Alcohol & Alcoholism Vol. 35, No. 4, pp. 336–343, 2000

ALTERATIONS IN PREIMPLANTATION *IN VIVO* DEVELOPMENT AFTER PRECONCEPTIONAL CHRONIC MODERATE ALCOHOL CONSUMPTION IN FEMALE MICE

ELISA CEBRAL*, ANDREA LASSERRE, VALERIA RETTORI and MARTHA A. DE GIMENO

Centro de Estudios Farmacológicos y Botánicos (CEFYBO-CONICET), Serrano 669, (1414) Capital Federal, Argentina

(*Received* 14 *December* 1999; *in revised form* 24 *February* 2000; *accepted* 24 *March* 2000)

Abstract — Although many studies have explored the effects of acute or chronic ethanol exposure during the postimplantation period on embryo/fetal development, few reports have described the ethanol effects on preimplantation embryo development. Little is known about the effects of ethanol consumption prior to gestation on embryo growth. Recently, we have shown that chronic moderate ethanol intake by prepubertal female mice reduces the ovulatory response and impairs *in vitro* fertilization and *in vitro* embryo preimplantation development. The purpose of the present work was to evaluate the effects of preconceptional chronic moderate ethanol ingestion on preimplantation embryo morphology and differentiation, the timing of cleavage and embryo growth *in vivo*, and to determine the time pattern in which alterations appear. Prepubertal female mice were treated with 10% (w/v) ethanol for 30 days prior to conception. After inducing ovulation on day 27 and 29 of the ethanol treatment, females were mated with control males and the day of presence of vaginal plug was day 1. On day 1, a decreased percentage of normal fertilized oocytes, elevated parthenogenetic oocyte activation and unfertilized eggs with abnormal metaphase II were found in ethanol-treated, compared to control females. On day 2, while any differences in the total percentage of 2-cell embryos were observed, the treated females had a significantly higher percentage of morphologically abnormal embryos, compared to control females. On day 3, the preconceptional consumption of ethanol produced significantly reduced percentages of compacted morulae and an increased percentage of uncompacted morulae. The total percentage of morulae in the treated females was lower than in controls. On day 4, ethanol-treated females showed significantly decreased percentages of hatched attached blastocysts and increased early blastocyst and morula percentages, compared to controls. Thus, preconceptional chronic moderate ethanol ingestion by prepubertal female mice produced retarded development, impaired blastocyst hatching, abnormal embryo morphology and embryo loss by fragmentation due to alterations induced in the female gamete.

INTRODUCTION

Animal models of alcohol consumption have used high doses of ethanol at different stages of the organogenetic period in an attempt to induce the anatomical defects of the human fetal alcohol syndrome (FAS) (Weston *et al.*, 1994; Abel, 1995). FAS can occur in infants born of mothers who are chronic heavy daily drinkers (Kaufman, 1997). However, the susceptibility of the preimplantation stages of embryo development to maternal alcohol exposure still remain relatively unexplored. In this regard, Checiu and Sandor (1982, 1986) reported reduced implantation with impaired oviductal embryo transport, and retarded and abnormal development of mouse embryos associated with acute maternal ethanol intoxication during the preimplantation period. But ethanol consumption by the mother both prior to and at the time of conception may be potentially even more harmful than ethanol consumption during pregnancy.

At present, it is unclear whether alcohol has actions at the cellular or subcellular level, or if the underlying mechanisms vary according to the organ system, the dose, the time and/or the duration of the exposure. Ethanol might act as a mutagen directly or indirectly through its first metabolite, acetaldehyde (Kaufman and Bain, 1984*a*), which can interfere with microtubule integrity and tubulin polymerization, causing chromosome segregation errors (Tuma and Sorrell, 1987; Tuma *et al.*, 1991).

Most studies with animal models were conducted with high doses of ethanol in liquid diets that resulted in elevated ethanol-blood/tissue levels. However, models based on alcohol intake in drinking water were seldom used, because they produced low blood-alcohol levels. The administration of ethanol by mouth to female mice at periconception was capable of inducing a relatively high incidence of aneuploidy in the resultant zygotes (Kaufman, 1983; Kaufman and Bain, 1984*a*). Alcohol given i.p. at specific times after ovulation can alter the quality of oocytes, increasing parthenogenesis (Dyban and Khozai, 1980). Chronic ethanol consumption impairs the reproductive cycle and alters ovarian function (Van Thiel *et al.*, 1978; Eskay *et al.*, 1981; Cebral *et al.*, 1998*a*). Alcohol also produces an increased risk of infertility in women (Mello, 1988; Grodstein *et al.*, 1994), altered oocyte quality (Cebral *et al.*, 1998*b*), reduced *in vitro* fertilization (Cebral *et al.*, 1997), and retarded and impaired preimplantation development *in vitro* (Cebral *et al.*, 1999).

There have been very few studies about the effects of pregestational ethanol intake on the morphology and growth of embryos. The purpose of this work was therefore to investigate if the preconceptional chronic consumption of moderate amounts of ethanol affects *in vivo* fertilization (the pronucleous formation and/or the nuclear status of the zygotes), and if previous intake of ethanol has deleterious effects on different stages of preimplantation embryo development *in vivo*.

MATERIALS AND METHODS

Animals

Groups of three to four hybrid F1 mice (C57/B1 × CBA) from our colony were kept in plastic cages and maintained under controlled room temperature (25°C) and light cycles (14 h light: 10 h dark, lights on at 06 : 00). They were fed *ad libitum* with a commercial mouse chow diet (Diet No. 1, Nutrimentos S.A., Buenos Aires, Argentina). Daily caloric

*Author to whom correspondence should be addressed.

intake was estimated by the caloric value of the diet used (2900 kcal/kg). The amount of food consumed by female mice was determined by the difference between the offered chow and the remaining food plus the amount spilled.

Adult 75-day-old male mice (average body weight ± SEM: 29.4 ± 0.68 g) were used. Females were immature (prepubertal, 30-day-old, average body weight: 17.6 ± 0.35 g) at the start of the ethanol administration period. Control female mice were the same age and weight as the ethanol-treated mice at the beginning of the ethanol treatment.

Ethanol treatment

Immature female mice were treated with 10% (w/v) ethanol in drinking water for 30 days. The ethanol exposure was stopped at the beginning of pregnancy. Controls received water with maltose–dextrin (3.8 kcal/g) in a proportion of 56 g to 300 ml water, to be isocaloric with 10% ethanol. The body weights were recorded daily throughout the treatment in all females used. The amount of daily liquid intake was determined by volume differences between the offered and the remaining volume. Calories derived from ethanol 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 in five female mice for each experimental group.

The effects of preconceptional chronic moderate ethanol ingestion were examined on *in vivo* fertilization (day 1, pronucleus formation) and on *in vivo* development (from day 2 to day 4).

Blood-ethanol measurement

A group of five immature female mice chronically treated with ethanol as described above were decapitated and trunk blood was collected into heparinized Eppendorf tubes at 06 : 00 on day 30 of treatment. Samples were maintained at 4°C, for blood-ethanol determinations within 4 h of collection. Ethanol was measured by gas chromatography as described previously (Cebral *et al.*, 1997, 1998*a*).

In vivo fertilization

Female mice were induced to superovulate with 10 IU of pregnant mare's serum gonadotrophin (PMSG, Sigma Chemical Co., St Louis, MO, USA) given at 13 : 00 on day 28 of the ethanol treatment and 10 IU of human chorionic gonadotrophin (HCG, Sigma) 48 h later (day 30). One female was than caged immediately with one adult non-treated male, previously isolated. On the morning of the next day, mating was confirmed by the presence of the vaginal plug (day 1). The following groups were studied (1) control group: control females mated with control males; and (2) ethanol-treated group: ethanol-treated females mated with control males.

In vivo fertilization was evaluated at 24–32 h post-HCG. The zygotes from the oviducts of each female were recovered by flushing with M2 medium (Quinn *et al.*, 1982) and washed to remove the cumulus cells and the spermatozoa. They were placed into 100 µl of M16 medium (Whittingham, 1971), supplemented by 3% bovine serum albumin (BSA) and overlaid with mineral oil. The medium contained the vital fluorocrome Hoechst 33342 (0.5 µg/ml, Sigma). The embryos and/or oocytes were incubated for 1 h, washed and mounted for observation under a fluorescence microscope. They were classified as *fertilized oocytes* when they presented the second polar body (II PB) with two pronuclei (2PN) (fertilized normally). The triploid oocytes (polyspermic, with II PB and 3 PN) and oocytes with II PB and 2 PN were also considered as fertilized. *Unfertilized activated oocytes* were parthenogenetically activated and appeared with: II PB and 1 PN, II PB and dispersed chromosomes (DC) in the cytoplasm (metaphase plate disruption), 0 PB (without second polar body) and 1 PN and 2-cell embryo from immediate cleavage. *Unfertilized intact oocytes* were oocytes with normal metaphase II plate (Me II) or oocytes with abnormal metaphase (disruption of metaphase plate with decondensed and DC). Fragmented and/or necrotic (lysed) oocytes were recorded as *degenerated*.

In vivo development

Females were induced to superovulate with PMSG and HCG (10 IU) and caged with one male previously isolated at the time of HCG injection, as described before. On the following morning, mating was confirmed by the presence of the vaginal plug (day 1 of pregnancy). The same groups as indicated previously were studied.

(a) Collection of embryos. Females were killed by cervical dislocation at varying days of *in vivo* development. The eggs, embryos and other elements (intact, fragmented and/or dead oocytes, empty zona pellucida) from each female were recovered from the oviducts and/or uteri by flushing with M2 medium. The cells were washed to remove pieces of tissue and placed in a 100-µl drop of M16 medium supplemented with BSA 3% and covered with mineral oil, for examination under an inverted phase contrast microscope and for further studies. Results were expressed as the mean percentage of embryos calculated with the total number of elements (intact or degenerated eggs, embryos and/or oocytes) recovered per female.

(b) Evaluation of general development. On day 2 (48 h post-HCG), 2-cell embryos were quantified from the oviducts. Other elements were recorded: II PB (one cell with second polar body), 4-cell embryos (embryos derived from activated oocytes or immediatelly cleaved embryos), oocytes without zona pellucida and/or empty zona pellucida.

On day 3 (74–76 h post-HCG), embryos recovered from oviducts were at the morula stage (8-cell uncompacted and compacted embryos). Other elements can also appear: 1-, 2- or 4-cell embryos and/or unfertilized eggs.

On day 4 (96 and 99 h post-HCG), the following were obtained: morulae, no hatched blastocysts (non-extruded embryos from the zona pellucida) and hatched blastocyst (zona-free embryos). The blastocyst differentiation grade was assessed by quantification under phase-contrast microscopy as the very early blastocyst (embryo with small vesicles in blastomeres), early blastocyst (with an initial visible blastocele), expanded blastocysts (with expansion of the cavity), hatched early blastocysts (zona-extruded blastocysts with visible cavity), and hatched attached blastocysts (extruded from the zona pellucida without a visible cavity or implanting blastocyst appeared). This embryo classification was completed and confirmed by counting the number of nuclei/embryo, as indicated above. Non-fragmented embryos recovered from oviducts or uteri were quantified to evaluate the utero-tubaral distribution of embryos 99 h post-HCG.

(c) Evaluation of embryo quality. Embryo quality was determined by the following parameters: extracellular

fragmentation (presence of small clumps of cytoplasm surrounded by membrane in the perivitelline space); no intact blastomeres (with necrotic signs or lysis); blastomeres with unequal size; decompacted blastomeres in compacted morulae; abnormal cavitation (two or more blastoceles); vesicles in ectoderm; small inner cell mass. When the embryo presented with more than 50% of fragmentation, it was considered to be fragmented. Embryos with high rates of lysis were considered to be dead embryos. Both fragmented and dead embryos were classified as 'degenerated'.

(d) Evaluation of embryo growth. On days 3 and 4, the number of cells/embryo was determined by counting the nuclei of individual embryos recovered from each group, according to the air-drying technique of Tarkowski (1966). Briefly, the embryos were swollen in drops of 0.9% sodium citrate for 8–10 min and transferred to clean slides to fix for 30 s by dropping a single drop of methanol : acetic acid (3 : 1 v/v) over the embryos, and air dried. The cytoplasm of the embryos was dispersed by this technique leaving only chromatin behind. The cells were stained with Giemsa solution (2 : 1, v/v) for 10 min and the nuclei were examined under light microscopy.

On day 3, the mean number of cells/embryo, mean number of mitoses/embryo and the percentage of embryos with mitotic cells were quantified in the embryos recovered in each group. The mitotic index (MI) was calculated as the total number of metaphases/total number of cells. On day 4 (96–99 h post-HCG), the distribution of nuclei/embryo was assessed in a total of 56 embryos from seven control females and 54 embryos from seven ethanol-treated females. The result was expressed as the percentage of embryos vs the number of cells/embryo range.

Statistics

The mean percentages of developed embryos and SEM were calculated with the total elements recovered/treated female (intact embryos, eggs, zygotes, oocytes and/or others) (n = no. of females used). The data from these two groups were analysed by Student's t-test. The percentages of embryos in the evaluation of the embryo growth were examined by the χ^2 test. The Instat Program (GraphPAD software, San Diego, CA, USA) was used for calculations. A $P < 0.05$ was considered as significant.

RESULTS

Average daily intakes of food, fluid, and ethanol in females, expressed in g, ml, and/or kcal/mouse respectively, are shown in Table 1. These data were recorded in five female mice for each group. The percentage of ethanol derived calories (% EDC) was 26.8%. The body weight reached at the end of the treatment was similar in the control and ethanol-treated females used in all studies. The total caloric intake was similar in both groups. Blood-ethanol levels on the morning of the last day of treatment were 48.1 ± 1.5 mg/dl.

Table 1. Daily intake of food, fluid, and percentage of ethanol-derived calories in female mice chronically treated with 10% ethanol

	Food intake g (kcal)	Fluid intake (ml)	Ethanol intake g (kcal)	% EDC	Body weight (g)
Control females	3.7 ± 1.1 (10.7 ± 0.7)	9.4 ± 0.7	—	—	25.4 ± 0.5
Treated females	5.3 ± 0.5 (15.3 ± 0.1)***	7.8 ± 1.1	0.8 ± 0.3 (5.6 ± 0.5)	26.8	26.8 ± 1.2

Daily food and fluid intake was assessed in five control and five ethanol-treated females. The amounts given in g, ml or kcal are mouse/day. The kcal values are in parentheses. % EDC, percentage of ethanol-derived calories. ***$P < 0.001$, Student's t-test.

In vivo fertilization

Table 2 shows that chronic 10% ethanol treatment produced a significantly decreased percentage of normal fertilized oocytes (with II PB and 2 PN), as compared to the control females [$P < 0.01$, Table 2(a)]. Unfertilized activated eggs (with II PB and 1 PN) were present significantly more in the treated females than in the controls [$P < 0.01$, Table 2(b)], but also a significantly increased percentage of oocytes with 0 PB and 1 PN (diploid eggs) could be seen in the treated group [$P < 0.05$, Table 2(b)]. A significantly elevated percentage of unfertilized intact oocytes with abnormal metaphase plates was found in the treated females, as compared to the controls [$P < 0.05$, Table 2(c)]. The total percentage of fragmented and/or dead oocytes was similar between the control and the ethanol-treated group (data not shown).

In vivo development

On day 2, there was a similar percentage of 2-cell embryos in both control and ethanol-treated females [Table 3(a)], but the percentage of morphologically abnormal 2-cell embryos was significantly increased in the treated, compared to the control, group ($P < 0.05$). No significant group differences were observed in other cells [Table 3(b)].

On day 3 (76 h post-HCG), the total percentage of morulae was significantly decreased in the ethanol-treated, as compared to the control, females [$P < 0.01$, Table 4(a)]. The compacted morula stage was significantly reduced ($P < 0.001$) and uncompacted morulae were increased ($P < 0.05$) in the ethanol-treated group compared to the control group. The percentage of degenerated embryos and others (1-, 2- or 4-cell embryos, unfertilized eggs) recovered from the oviducts was significantly increased in the ethanol-treated females compared to the control females [$P < 0.05$ and $P < 0.01$, Table 4(b)]. The growth of embryos was analysed and the mitotic cells were assessed to evaluate the timing of division. The mean number of cells/embryo was significantly decreased in the ethanol-treated, as compared to the control, group [$P < 0.05$, Table 4(c)]. The treated females presented a significantly higher mitotic index than the control females ($P < 0.05$) [Table 4(c)].

On day 4, the effects of 10% ethanol consumption on the *in vivo* development was first studied at 96 h post-HCG. In the control females, almost all embryos were at the hatched blastocyst stage and very few embryos were still morulae, whereas in the ethanol-treated females, the percentage of hatched blastocysts was significantly decreased ($P < 0.05$) and the percentage of morulae increased ($P < 0.05$) (data not shown). In the ethanol-treated females, the mean total

Table 2. Effect of chronic 10% ethanol intake on *in vivo* fertilization

(a) Fertilized eggs

Group	*N*	*n*	II PB + 2 PN	II PB + 3 PN	2 PB + 2 PN	Total fertilized
Control	6	219	77.4 ± 2.4	0	1.2 ± 0.7	78.5 ± 2.6
Ethanol	5	174	61.4 ± 4.9**	0	9.2 ± 3.9*	70.5 ± 3.6*

(b) Unfertilized activated eggs

Group	*N*	II PB + 1 PN	II PB + DC	0 PB + PN	2-cell	Total activated
Control	6	1.1 ± 0.6	0.5 ± 0.5	0	0.6 ± 0.6	2.2 ± 0.5
Ethanol	5	10.4 ± 2.7**	0	0.6 ± 0.2*	1.7 ± 1.3	12.8 ± 3.4**

(c) Unfertilized intact eggs

Group	*N*	Me II	Abnormal Me	Total intact
Control	6	2.1 ± 0.9	0	3.4 ± 0.8
Ethanol	5	2.8 ± 2.0	1.9 ± 0.9*	4.7 ± 1.4

In vivo fertilization was evaluated at 24–26 h post-HCG (day 1). Recovered eggs were analysed after incubation in Hoechst 33342 and observation under a fluorescence microscope. The results are expressed as the mean percentage of eggs with respect to the total number of elements recovered/female ± SEM. *N*, number of females used; *n*, total number of eggs and other elements recovered, II PB + 2 PN, percentage of normal fertilized eggs; II PB + 3 PN, triploid eggs; 2 PB + 2 PN, with two polar bodies; II PB + 1 PN, second polar body and one pronucleus; II PB + DC, second polar body with dispersed chromosomes in the cytoplasm; 0 PB + PN, absence of second polar body and one or two pronuclei; 2-cell, embryos with two cells from immediate cleavage; Me II, oocytes with normal metaphase II plate; Abnormal Me, oocytes with dispersed chromosomes in the cytoplasm and/or disrupted metaphase plate; control, embryos from control females mated with control males; Ethanol: ethanol-treated females. *$P < 0.05$, **$P < 0.01$, Student's *t*-test.

percentage of embryos/female was significantly reduced compared to controls (59.8 ± 11.2 vs 94.6 ± 3.6, $P < 0.01$) (data not shown).

Table 5 shows the embryo stages developed on day 4 at 99 h post-HCG. In the ethanol-treated group, the percentage of morulae and un-hatched blastocysts was significantly increased compared to the control group [$P < 0.05$, Table 5(a)], but the hatched blastocyst and the total number of blastocysts was significantly decreased in the ethanol-treated group, compared to the control group ($P < 0.05$). When the blastocyst differentiation was analysed, the percentage of early blastocyst was increased significantly ($P < 0.01$), and the percentage of attached blastocyst stage was significantly reduced in the ethanol-treated females, compared to the control females [$P < 0.01$, Table 5(b)]. The total percentage of fragmented and/or dead embryos and the percentage of abnormal embryos were significantly higher in the ethanol-treated, than in the control, group [$P < 0.01$ and $P < 0.05$, Table 5(c)].

The utero-tubaral distribution of embryos recovered at 99 h post-HCG is shown in Table 6. As can be seen, in the ethanol-treated females, almost all non-fragmented embryos of the total percentage of embryos recovered (71.5%) were found in the uteri, and the percentage was significantly reduced, compared to the control females ($P < 0.05$). The rest of the embryos were found in the oviduct tract, but we did not observe significant differences between the control and the ethanol-treated groups.

Embryo growth was analysed by counting the number of cells/embryo recovered on day 4. This evaluation allowed the correct classification of embryos in stages of morulae (less than 32 cell embryos), very early blastocyst, early blastocyst, expanded blastocyst, and hatched blastocyst (both early and attached) (Table 7). In the ethanol-treated females, the percentage of embryos with 31–40 cells/embryo (very early and early blastocyst) was significantly increased, compared to the controls ($P < 0.05$) (Fig. 1). The percentage of embryos with more than 60 cells (preferentially hatched blastocysts) was significantly lower in the treated group than in the control group ($P < 0.001$) (Fig. 1).

Table 3. Effects of 10% ethanol consumption on day 2

(a) 2-cell embryos

Group	*N*	*n*	Total embryos	Abnormal 2-cells
Control	6	204	79.1 ± 4.4	2.4 ± 1.6
Ethanol	6	240	78.8 ± 5.0	6.5 ± 1.6*

(b) Others

Group	*N*	II PB	4-cells	Fragm/Dead	Others
Control	6	3.1 ± 1.9	0	10.7 ± 3.6	7.1 ± 0.7
Ethanol	6	1.4 ± 0.6	0	14.5 ± 3.8	5.3 ± 1.8

On day 2 (48 h post-HCG), the total embryos and other elements were recovered from the oviducts of each female and quantified and expressed as the mean percentage ± SEM. *N*, number of females; *n*, total number of eggs, embryos and other elements recovered; Mean total percentage ± SEM of two-cells, morphologically normal embryos; abnormal 2-cells, morphologically abnormal embryos; 4-cells, percentage of 4-cell embryos derived from activated eggs (immediately cleaved embryos); II PB, percentage of 1-cell embryos with second polar body; Fragm/Dead, degenerated embryos; Others, intact oocytes, oocytes without zona pellucida (ZP) and/or empty ZP. *$P < 0.05$, Student's *t*-test.

Table 4. Effects of 10% ethanol consumption on day 3

(a) Morula stage

Group	*N*	*n*	Normal morulae		Abnormal mor. (UM + CM)	Total morulae
			Un.mor.	C.mor.		
Control	9	230	4.7 ± 2.1	67.4 ± 3.2	0.3 ± 0.3	72.4 ± 4.0
Ethanol	8	133	15.3 ± 7.5*	15.3 ± 9.0***	0.9 ± 0.5	31.6 ± 10.3**

(b) Other elements

Group	*N*	Fragmented	Dead	Total fragm/Dead	Others
Control	9	8.3 ± 3.0	16.2 ± 3.4	24.7 ± 3.5	2.1 ± 1.3
Ethanol	8	17.9 ± 3.2*	25.6 ± 6.6	43.5 ± 7.6*	24.9 ± 7.5**

(c) Embryo growth: cell number/embryo and mitotic index

Group	*N*	*ne*	Cells/emb.	Mit./emb.	Mit.emb. (%)	Mit.Index (MI)
Control	6	60	13.2 ± 0.5	0.8 ± 0.2	21/60 (35)	49/781 (0.06)
Ethanol	5	39	11.5 ± 0.6*	1.1 ± 0.3	16/39 (41)	42/447 (0.09) a

The embryos recovered on day 3 (74–76 h post-HCG) from oviducts of each female were quantified under a phase contrast microscope and expressed as the mean percentage ± SEM/female. Morulae were analysed for the number of cells and mitotic nuclei by the Tarkowski (1966) method. Un.mor, uncompacted morulae; C.mor, compacted morulae. Fragmented and/or dead embryos and other elements recovered; (1-, 2- or 4-cell embryos, unfertilized eggs) were assessed. *N*, number of females; *n*, total number of embryos and others recovered; *ne*, number of embryos analysed for the number of cells/embryo (Cells/emb.); Mit./emb., mean number ± SEM of mitosis/embryo; Mit.emb., percentage of embryos with mitotic cells; Mit.Index (MI), Mitotic Index (total number of metaphases/total number of cells); Control, embryos from control females mated with control males. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's *t*-test; $^{a}P < 0.05$, χ^2-test.

Table 5. Effects of 10% ethanol consumption on day 4 of development: embryos from 99 h post-HCG

(a) Developed embryos

Group	*N*	Morulae	Blastocyst			Total embryos
			Unhatched	Hatched	Total Blast.	
Control	5	2.6 ± 1.5	9.2 ± 6.0	79.7 ± 10.0	88.8 ± 7.6	91.4 ± 5.4
Ethanol	5	12.6 ± 5.0*	28.3 ± 7.6*	31.1 ± 14.7*	58.8 ± 9.2*	71.5 ± 8.5*

(b) Blastocyst differentiation

Group	*N*	E.Blast.	Exp.Blast.	Hatched Blast.		
				Early	Attached	Total
Control	5	1.4 ± 1.4	7.8 ± 5.7	20.0 ± 9.4	59.7 ± 7.3	79.6 ± 10.3
Ethanol	5	11.5 ± 2.8**	16.7 ± 5.7	11.7 ± 5.5	19.4 ± 11.0**	31.1 ± 10.5*

(c) Quality of embryos

Group	*N*	Total fragm/Dead	Abnormal embryos
Control	5	8.5 ± 2.1	1.2 ± 1.1
Ethanol	5	23.0 ± 7.5*	30.2 ± 9.4**

The embryos were recovered by flushing from oviducts and uteri of each female and analysed under a phase contrast microscope for development, embryo differentiation, and quality (morphologically abnormal and fragmented, and/or dead embryos). *N*, number of females; Total embryos (%), morulae plus blastocysts; results are the mean percentage ± SEM. E., early blastocyst; Exp., expanded blastocyst; Hatched early, extruded blastocyst with blastocele; Hatched attached, implanting blastocyst (hatched and without cavity embryo); Control, embryos from control females mated with control males. $*P < 0.05$, $**P < 0.01$, Student's *t*-test.

Table 6. Utero-tubaral distribution of embryos

Group	*N*	Oviducts	Uteri
Control	5	0	91.5 ± 5.4
Ethanol	5	5.3 ± 4.4	68.5 ± 8.6*

The non-fragmented embryos recovered from the oviducts or uteri from each female were quantified for the utero-tubaral distribution. *N*, number of females used; Control, embryos from control females mated with control males. Results are expressed as the mean percentage ± SEM/female. *$P < 0.05$, Student's *t*-test.

Table 7. Stages of embryo development *in vivo* in relation to the number of nuclei on day 4

Embryo stage	No. of cells/embryo	Optical characteristics
Morula	<32	8–16 cells: uncompacted embryo 16–32 cells: compacted blastomeres
Very early blastocyst	32–35	Formation of fluid-filled vesicles in the cytoplasm
Early blastocyst	36–40	With one or two vacuoles which enlarge and subsequently coalesce and formation of visible initial small size of embryo
Expanded blastocyst	41–50	Blastocele in the overall size of embryo Distinguishable inner cell mass from the trophoblastic cells
Hatched blastocyst	>50	Early hatched blastocyst: without visible zona pellucida, blastocele not fully expanded. Attached hatched blastocyst: blastocele not visible, smaller embryo

The correct classification of embryo stages on day 4 of *in vivo* development was done by assessing the number of cells/embryo. The general characteristics of each embryo were observed under a phase contrast microscope before placing the embryo on slides to count the nuclei by the Tarkowski (1966) method, as described in the Materials and methods section.

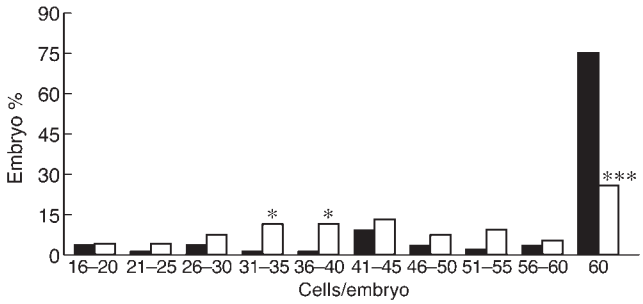

Fig. 1. Embryo growth: number of cells/embryo on day 4.
The total number of nuclei/embryo was determined by the Tarkowski (1966) method. Columns represent the percentage of embryos in each range of number of cells assessed. Black columns: control females, white columns: ethanol-treated females mated with control males. *$P < 0.05$, ***$P < 0.001$, χ^2-test.

DISCUSSION

We postulated that prolonged ingestion of moderate doses of ethanol by young female mice prior to gestation could affect *in vivo* fertilization and the preimplantation *in vivo* development. We considered it relevant to evaluate the zygotes after mating, on day 1 of development, in order to study not only the *in vivo* fertilization rates, but also the nuclear status of the eggs. We found that the normal fertilized oocyte rates were reduced in the treated females, because elevated parthenogenetic activation values were observed. These results are in agreement with our previous studies, in which chronic consumption of 10% ethanol produced a high percentage of oviductal activated oocytes (1 PN) in the cumulus–oocyte complexes at 16 and 20 h post-HCG without fertilization (Cebral *et al.*, 1998*b*). Moreover, we have seen that chronic moderate ethanol exposure produced a reduced percentage of fertilized oocytes after *in vitro* insemination, because there was an elevated percentage of haploid oocytes (1 PN) (Cebral *et al.*, 1999). It has been suggested that ethanol can induce parthenogenetic activation of oocytes (meiosis resumption) (Kaufman, 1978) by releasing internal calcium pools (Whittingham, 1980). Calcium signalling during parthenogenetic egg activation was postulated to affect preimplantation development, even several cell divisions after Ca^{2+} oscillations occur (Vitullo and Ozil, 1992; Bos-Mikich *et al.*, 1997). Further, calmoduline activation by Ca^{2+} initiates several cellular events (Lu and Means, 1993), including altered gene expression (Hama *et al.*, 1995). Prolonged ethanol exposure could not only trigger Ca^{2+} signals and activation of the oocyte, but also alter gene transcription, as occurs in neuronal (Wilke *et al.*, 1994; Ghosh and Greenberg, 1995) and non-neuronal (Fatayerji *et al.*, 1996) cell types. Ethanol treatment for a long period of time was capable of inducing abnormal metaphase plates and therefore chromosomes could be dispersed in the cytoplasm of intact oocytes after fertilization. Some mechanism(s) of these ethanol effects may be related to the involvement of abnormal chromosome segregation (Kaufman, 1983). Multipolar spindles and some microtubules at the periphery of the spindle, that may be associated with disorganized alignment of the chromosomes, were encountered following ethanol exposure (O'Neill and Kaufman, 1989). High incidence of aneuploidy due to ethanol intake around the ovulation time (Kaufman and Bain, 1984*a*; Kaufman, 1985), interference with cell division (Kaufman, 1997), and cytoskeletal structure and function of the spindle apparatus (Kaufman, 1985; O'Neill and Kaufman, 1989), disrupted regulation of normal calcium concentrations in the proximity of the spindle apparatus during cytokinesis (Kaufman, 1997) is also attributed to ethanol exposure.

We have found previously that 10% ethanol produced elevated fragmentation in the oocyte–cumulus complex recovered at 16 h post-HCG and maintained for 5 h in culture medium with or without spermatozoa (Cebral *et al.*, 1998*b*, 1999). In the present study, the treated females did not show increased fragmentation rates at 32 h post-HCG. Perhaps, even when blood-ethanol levels of 50 mg/dl were considered by us to be toxic to the ovarian oocyte (Cebral *et al.*, 1998*a*), the oviductal environment could protect against increased

fragmentation in the eggs. This is in agreement with our previous study which showed that the percentage of fragmented oocytes recovered from the ampullae 20 h post-HCG was similar in both groups. We believe that the altered oocyte quality and fertilization are more clearly manifested in *in vitro* conditions. Moreover, the oocyte susceptibility to the deleterious effects of ethanol for increasing apoptosis is more evident by *in vitro* manipulation of the gamete.

We investigated whether ethanol treatment could alter the embryo growth and/or morphology at earlier days of development *in vivo*. On day 2, although development of 2 cell embryos was similar in both groups, embryos from ethanol-treated females presented different blastomere size or absence of polar body or fragments in the perivitelline space. These abnormal embryos could undergo arrest later, because on day 3, 2- or 4-cell embryos were also observed. However, since the percentage of 2-cell embryos developed *in vivo* was not found to be reduced, compared to controls, but significantly reduced when developed *in vitro* (Cebral *et al.*, 1999), we believe that the embryos from alcoholic females have severely impaired development *in vitro*.

On day 3 of gestation, a delayed development accompanied by augmented embryo fragmentation was also seen, since reduced numbers of compacted morulae and increased numbers of uncompacted embryos were found in the ethanol-treated females. The decreased number of cells/embryo and the elevated mitotic index suggest that even the smaller morulae from ethanol-treated females had cells undergoing division and therefore late cell division. It has been observed that haploid or diploid parthenogenetic embryos can develop more slowly than control fertilized diploid ones (Kaufman, 1990; Henery and Kaufman, 1992). We think that this delay is due to the nuclear status of embryos induced earlier by ethanol exposure. This is also suggested by the observation of the nuclear status of the oocytes post *in vivo* fertilization (day 1). Moreover, previous reports have demonstrated that monosomic or trisomic embryos induced by maternal exposure to ethanol are capable of reaching at least the morula stage (Kaufman and Bain, 1984*b*). Indeed, when embryo growth was explored 96 and 99 h post-HCG (day 4), we found that the ethanol-treated females had reduced quantities of embryos (particularly the blastocyst stages), despite the similar rate of recuperation, compared to control females. A high number of these embryos showed morphological abnormalities, such as smaller size, presence of vesicles in the cytoplasm or fragments in several blastomeres and/or in the perivitelline space. We hypothesized that these embryo abnormalities may be caused by the prolonged pregestational ethanol consumption. Moreover, another adverse effect of ethanol could be the induction of apoptotic processes later in the development via the increase of cell fragmentation, as seen in the treated females. We think that morphologically abnormal embryos died later, because of fragmentation or cytoplasmic lysis, and that only the completely normal embryos survived and were implanted. It was suggested that parthenogenetic embryos can undergo apoptosis (Takase *et al.*, 1995), although little is known about the mechanisms underlying the degeneration of oocytes and embryos. Since only a few embryos reached the hatched blastocyst stage, we believe that ethanol consumption could impair not only the hatching process but also the development of competent implanting blastocysts. Furthermore, the lower number of cells in the embryos from the ethanol-treated females confirms reduced blastocyst development. These results suggest that severe embryo growth retardation could be induced by pregestational ethanol ingestion, and that these effects can be detected later in development, even when ethanol consumption is stopped. In consequence, the implantation might be reduced or delayed in ethanol-treated females. We have found similar results in the *in vitro* development model (Cebral *et al.*, 1999), when not only the process of blastocyst expansion was impaired or retarded, but also the total extrusion of the embryo from the zona pellucida was strongly affected by ethanol consumption.

The total percentage of non-fragmented/dead embryos recovered from each female was analysed for utero-tubaral distribution on day 4. The observation of reduced quantities of embryos in the uteri and a few in the oviduct tract in ethanol-treated females suggests retarded embryo transport and therefore impaired functioning of the maternal tissues. We believe that preconceptional ethanol consumption adversely affected the embryo and its capacity of development *in vivo* and *in vitro*, the functioning of maternal genital tract, and the embryo–maternal communication necessary for implantation.

In conclusion, this study shows that several stages of embryos developed *in vivo* were affected by chronic moderate ethanol ingestion when female mice were prepubertal and were treated before gestation. The retarded embryo development, morphological abnormalities, embryo losses, and impaired fertilization could be consequences of abnormal pronuclei formation due to impaired chromosome segregation during oocyte maturation.

Acknowledgements — The authors thank Dr Marcelo Viggiano for revising this paper and for suggestions regarding the statistical analysis. This work was supported by Grant PIP 4076 from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Bago S.A. (Argentina).

REFERENCES

Abel, E. L. (1995) An update on incidence of FAS: FAS is not an equal opportunity birth defect. *Neurotoxicology and Teratology* **17**, 437–443.

Bos-Mikich, A., Whittingham, D. G. and Jones, K. T. (1997) Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Developmental Biology* **182**, 172–179.

Cebral, E., Lasserre, A., Rettori, V. and Gimeno, M. A. (1997) Impaired mouse fertilization by low chronic alcohol treatment. *Alcohol and Alcoholism* **32**, 563–572.

Cebral, E., Lasserre, A., Faletti, A. and Gimeno, M. A. (1998*a*) Response to ovulatory induction following moderate chronic ethanol administration in mice. *Medical Science Research* **26**, 29–31.

Cebral, E., Lasserre, A., Motta, A. and Gimeno, M. A. (1998*b*) Mouse oocyte quality and prostaglandin synthesis by cumulus oocyte complex after moderate chronic ethanol intake. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **58**, 381–387.

Cebral, E., Lasserre, A., Rettori, V. and Gimeno, M. A. F. (1999) Deleterious effects of chronic moderate alcohol intake by female mice on preimplantation embryo growth *in vitro*. *Alcohol and Alcoholism* **34**, 551–558.

Checiu, M. C. and Sandor, S. (1982) The effect of ethanol upon early development in mice and rats. IV. The effect of acute ethanol intoxication on day 4 of pregnancy upon implantation and early post-implantation development in mice. *Morphology and Embryology* **28**, 127–133.

Checiu, M. and Sandor, S. (1986) The effect of ethanol upon early development in mice and rats. IX. Late effect of acute

preimplantation intoxication in mice. *Morphology and Embryology* **32**, 5–11.
Dyban, A. P. and Khozai, L. I. (1980) Parthenogenetic development of ovulated mouse ova under influence of ethyl alcohol. *Bulletin of Experimental Biology and Medicine* **89**, 528–530.
Eskay, R. L., Ryback, R. S., Goldman, M. and Majchrowicz, E. (1981) Effect of chronic ethanol administration on plasma levels of LH and the estrous cycle in the female rat. *Alcoholism: Clinical and Experimental Research* **5**, 204–206.
Fatayerji, N., Engelman, G. L., Myers, T. and Handa, R. J. (1996) In-utero exposure to ethanol alters mRNA for insulin-like growth factors and insulin-like growth factor-binding proteins in placenta and lung of fetal rats. *Alcoholism: Clinical and Experimental Research* **20**, 94–100.
Ghosh, A. and Greenberg, M. E. (1995) Calcium signaling in neurons: molecular mechanism and cellular consequences. *Science* **268**, 239–247.
Grodstein, F., Goldman, M. B. and Cramer, C. W. (1994) Infertility in women and moderate alcohol use. *American Journal of Public Health* **84**, 1429–1432.
Hama, N., Paliogianni, F., Fessler, B. J. and Boumpas, D. T. (1995) Calcium/calmodulin-dependent protein kinase II downregulates both calcineurin and protein kinase C-mediated pathways for cytokine gene transcription in human T cells. *Journal of Experimental Medicine* **181**, 1217–1222.
Henery, C. C. and Kaufman, M. H. (1992) Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. *Molecular Reproduction and Development* **31**, 258–263.
Kaufman, M. H. (1978) The experimental production of mammalian parthenogenetic embryos. In *Methods in Mammalian Reproduction*, Daniel, J. C. ed., pp. 21–47. Academic Press, New York.
Kaufman, M. H. (1983) Ethanol-induced chromosomal abnormalities at conception. *Nature* **302**, 258–260.
Kaufman, M. H. (1985) An hypothesis regarding the origin of aneuploidy in man: indirect evidence from an experimental model. *Journal of Medical Genetics* **22**, 171–178.
Kaufman, M. H. (1990) *Early Mammalian Development: Parthenogenetic Studies*, pp. 144–145. Cambridge University Press, Cambridge.
Kaufman, M. H. (1997) The teratogenic effects of alcohol following exposure during pregnancy and its influence on the chromosome constitution of the pre-ovulatory egg. *Alcohol and Alcoholism* **32**, 113–128.
Kaufman, M. H. and Bain, I. M. (1984*a*) Influence of ethanol on chromosome segregation during the first and second meiotic divisions in the mouse egg. *Journal of Experimental Zoology* **230**, 315–320.
Kaufman, M. H. and Bain, I. M. (1984*b*) The development potential of ethanol-induced monosomic and trisomic conceptus in the mouse. *Journal of Experimental Zoology* **231**, 149–155.
Lu, K. P. and Means, A. R. (1993) Regulation of the cell cycle by calcium and calmodulin. *Endocrinology Reviews* **14**, 40–58.
Mello, N. K. (1988) Effects of alcohol abuse on reproductive function in women. *Recent Developments in Alcoholism*, pp. 253–276. Plenum, New York.
O'Neill, G. T. and Kaufman, M. H. (1989) Cytogenetic analysis of ethanol-induced parthenogenesis. *Journal of Experimental Zoology* **249**, 182–192.
Quinn, P., Barros, C. and Whittingham, D. G. (1982) Preservation of hamster oocytes to assay the fertilization capacity of human spermatozoa. *Journal of Reproduction and Fertility* **66**, 161–168.
Takase, K., Ishikawa, M. and Hoshiai, H. (1995) Apoptosis on degeneration process of unfertilized mouse ova. *Tohoku Journal of Experimental Medicine* **175**, 69–76.
Tarkowski, A. K. (1966) An air drying method for chromosome preparations from mouse eggs. *Cytogenetics (Basel)* **5**, 394–400.
Tuma, D. J. and Sorrell, M. F. (1987) Functional consequences of acetaldehyde binding to proteins. *Alcohol and Alcoholism* **22** (Suppl. 1), 61–66.
Tuma, D. J., Smith, S. L. and Sorrell, M. F. (1991) Acetaldehyde and microtubules. *Annals of the New York Academy of Sciences* **625**, 786–792.
Van Thiel, D. H., Gavaler, J., Lester, R. and Sherins, R. J. (1978) Alcohol-induced ovarian failure in the rat. *Journal of Clinical Investigation* **61**, 624–632.
Vitullo, A. D. and Ozil, J. P. (1992) Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. *Developmental Biology* **151**, 128–136.
Weston, W. M., Greene, R. M., Uberti, M. and Pisano, M. M. (1994) Ethanol effects on embryonic craniofacial growth and development: implications for study of the fetal alcohol syndrome. *Alcoholism: Clinical and Experimental Research* **18**, 177–182.
Whittingham, D. G. (1971) Culture of mouse ova. *Journal of Reproduction and Fertility* **14**, (Supp.), 7–21.
Whittingham, D. G. (1980) Parthenogenesis in mammalians. In *The Oxford Reviews of Reproductive Biology*, Finn C. A. ed., pp. 205–231, Clarendon Press, Oxford.
Wilke, N., Sganga, M., Barhite, S. and Miles, M. F. (1994) Effects of alcohol on gene expression in neural cells. *EXS* **71**, 49–59.