Abstract — Little is known about the effects of low chronic alcohol intake on fertility, particularly in females. Recently, we have shown that chronic 10% (w/v) ethanol treatment affects in-vitro fertilization of mouse female gamete. The aim of this study was to solve questions concerning the lowest dose and duration of ethanol treatment required to alter the fertility of immature and adult female and adult male mouse. Mice were treated with 5% and 2.5% (w/v) ethanol in drinking water for 4 weeks. The in-vitro fertilization rates were significantly decreased with the 5% ethanol when oocytes from prepubertal and pubertal ethanol-treated females were inseminated with spermatozoa from adult control males. The in-vitro fertilization rates were not diminished when oocytes from control females were inseminated with spermatozoa from adult ethanol-treated males. Haploid oocytes were increased when oocytes came from immature females treated with ethanol. The in-vitro fertilization rates were not decreased in adult treated females. The in-vivo fertilization rates were not modified when prepubertal ethanol-treated females were mated with adult control males. Fragmented oocytes, in the in-vitro fertilization experiments, were significantly increased when they came from prepubertal and adult treated females inseminated with ethanol-treated males. These results show that there is a threshold of the ethanol dose to produce an effect. Chronic low ethanol ingestion by immature female mice has a deleterious effect on their in-vitro fertilization. Furthermore, acute ethanol ingestion by adult females during the induction of ovulation resulted in high parthenogenetic activation and fragmentation of mouse oocytes.

INTRODUCTION

Excessive use of alcoholic beverages results in a variety of medical and psycho-sociological disturbances that identify alcoholism as one of modern society's major problems. Pathophysiology is dependent upon the concentration and the duration of ethanol exposure (Willis et al., 1983; Anderson et al., 1983). Ethanol may have a direct action on gonads (Van Thiel et al., 1978, 1979; Dees and Skelley, 1990) as well as on the hypothalamic–pituitary axis (Cicero and Badger, 1977; Dees et al., 1990; Valimaki et al., 1990). In the human male, the clinical consequences of delayed sexual maturation are substantial, affecting not only fertility, but also the development of secondary male characteristics (Anderson et al., 1987). In women, there is an increased risk of infertility with high alcohol consumption (Mello, 1988) and even moderate alcohol use can affect fertility (Grodstein et al., 1994; Cebral et al., 1995). The inability of apparently competent spermatozoa to penetrate the zona pellucida is often associated with ooplasmic anomalies (one or more pronucleus, with one or no polar bodies and other occasional anomalies) (Bedford and Kim, 1993).

Ethanol seems to be able to inhibit fertilization in vitro and in vivo (Anderson et al., 1981, 1983) by inhibiting essential sperm processes (capacitation or acrosomal reaction), that precede sperm penetration of the oocyte. Acute paternal alcohol administration to rats, 24 h prior to breeding, does not affect mating behaviour, but results in a diminished fertility rate (Cicero et al., 1994). Ethanol can interfere with membrane-associated signal transduction mechanisms that trigger intracellular responses (Hoek and Rubin, 1990).

There may be thresholds for ethanol to produce its effects, with respect to blood/tissue levels and duration of exposure. However, questions concerning the dose and duration of ethanol exposure required to cause alterations in fertility remain
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unresolved, though experiments on laboratory animals can be helpful (Anderson et al., 1983).

Most studies of ethanol-induced fertility alterations have been conducted with the male gender of both man and laboratory animals. The effects of ethanol on pubertal processes are poorly understood, and only a few studies have been conducted in this respect. Some reports examined the effect of chronic ethanol ingestion on male reproductive function by sexually immature male mice, and it was observed that ethanol delays certain aspects of sexual maturation (Ramaley, 1982; Anderson et al., 1987). In immature female rats, the detrimental effects of this drug include a delay in vaginal opening, decreased uterine and ovarian weights and depressed ovarian function (Bo et al., 1982). However, periods of development which may differ in their sensitivity to the effects of ethanol on the female gamete and its fertility have not been identified.

The main object of this work was to study the effects of a prolonged low alcohol ingestion by female and/or male mice on fertilization rates in vitro and in vivo and on the quality of oocytes post-insemination. We also evaluated the effect of chronic ethanol ingestion throughout several stages of sexual maturation of females in relation to its fertility. We additionally examined the possibility that an acute ethanol treatment given to females during the induction of ovulation can affect the quality of the gamete.

MATERIALS AND METHODS

Animals

Hybrid F₁ mice (C57/B1 × CBA) from our own colony were maintained under automatically controlled temperature (25°C) and a 14 h light/10 h dark cycle. Groups of three animals each were kept in plastic cages, and were fed ad libitum with a commercial mouse chow diet (Diet No. 1 from Nutrimentos S.A., Buenos Aires, Argentina). Daily caloric intake was estimated by the calorific value of the diet used (2900 kcal/kg). The amount of food consumed by the animals was determined by the daily offered chow and the remaining food, less the amount spilled.

Males were adult 75 days old (average body weight: 29.50 ± 0.88 g). Females were immature (prepubertal 30 days old, average body weight: 16.80 ± 0.55 g, and pubertal 40 days old, average body weight: 20.00 ± 0.45 g), and adult 80 days old (average body weight: 30.50 ± 0.64 g), at the start of the alcohol experiments.

Ethanol treatments

Chronic ethanol administration. Immature and adult female and adult male mice were treated with 5% (w/v) ethanol in drinking water, and 2.5% (w/v) ethanol was administered to prepubertal females. Animals were maintained on treatment for 4 weeks. Controls received water and ethanol was isocalorically substituted by maltose–dextrin (3.8 kcal/g). The body weights were recorded daily throughout the treatment. The amount of daily liquid intake was determined by volume differences between the offered and remaining volume. The ethanol calories were estimated as 7.1 kcal/g. From these data, daily patterns of caloric intake and the percentage of ethanol-derived calories (%EDC) were determined.

The effects of chronic ethanol on fertilization in vitro and in vivo and on the quality of the male gamete were examined.

Acute ethanol administration. Prepubertal females (30 days old) were kept under controlled conditions and food and water were provided ad libitum for 27 days. Acute ethanol treatment began at 10:00 on day 27 when they received 5% (w/v) ethanol in drinking water. Controls were as before. Females were induced to superovulate, while being exposed to ethanol, with 10 IU of pregnant mare’s serum gonadotrophin (PMSG, Sigma, St Louis, MO, USA) given i.p. at 18:00 on day 27 and 48 h later (day 29) with 10 IU of human chorionic gonadotrophin (HCG, Sigma). Here, the ethanol treatment was delayed to day 30 (at 10:00) when females were killed (at 16:30 post-HCG) to remove the cumulus masses and to assess the quality of oocytes.

Blood ethanol measurement

In a separate trial, five pairs of adult male mice were chronically treated, as described above. At 06:00 on day 29, mice were decapitated and trunk blood was collected into heparinized Eppendorf tubes. Samples were maintained at 4°C, for blood ethanol to be measured within 4 h of the collection. Blood ethanol determination was done using a Hewlett-Packard gas chromatograph (model
alcohol and mouse fertilization

5840, equipped with a flame ionization detector, a head-space injection device and an integrator and recorder. Ethanol levels were quantified from standard curves of known ethanol concentration versus detector response and were expressed as mg/dl of blood.

In vitro fertilization

Source and collection of spermatozoa. Male mice were killed by cervical dislocation on the morning of day 29. One epididymis of a male was dissected and the cauda placed into a 200 μl drop of a modified fertilization medium (FM) (Fraser and Drury, 1975), supplemented with 30 mg/ml of bovine serum albumin (BSA 3%, Sigma), and overlaid with mineral oil (Sigma). Spermatozoa were obtained by making small cuts in the cauda. The dense mass of spermatozoa was allowed to flow out freely for 5 min. The tissue was removed and sperm concentration was determined using a Neubauer chamber. Sperm suspension was then incubated for 90 min in the same medium in a humidified tissue culture incubator (37°C, 5% CO₂ in air) to allow capacitation.

Source and collection of oocytes. Immature and adult female mice were induced to superovulate with 10 IU PMSG at 18:00 on day 26 of the ethanol treatment and with 10 IU HCG 48 h later. Females were killed by cervical dislocation 16–17 h after HCG injection. Both oviducts were immediately removed and placed in phosphate-buffered saline (PBS). Cumulus masses containing oocytes were released from one ampulla into a 150 μl drop of FM (one cumulus mass per drop), and overlaid with mineral oil.

In vitro insemination. Cumulus–oocytes of two drops obtained from one female were inseminated with 1–2 x 10⁶ spermatozoa/ml that came from one male. The following groups were studied:

- Group I: oocytes of control females inseminated with spermatozoa of control males.
- Group II: oocytes of control females inseminated with spermatozoa of ethanol-treated males.
- Group III: oocytes of ethanol-treated females inseminated with spermatozoa of control males.
- Group IV: oocytes of ethanol-treated females inseminated with spermatozoa of ethanol-treated males.

Evaluation of events after fertilization. Oocytes were recovered 5 h after insemination and washed to remove the cumulus cells and the adherent spermatozoa. They were then placed in a 100 μl drop of M16 medium (Whittingham, 1971) supplemented with BSA 3%, overlaid with mineral oil. Oocytes were classified as being ‘activated’ when the second polar body (II PB) was visible under an inverted phase contrast microscope.

After 7–8 h post-insemination, a fraction of the total activated oocytes was incubated for 1 h with Hoechst 33342 (0.5 μg/ml, Sigma) in M16, to examine under fluorescence microscope the pronucleus formation. Oocytes were classified as being normally fertilized when II PB was present with two pronuclei (2 PN). Haploid (activated oocytes with one pronucleus (1 PN), triploid (polyspermic, with II PB and 3 PN) and anucleate (with 0 PN) oocytes were assessed.

In-vivo fertilization

Mating. Prepubertal females were used at the beginning of the treatment. They were induced to superovulate on day 26 and 28 (at 16:30) of the ethanol treatment. After HCG injection, one female was immediately caged with one adult male, isolated previously. Mating was confirmed by the presence of vaginal plug 14–18 h later.

The following groups were studied:

- Group I: control females mated with control males.
- Group II: control females mated with ethanol-treated males.
- Group III: ethanol-treated females mated with control males.
- Collection of embryos. Females were killed 24–32 h after mating (day 2 after HCG injection). Fertilized 1 and 2-cell embryos were recovered from the oviductal ampullae by dissection into M2 medium (Quinn et al., 1982). The cells were washed to remove pieces of tissue and placed in a 100 μl drop of M16 covered with mineral oil, to be examined under an inverted phase contrast microscope.

Mouse gamete quality

Oocyte quality evaluation in chronic and acute ethanol experiments. The quality of the oocytes was assessed by counting fragmented oocytes in in-vitro and in-vivo fertilization experiments. Intact, activated, fragmented, immature and abnormal oocytes (morphologically altered) from the acute ethanol-treated females were assessed.
Table 1. Daily intake of food, fluid and percentage of ethanol-derived calories in mice chronically treated with 5% ethanol

<table>
<thead>
<tr>
<th>Sex and treatment</th>
<th>Food intake g (kcal)</th>
<th>Drinking volume ml</th>
<th>Ethanol intake g (kcal)</th>
<th>%EDC (mean % of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>137 ± 6 (394)</td>
<td>232 ± 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol-treated</td>
<td>175 ± 20 (507)</td>
<td>288 ± 22</td>
<td>14.4 ± 1.1 (102)</td>
<td>16.7</td>
</tr>
<tr>
<td>Adult females</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>174 ± 23 (505)</td>
<td>384 ± 42</td>
<td>8.5 ± 0.8 (60)</td>
<td>14.7</td>
</tr>
<tr>
<td>Ethanol-treated</td>
<td>120 ± 11 (348)</td>
<td>169 ± 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pubertal females</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>322 ± 12 (935)</td>
<td>318 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol-treated</td>
<td>257 ± 20 (745)</td>
<td>278 ± 10</td>
<td>13.9 ± 0.5 (99)</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Daily food and fluid intake was assessed in control and ethanol-treated animals. The amounts given in g, ml or kcal are per kg body weight and per day. The kcal values are in parentheses. Adult mice were 75-80 days old, and they had an average body weight of 30 g (described in Materials and methods). Pubertal females were 40 days old and weighed 20 g. %EDC: percentage of ethanol-derived calories.

Results were expressed as percentage of oocytes per female.

Epididymal spermatozoa quality evaluation. Cauda of one epididymis from control and ethanol-treated males was dissected in a 200 µl drop of FM. The total sperm content was allowed to disperse by making small cuts, and then the tissue was removed. Sperm quality was assessed by direct microscopic evaluation as the percentage of motility and hyperactivation after 90 min of capacitation.

Statistics

Results are expressed as means ± SEM and were evaluated using analysis of variance (ANOVA): Dunnett test, and Student's t-test by means of the Instat program (GraphPAD software, San Diego, CA, USA). A P < 0.05 was considered to indicate significance.

RESULTS

Average daily intakes of food, fluid and ethanol by mice are expressed per kg body weight and are shown in Table 1. The pubertal females consumed an average of 278 ± 9 ml of ethanol/kg/day (ethanol derived calories: 99 kcal/kg/day), and the percentage of ethanol-derived calories (%EDC) was 11.7%. The adult females consumed 169 ± 18 ml ethanol/kg/day (ethanol derived calories: 60 kcal/kg/day), and the %EDC was 14.7%. The males consumed an average of 288 ± 22 ml ethanol/kg/day (ethanol derived calories: 102 kcal/kg/day), and the %EDC was 16.7%.

The body weights of mice were not significantly changed at the end of the treatment, in control or ethanol-treated animals.

Blood ethanol levels in the morning (06:00) of the last day of treatment were almost non-detectable, being below the detection limit used in the assay.

In-vitro fertilization

Activation events in prepubertal females. The chronic administration of 5% (w/v) ethanol in drinking water to females induced a lower activation rate (oocytes with II PB) as compared to control females (Table 2). The percentage of activated oocytes in group III (oocytes of ethanol-treated females inseminated with spermatozoa of control males) and group IV (oocytes of ethanol-treated females inseminated with spermatozoa of ethanol-treated males) was significantly decreased, as compared to the control groups (P < 0.05, Dunnett test). There were no significant differences in activation rates in group II (oocytes of control females inseminated with spermatozoa of ethanol-treated males) as compared to the control group.

When 2.5% (w/v) ethanol was chronically administered, the percentage of activated oocytes in group III (oocytes of ethanol-treated females inseminated with spermatozoa of control males) was not altered as compared to group I (oocytes of
Table 2. Activation rates of prepubertal females, in-vitro fertilization, with chronic 5 and 2.5% (w/v) ethanol: expulsion of second polar body (II PB), 5 h post-insemination

<table>
<thead>
<tr>
<th>Group</th>
<th>5% ethanol</th>
<th>2.5% ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>II PB</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>C. females × C. males</td>
<td>721</td>
<td>76.6 ± 3.4</td>
</tr>
<tr>
<td>C. females × E. males</td>
<td>580</td>
<td>67.6 ± 5.0</td>
</tr>
<tr>
<td>E. females × C. males</td>
<td>608</td>
<td>56.1 ± 6.3*</td>
</tr>
<tr>
<td>E. females × E. males</td>
<td>306</td>
<td>57.3 ± 6.7*</td>
</tr>
</tbody>
</table>

In-vitro fertilization was performed in a 150 µl culture medium (FM) containing 3% BSA, with cumulus-oocyte complexes from the oviducts inseminated with a final concentration of $10^6$ spermatozoa/ml from a male. After 5 h, oocytes were transferred to 100 µl M16 medium to record the extrusion of II PB.

N: total number of oocytes; II PB: percentage of oocytes with second polar body, expressed as mean ± SEM (n: number of mice); C.: control; E.: ethanol-treated; *P < 0.05, Dunnett test.

control females inseminated with spermatozoa of control males). Group II was not included, since the IVF rate with 5% ethanol was not altered when oocytes of control females were inseminated with spermatozoa of ethanol-treated males. The in-vitro fertilization rate did not decrease with 2.5% ethanol in group III (oocytes of ethanol-treated females inseminated with spermatozoa of control males); therefore group IV (oocytes of ethanol-treated females inseminated with spermatozoa of ethanol-treated males) was also omitted.

PN formation in prepubertal females (8 h post-insemination). The percentage of fertilized oocytes (2 PN and II PB) was significantly (P < 0.01) decreased in both groups III and IV, as compared to the control group I (Dunnett test). There were no significant differences between group II and group I. Haploid oocytes (1 PN and II PB) were higher in group III and group IV than in the control group I (P < 0.05). Polyspermic (II PB and 3 PN) and anucleate oocytes (II PB and 0 PN) percentages were very small and there were no differences between the groups (Table 3A). The in-vitro fertilization rates (oocytes with II PB and 2 PN) were significantly decreased when prepubertal females were treated with 5% (w/v) ethanol.

When 2.5% (w/v) ethanol was administered to females, the percentage of fertilized oocytes in

Table 3. In-vitro fertilization rates of prepubertal females: presence of pronucleus in activated oocytes (with II PB), 8 h post-insemination

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>n</th>
<th>3 PN</th>
<th>2 PN</th>
<th>1 PN</th>
<th>0 PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) 5% (w/v) ethanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. females × C. males</td>
<td>238</td>
<td>6</td>
<td>1.7 ± 0.8</td>
<td>74.9 ± 3.3</td>
<td>4.1 ± 1.6</td>
<td>0.9 ± 0.6</td>
</tr>
<tr>
<td>C. females × E. males</td>
<td>183</td>
<td>5</td>
<td>2.5 ± 1.0</td>
<td>56.1 ± 2.7</td>
<td>6.4 ± 1.7</td>
<td>1.1 ± 0.7</td>
</tr>
<tr>
<td>E. females × C. males</td>
<td>138</td>
<td>5</td>
<td>1.3 ± 0.8</td>
<td>32.2 ± 9.4**</td>
<td>16.6 ± 6.4*</td>
<td>3.5 ± 1.5</td>
</tr>
<tr>
<td>E. females × E. males</td>
<td>154</td>
<td>6</td>
<td>1.5 ± 0.9</td>
<td>39.3 ± 5.1**</td>
<td>16.0 ± 1.6*</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>(B) 2.5% (w/v) ethanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. females × C. males</td>
<td>146</td>
<td>4</td>
<td>1.9 ± 0.8</td>
<td>81.0 ± 5.4</td>
<td>4.0 ± 2.1</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>E. females × C. males</td>
<td>221</td>
<td>6</td>
<td>2.1 ± 0.6</td>
<td>80.0 ± 6.1</td>
<td>5.1 ± 2.7</td>
<td>1.2 ± 0.5</td>
</tr>
</tbody>
</table>

Fertilized oocytes were placed in 100 µl M16 medium 5 h post-insemination and were incubated with Hoechst 33342 for 1 h. At 8 h post-insemination, activated oocytes (with II PB) were washed three times and mounted to examine for the presence of pronuclei with fluorescence microscopy. Fragmented, dead and unfertilized oocytes were not included in the evaluation.

N: total number of oocytes; n: number of mice; PN: percentage of oocytes with pronucleus (mean ± SEM); C.: control; E.: ethanol-treated. *P < 0.05, **P < 0.01, Dunnett test.
Table 4. Activation and pronucleus formation of pubertal and adult females, in in-vitro fertilization, with chronic 5% (w/v) ethanol

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>II PB</th>
<th>+3 PN</th>
<th>+2 PN</th>
<th>+1 PN</th>
<th>+0 PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pubertal females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. females × C. males</td>
<td>5</td>
<td>87.2 ± 3.4</td>
<td>2.1 ± 0.8</td>
<td>80.6 ± 6.4</td>
<td>4.7 ± 2.7</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>E. females × C. males</td>
<td>4</td>
<td>79.2 ± 7.4</td>
<td>1.3 ± 0.8</td>
<td>62.5 ± 7.2*</td>
<td>14.7 ± 2.6*</td>
<td>1.6 ± 1.0</td>
</tr>
<tr>
<td>Adult females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. females × C. males</td>
<td>5</td>
<td>84.2 ± 2.6</td>
<td>1.8 ± 0.4</td>
<td>75.6 ± 4.5</td>
<td>5.5 ± 1.5</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>E. females × C. males</td>
<td>5</td>
<td>81.5 ± 7.8</td>
<td>1.4 ± 0.7</td>
<td>70.5 ± 5.6</td>
<td>7.3 ± 2.0</td>
<td>2.3 ± 1.1</td>
</tr>
</tbody>
</table>

Activated oocytes (with II PB) from pubertal and adult ethanol-treated females were placed in 100 μl M16 medium 5 h post-insemination and were incubated with Hoechst 33342 for 1 h. At 8 h post-insemination, oocytes were washed and mounted to examine the presence of pronuclei with fluorescence microscopy. Fragmented, unfertilized oocytes (metaphase II) were not included in the observation.

n: number of mice; II PB (second polar body): percentage of activated oocytes, from the total recovered oocyte number, expressed as mean ± SEM; +3 PN: oocytes with II PB + 3 pronuclei (PN) (triploids) (mean ± SEM); +2 PN: percentage of fertilized oocytes (with II PB + 2 pronuclei); +1 PN: percentage of activated oocytes (haploid cell); +0 PN: percentage of anucleate oocytes; C: control; E.: ethanol-treated; *P < 0.05, Dunnett test.

Quality of gametes

Quality of oocytes in in-vitro and in-vivo fertilization: chronic treatment. At 5 h post-insemination, the fragmented oocytes from prepubertal, pubertal and adult females in in-vitro fertilization experiments, and oocytes from prepubertal females in in-vivo experiments, were assessed. Table 6 shows the results for the prepubertal and adult females in IVF experiments, since similar findings were observed with pubertal ethanol-treated females inseminated with spermatozoa of control males. When females were prepubertal, group IV (oocytes of ethanol-treated

In-vivo fertilization

The fertilization rates of control females mated with ethanol-treated males were not significantly reduced (Table 5). The fertilization percents of prepubertal ethanol-treated females mated with control males were not decreased, as compared to controls. The value of this group III was not significantly lower than that of the control group because of high dispersion (Table 5).
females inseminated with spermatozoa of ethanol-treated males) showed increased fragmentation rates as compared to the control group (22.2 ± 6.7% vs 8.7 ± 1.6%; \( P < 0.01 \), Dunnett test). When females were adult, the percentage of fragmented oocytes was higher than in the control group (11.5 ± 1.3% vs 7.8 ± 1.4%; \( P < 0.05 \)).

At 24 h post-insemination, in in-vivo fertilization experiments, no increments in fragmented oocyte per cents were observed in the ethanol-treated groups, as compared to the control.

Quality of oocytes of acute ethanol-treated females. When 60-day-old females were treated with 5% ethanol for 72 h, the quality of the superovulated oocytes was impaired (Table 1). The quantity of intact oocytes was significantly reduced in ethanol-treated females as compared to control females (\( P < 0.01 \), Dunnett test). Furthermore, percentages of activated and fragmented oocytes were also significantly increased in ethanol-treated females (\( P < 0.05 \)), as observed in prepubertal ethanol-treated females. No differences were observed in the percentages of immature and abnormal oocytes from the treated females.

Quality of male gamete. Adult males were chronically treated with 5% ethanol and cauda sperm motility was assessed. There were no significant differences in sperm motility and hyperactivation between ethanol-treated and control males (Student’s \( t \)-test) (data not shown).

DISCUSSION

In the present study, the treatment was carried out for periods that included the age of onset of sexual maturity in female mice, and also the late pubertal development, during which females

### Table 6. Chronic ethanol effects on the quality of oocytes from in-vitro and in-vivo fertilization (prepubertal and adult females, 5% ethanol)

<table>
<thead>
<tr>
<th>Group</th>
<th>In vitro</th>
<th></th>
<th>In vivo</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prepubertal</td>
<td>Adult</td>
<td>Prepubertal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fragmented (%)</td>
<td></td>
<td>Fragmented (%)</td>
</tr>
<tr>
<td>C. females × C. males</td>
<td>15</td>
<td>8.7 ± 1.6</td>
<td>5</td>
<td>7.8 ± 1.4</td>
</tr>
<tr>
<td>C. females × E. males</td>
<td>12</td>
<td>14.7 ± 1.8</td>
<td></td>
<td>11.5 ± 1.3*</td>
</tr>
<tr>
<td>E. females × C. males</td>
<td>13</td>
<td>7.9 ± 0.8</td>
<td>5</td>
<td>11.5 ± 1.3*</td>
</tr>
<tr>
<td>E. females × E. males</td>
<td>10</td>
<td>22.2 ± 6.7***</td>
<td></td>
<td>8.4 ± 4.0</td>
</tr>
</tbody>
</table>

At 5 h after in-vitro insemination, all oocytes in each 150 \( \mu l \) of FM medium were transferred to 100 \( \mu l \) of M16 medium to examine the events of fertilization and quality of the eggs, under a phase contrast microscope. The groups are the same as in earlier Tables. At 24 h after mating, the total oocytes and embryos were recovered from the oviduct of control or ethanol-treated groups, placed in 100 \( \mu l \) of M16 medium and the quality was assessed using a phase contrast microscope.

At 27 day (10:00) the ethanol-treated females (60 days old) received 5% (w/v) ethanol in drinking water. Female mice were injected to induce superovulation on day 27 (PMSG) and on day 29 (HCG). The ethanol administration was prolonged to day 30 (10:00), when females were killed (16:30 h post-HCG) to assess the quality of the gametes: intact, activated, fragmented, immature and abnormal (oocytes with morphological alterations).

### Table 7. Acute ethanol effects on the quality of oocytes (5% ethanol for 72 h)

<table>
<thead>
<tr>
<th>Group</th>
<th>( n )</th>
<th>Intact</th>
<th>Activated</th>
<th>Fragmented</th>
<th>Immature</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. females</td>
<td>5</td>
<td>76.8 ± 3.7</td>
<td>10.9 ± 1.6</td>
<td>6.8 ± 1.3</td>
<td>3.7 ± 2.3</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>E. females</td>
<td>5</td>
<td>44.8 ± 8.9**</td>
<td>32.0 ± 10.1*</td>
<td>15.4 ± 4.4*</td>
<td>1.8 ± 1.8</td>
<td>5.7 ± 3.3</td>
</tr>
</tbody>
</table>

At day 27 (10:00) the ethanol-treated females (60 days old) received 5% (w/v) ethanol in drinking water. Female mice were injected to induce superovulation on day 27 (PMSG) and on day 29 (HCG). The ethanol administration was prolonged to day 30 (10:00), when females were killed (16:30 h post-HCG) to assess the quality of the gametes: intact, activated, fragmented, immature and abnormal (oocytes with morphological alterations).

\( n \): number of females; C.: control; E.: ethanol-treated; \*\( P < 0.05 \), **\( P < 0.01 \), Dunnett test.
become fully reproductively competent. The results show that low chronic alcohol ingestion affects the in-vitro fertilization rates of immature female mice. Percentages of activated oocytes were reduced when they came from prepubertal ethanol-treated females. When spermatozoa from control or ethanol-treated adult males were used to inseminate oocytes from control females, the percentages of activated oocytes were not altered. At 8 h post-insemination, a fraction of the activated oocytes had only 1 PN (haploid cells), and they were significantly increased when oocytes came from prepubertal ethanol-treated females. Also, when females were pubertal and treated with ethanol, the percentages of haploid oocytes were higher than in the control group. It is known that oocytes of most mammalian species are arrested at the second metaphase of meiosis after ovulation, but they can be activated and disarranged by spermatozoa or other agents to resume meiosis and to form diploid or haploid zygotes, respectively (Shina et al., 1993; Balakier and Casper, 1993). If the male genome is eliminated, then it takes no further part in the development of the embryo, which becomes gynogenetic (Henery and Kaufman, 1992). On the other hand, previous work shows that high concentrations of ethanol are required to induce parthenogenetic activation of oocytes (Gulyas and Yuan, 1985; Kubiak, 1989; Marcus, 1990). Also, Dyban and Khozhai (1980) have demonstrated that an i.p. injection of 0.35 ml of a 25% ethanol solution given at appropriate times after spontaneous ovulation can activate the oocytes. Cuthbertson (1983) reported that ethanol concentrations of ≤1% were unable to produce activation of eggs. In spite of these data, we think that a prolonged exposure to ethanol could induce parthenogenetic activation of the oocytes, when females are immature, since adult ethanol-treated females did not have higher oocyte activation rates. Perhaps, oocytes from immature females are more sensitive to the effects of low concentration of ethanol given chronically than those of adults. When females were prepubertal, the oocyte activation rate was less than in the control group. It might be because the remaining oocytes could not be fertilized, or underwent parthenogenetic activation. It is known that oocytes with morphological alterations and/or membrane damage undergo degeneration, fragmentation and death. Moreover, it has been reported that alcohol can enhance free radical generation in the oocyte (Dawson et al., 1994; Henderson et al., 1995; Mira et al., 1995), and cause damage by acting on phospholipids, carbohydrates, metalloproteins and DNA (Nordmann, 1994; Santiard et al., 1995). Thus, alcohol treatment may affect the viability of apparently normal oocytes and/or the fertilization rates by increasing free radical generation. When oocytes came from pubertal females, the activation rate was not different from controls; however there were less fertilized oocytes because haploid cells were increased.

Our hypothesis was that chronic ethanol treatment could affect in-vitro fertilization. To rule out the possibility that the presence of ethanol during ovulation could affect oocyte quality, we gave ethanol for only 72 h, during which period superovulation was induced. We found that this regimen also produced effects on the quality of oocytes (more activation and fragmentation) similar to the chronic treatment. These results suggest that, not only the dose of ethanol is critical, but also the time when alcohol is given.

The ethanol treatment did not decrease the in-vivo fertilization rates of group III (prepubertal ethanol-treated females mated with control adult males), as compared to the control group. It is known that chronic alcohol treatment impairs sexual behaviour with diminished copulatory activity of the male, and 'in-vivo' matings are often unsuccessful (Anderson et al., 1983). But, our results show that the fertilizing capacity of spermatozoa that came from alcohol-treated males was not affected. Also, the in-vivo fertilization rates of prepubertal alcoholic females were not altered.

Anderson et al. (1983) reported that 5 and 10 weeks of exposure to 5% (v/v) ethanol are the less severe alcohol treatments, compared to 20 weeks of exposure to 5% (v/v) ethanol or 5 weeks of exposure to 6% (v/v) ethanol. The former treatments did not produce significant changes in the relative numbers of dysmorphic spermatozoa, but the latter regimens resulted in significant reduction in sperm content, testicular damage and poor quality of spermatogenesis. In our study, treatment with 5% (w/v) ethanol for 4 weeks could be considered as the less severe treatment, because it produced lower fertilization rates neither in-vivo (group II; control females mated with ethanol-treated males), nor in vitro, and the content and the
quality of cauda spermatozoa were not affected in these experiments.

The numbers of fragmented oocytes in in-vitro fertilizations were significantly higher in group IV (oocytes of prepubertal ethanol-treated females inseminated with spermatozoa of ethanol-treated males). Perhaps this group had more fragmented oocytes because both gametes came from ethanol-treated mice, and this association could produce a deleterious effect in maintaining oocyte quality after fertilization. However, it is known that, if fertilization does not occur within the short programmed period, the ovum loses its capacity to be fertilized and starts to degenerate (Takase et al., 1995). Little is known about the mechanism underlying the degeneration of ova. DNA and cytoplasmic fragmentation are a consequence of 'programmed cell death' (PCD) with typical features of apoptosis (Gavrieli et al., 1992; Gorczyca et al., 1992; Jurisicova et al., 1996). It was reported that fragmentation can occur in cleaved unfertilized ova that undergo parthenogenetic activation (Takase et al., 1995), and also if delayed fertilization occurs. It is known that delayed fertilization results from polyspermic fertilization, from penetrated oocytes without development of the male pronucleus, premature sperm chromosome condensation without egg activation, inability of apparent normal spermatozoa to bind the zona surface (Bedford and Kim, 1993), and other chromosomal abnormalities (high degree of aneuploidy) (Zenzes and Casper, 1992; Munne and Cohen, 1994). We could observe few polyspermic oocytes in all groups. But we do not know whether fragmented oocytes observed in group IV were polyspermic after insemination or before undergoing fragmentation.

In summary, our results suggest that there are thresholds in dose and time for chronic alcohol ingestion to produce alterations in fertility. Thus, a low concentration of ethanol given chronically to immature female mice is able to decrease the in-vitro fertilization rates. Also, the acute ingestion of ethanol during the ovulation period could alter the quality of the oocytes. This work confirms the toxic and deleterious effect of alcohol consumption by females on reproduction.

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