrdU monoclonal antibody (Zymed Laboratories). Subsequently, the sections were treated with streptavidin peroxidase complex solution for 10 minutes, and the reaction sites were visualized with AEC. Corresponding hematoxylin-stained sections were used for clear assignment of labeled nuclei to gonocytes and for obtaining the number of unlabeled gonocytes. The BrdU labeling index (% BrdU labeled gonocytes) was calculated from the number of BrdU-labeled gonocytes divided by the total number of gonocytes scored in 20 fields and multiplied by 100.

Assay for Apoptosis

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed as described by the manufacturer of the apoptosis detection system (Promega Biotech Corporation, Madison, WI). This assay detects fragmented DNA in apoptotic cells by catalytic incorporation of fluorescein-12-dUTP at the 3′-OH ends of DNA using the enzyme. The fluorescein-12-dUTP labeled DNA can then be visualized directly under fluorescence microscope. To quantify the relative differences in the number of apoptotic cells between the control and treated testes, seminiferous tubule containing 3 or more TUNEL-positive cells were counted out of 80 tubules from each of the 3 sections from every treatment group per experiment.

Statistical Analysis

All values are means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by pairwise comparison of the means at $P = .05$ (Tukey-Kramer test, Minitab 10 Xtra, Minitab Inc., State College, PA).

Results

Testicular Cord Formation and MIS Expression Are Normal in the Presence of Ethanol

The effects of ethanol on testicular cord formation were assessed using E13 gonad plus mesonephros explant cultures. Both control gonad and gonad treated with variable doses of ethanol (50, 100, 150, and 200 mM) formed testicular cords normally at the end of 3-day culture. Figure 1A and B shows the whole organ images of control gonad and 200 mM ethanol-treated gonad, respectively. We measured the areas of E13 testes using a NIH Image Analysis Program. There is no significant difference between the vehicle- and ethanol-treated testes (data not shown). Further analysis of the sections from those organs, which were stained with hematoxylin and eosin, showed that the testicular cords were well developed and the types of cells present in the testicular cords were normal looking in either control testes or ethanol-treated testes (Figure 1C and D). To determine whether Sertoli cell differentiation was affected in the presence of ethanol, MIS immunohistochemistry was performed (Figure 2). MIS was found at high levels specifically within the Sertoli cells lining the testicular cords. The expression of MIS in the E13 testes treated with ethanol remained similar to the controls.

GATA-4 and MIS Are Expressed Normally in Sertoli Cells in E18 and P3 Testes

To assess the effect of ethanol on Sertoli cells in the testes from E18 fetus and P3 rats, immunohistochemistry of 2

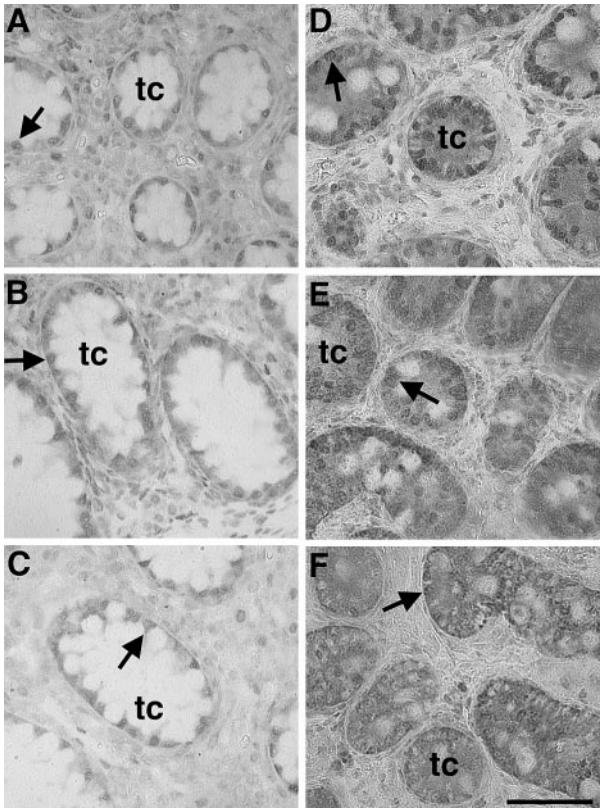

Figure 3. Effect of ethanol on GATA-4 protein expression in Sertoli cells. Embryonic day 18 **(A–C)** or P3 **(D–F)** testes were cultured for 3 days in the absence **(A, D)** or presence of 100 mM **(B, E)** and 200 mM **(C, F)** ethanol. Testicular sections were immunostained with an antibody against GATA-4. tc indicates testicular cord; arrows, Sertoli cells. Bar in F = 50 μm.

Sertoli cell markers, GATA-4 and MIS, was performed. GATA-4 protein was expressed normally in the nuclei of the Sertoli cells lining the seminiferous tubules and in the interstitial cells in both E18 and P3 testes treated with variable doses of ethanol (Figure 3). Similarly, MIS was expressed normally in the Sertoli cells of ethanol-treated E18 and P3 testes (data not shown).

Effect of Ethanol on the Number of Gonocytes

The gonocytes were counted after culturing testes from E13 and E18 embryos and P3 rats for 3 days in the absence or presence of variable concentrations of ethanol (50, 100, 150, or 200 mM). Ethanol did not affect the number of gonocytes in either E13 or E18 testes after 3 days of culture (data not shown). However, in P3 testes, the number of gonocytes was significantly reduced in the presence of 150 mM and 200 mM ethanol, compared with that in the controls (Figure 4). The number of Sertoli cells in the equivalent area did not change with ethanol treatment.

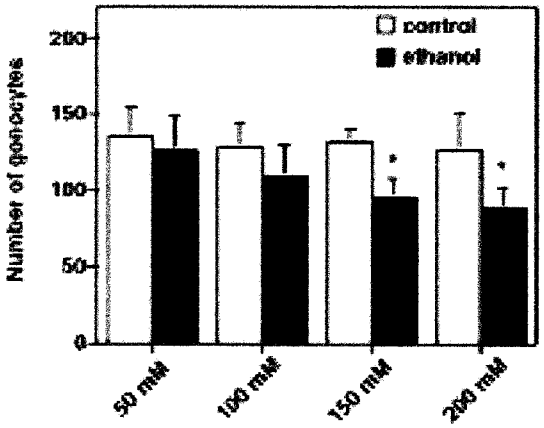

Figure 4. Effect of ethanol on the number of gonocytes in postnatal day 3 testes. Values are means ± SD of 3 independent experiments. Statistically significant differences ($P < .05$) are indicated by *.

Effect of Ethanol on Gonocyte Proliferation and Apoptosis in P3 Testes

To determine whether ethanol decreased the number of gonocytes by decreasing their proliferation in P3 testes, the effect of ethanol at concentrations of 150 and 200 mM, which significantly decreased the number of gonocytes in P3 testes, was evaluated. The proliferation of gonocyte was measured by the percentage of BrdU-labeled gonocytes. Ethanol at concentrations of 150 and 200 mM did not alter the proliferation of gonocytes in P3 testes after 3 days of culture (Figures 5 and 6).

To quantitatively evaluate the difference in gonocyte apoptosis between the control and ethanol-treated P3 testes, the percentages of seminiferous tubules containing 3 or more TUNEL-positive gonocytes per seminiferous tubule were determined. As shown in Figure 7A, only a few TUNEL-positive cells were observed in the control testes. In contrast, a dose-dependent increase in the TUNEL-positive cells was observed in the ethanol-treated testes (Figure 7B and C). The apoptotic cells appeared to be gonocytes according to their morphology and location in the seminiferous tubules. Ethanol at concentrations of 50 mM and 100 mM caused a slight, but not significant, increase in apoptosis. A significant increase in apoptosis was observed in the testes treated with ethanol at concentrations of 150 mM and 200 mM (Figure 8).

Effect of Ethanol on the Subcellular Localization of RARα in the Gonocytes of P3 Testes

In the control testes, RARα immunoreactivity was primarily found in the cytoplasm of gonocytes (Figure 9A). RARα immunoreactivity was similar in the testes treated with 50 mM ethanol to that in the control testes (data not shown). In the testes treated with 100 mM ethanol, immunostaining of RARα was primarily detected in the cy-

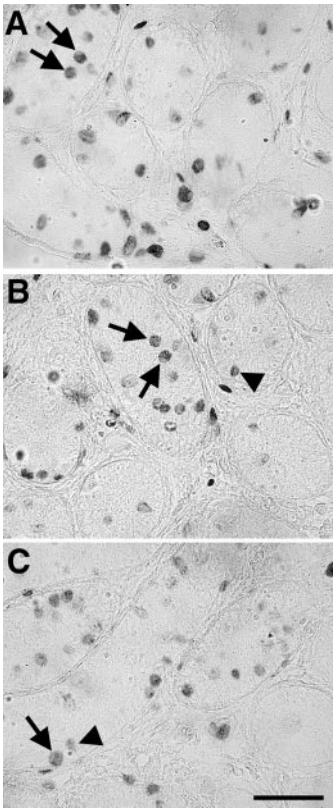

Figure 5. Effect of ethanol on proliferation of gonocytes in postnatal day 3 (P3) testes. P3 testes were cultured for 3 days in the absence **(A)** or presence of 150 mM **(B)** or 200 mM **(C)** ethanol. Arrows indicate 5-Bromo-2′-deoxyuridine (BrdU)-positive gonocyte; arrowheads, BrdU-positive Sertoli cells. Bar in C = 50 μm.

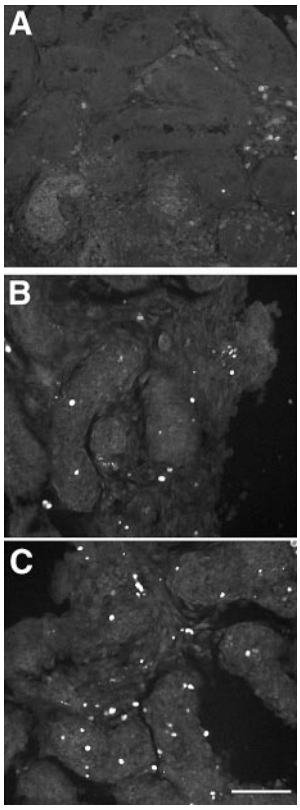

Figure 7. Effect of ethanol on gonocyte apoptosis in postnatal day 3 (P3) testes. P3 testes were cultured for 3 days in the absence **(A)** or presence of 100 mM **(B)** and 200 mM **(C)** ethanol. Apoptotic gonocytes were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. Bar in C = 50 μm.

toplasm of gonocytes, with a few gonocytes showing nuclear immunostaining (Figure 9B). The percentage of this nuclear staining of RARα increased in the testes treated with either 150 mM or 200 mM concentration of ethanol (Figure 9C and D).

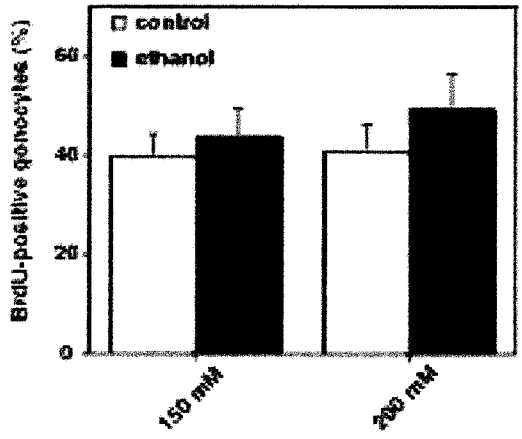

Figure 6. Quantitative analysis of 5-Bromo-2′-deoxyuridine incorporation into gonocytes in postnatal day 3 testes. Values are means ± SD of 3 independent experiments.

Discussion

Testicular development starts with seminiferous cord formation during sexual differentiation period of the embry-

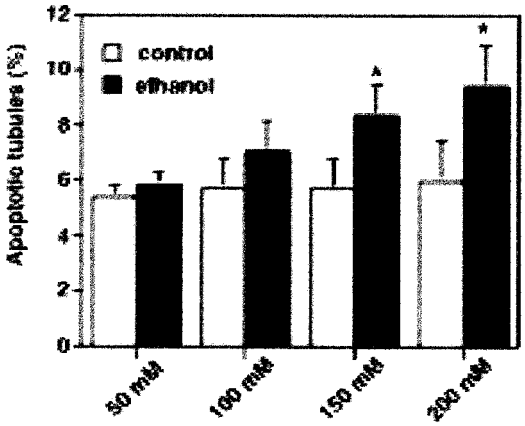

Figure 8. Quantitative analysis of apoptotic tubules in postnatal day 3 testes. Values are means ± SD of 3 independent experiments. Statistically significant differences ($P < .05$) are indicated by *.

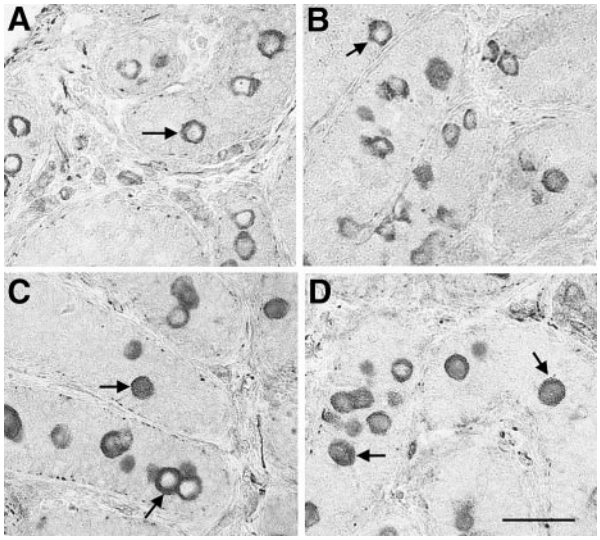

Figure 9. Effect of ethanol on subcellular localization of retinoic acid receptor alpha (RARα) in the gonocytes of postnatal day 3 (P3) testes. P3 testes were cultured for 3 days in the absence **(A)** or presence of 100 mM **(B)**, 150 mM **(C)**, and 200 mM **(D)** ethanol. Testicular sections were immunostained with an antibody against RARα. Arrows indicate gonocytes. Bar in D = 50 μm.

onic development. At this period, primordial Sertoli cells differentiate, controlled by functional Sry (Burgoyne et al, 1988; Koopman et al, 1991), and the cells from the mesonephros migrate into the adjacent gonad forming seminiferous cords (Buehr et al, 1993; Martineau et al, 1997). By late embryonic day E18, gonocytes enter into a mitotically quiescent stage until P3 to P5 after birth, whereas Sertoli cells remain proliferative (Pelliniemi et al, 1993). We found that ethanol, even at a high dose (200 mM), had no effect on seminiferous cord formation or on the expression of MIS in Sertoli cells of E13 testes. Similarly, the expression of both GATA-4 and MIS in Sertoli cells from E18 testes did not change after ethanol treatment compared to that of the controls. Moreover, the number of gonocytes in the ethanol-treated testes remained similar to that in the control testes from both E13 and E18 embryos. Our findings are in agreement with lack of evidence showing morphological anomalies of the testis in the fetal alcohol syndrome infants, in spite of the fact that ethanol is a well-established teratogenic agent and can cause many other anomalies in the fetus (Warren and Foudin, 2001).

However, the present study demonstrates that ethanol decreases the number of gonocytes in the P3 testis in culture by increasing apoptosis of gonocytes, and this effect is dose dependent. Although it has previously been reported that that ethanol can enhance the apoptosis of germ cells in adult rats (Zhu et al, 2000; Eid et al, 2002), to our knowledge, this is the first report that demonstrates that ethanol increases the apoptosis of immature germ cells (gonocytes). An increased apoptosis during this neonatal period may result in abnormal spermatogenesis in adults because it has been established that the normal development of germ cells in the first 3–5 postnatal days is critical to healthy spermatogenesis in adults (Orth et al, 1988; de Rooij, 1998).

The mechanism by which ethanol induces apoptosis in various cell types has not been explained. It has been hypothesized for some time that ethanol can competitively inhibit retinol oxidation to retinaldehyde catalyzed by alcohol dehydrogenase, effectively decreasing the concentration of retinoic acid (Duester et al, 1991). Thus, we wondered whether ethanol could decrease the retinoic acid level that, in turn, could decrease the activity of RARα. In this study, on the contrary, we found that ethanol increased the nuclear localization RARα in the gonocytes of P3 testes in a dose-dependent manner. This abnormal activation of RARα by ethanol could potentially elicit an irregular increase in the transcription of genes involved in apoptosis of the gonocytes of P3 testes. Previously, ethanol has been shown to increase TGFβ that induced macrophage apoptosis (Singhal et al, 1999). In testis, the treatment with TGFβ1 and TGFβ2 isoforms have decreased the number of gonocytes by increasing apoptosis in the organ cultures of testis from E13.5 fetus or from P3 rats but not in the organ culture of testis from E17.5 fetus (Olaso et al, 1998). Moreover, the expression of TGFβ isoforms was shown to be upregulated by retinoic acid and mediated by RARα (Cupp et al, 1999; Choudhury et al, 2000). Thus, TGFβ could very likely link RARα and apoptosis in ethanol-treated testes.

In addition, the ethanol-induced nuclear localization of RARα in the gonocytes of P3 testes is particularly interesting in light of our previous findings that retinoic acid (Braun et al 2000), protein kinase C, and mitogen-activated protein kinase (MAPK) (Braun et al, 2002) can increase the nuclear localization of RARα. These results suggest that ethanol could potentially increase the activities of protein kinase C and MAPK in germ cells, which could then regulate the subcellular localization of RARα. This possibility is compatible with recent evidence demonstrating that ethanol can increase the activity of MAPK in hepatocyte and pancreatic cells (Lee et al, 2002; Masamune et al, 2002). Further investigation is required to determine whether ethanol actually modulates the activities of protein kinase C or MAPK in germ cells.

In summary, we investigated the effect of ethanol on the rat testis at different developmental stages from embryonic to neonatal periods using an organ culture technique. We found that ethanol had no obvious effect on seminiferous cord formation, gonocyte numbers, or expression of MIS and GATA-4 in Sertoli cells in embryonic testes and that ethanol decreased gonocyte numbers by increasing apoptosis in a dose-dependent manner in

P3 testes. Concomitantly, the subcellular localization of RARα changed from more cytoplasmic to nuclear after ethanol treatment. Whether this change in the subcellular localization of RARα caused the ethanol-enhanced gonocyte apoptosis in P3 testis remains to be investigated.

Acknowledgment

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