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RESEARCH ARTICLE

Peri-implantational *in vivo* and *in vitro* embryo-trophoblast development after perigestational alcohol exposure in the CD-1 mouse

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Abstract

Long-term pregestational ethanol exposure induced altered fertilization and preimplantation embryogenesis. We evaluated preimplantational embryo-trophoblast differentiation, growth and invasiveness after perigestational ethanol 10% ingestion for 15 days preceding and up to day 4 (treated females [TF]: TF-D4 group) or 5 (TF-D5) of CD-1 gestation (control females [CF] with water). In TF-D4, expanded and hatched blastocyst numbers were significantly reduced ($p < 0.05$) versus CF-D4. Abnormal embryos and percentage of pyknotic nuclei were increased, and early blastocyst growth (nuclear number/embryo) and mitotic index was reduced ($p < 0.05$) versus CF-D4. On day 5 of gestation, TF-D5 presented significantly reduced total embryos and advanced embryo type 3 number versus CF-D5 ($p < 0.05$). During *in vitro* development, up to 72-hour culture, TF-D5 had reduced embryo type 1 (the least developed) and 3 percentages ($p < 0.05$) versus controls, whereas embryo type 2 percentage increased ($p < 0.05$) versus CF-D5. Embryo-trophoblast growth was studied during culture by morphometry. Embryo size ranges were classified as small, medium and large embryos. At 48-hour culture, small and medium embryos of TF had significantly increased mean area versus CF ($p < 0.05$), whereas large embryos had reduced mean area at 24-hour culture. Perigestational alcohol exposure up to days 4–5 induced embryo differentiation retardation, abnormal blastocyst growth and alterations of embryo-trophoblast growth and expansion during implantation, suggesting impaired regulation of trophoblast invasion and a relation with early pregnancy loss after mouse perigestational alcohol consumption.

Keywords

Alcohol, blastocyst, differentiation, growth, mouse, trophoblast

History

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Introduction

Maternal alcohol ingestion produces adverse embryo/fetal development and gestation outcomes, spontaneous abortion, prematurity, growth retardation and congenital malformations. These defects are collectively referred to as fetal alcohol spectrum disorders (FASDs) (Astley, 2004). According to the Institute of Medicine's revised classification system (Hoyme et al., 2005), there are currently six recognized diagnoses: fetal alcohol syndrome (FAS) with and without confirmed maternal alcohol exposure; partial FAS with and without confirmed maternal alcohol exposure; alcohol-related birth defects and alcohol-related neurodevelopmental disorder. After spontaneous abortion, FAS, the most adverse clinical outcome resulting from prenatal alcohol exposure, lies at the extreme end of this spectrum and is associated with three broad domains: prenatal and/or postnatal growth retardation; distinctive facial features and brain damage (Hoyme et al., 2005).

Ethanol effects in the embryo and gestation depend on doses and concentrations of ethanol, route of administration and time and period of exposure. In relation to this, epidemiological and animal studies clearly indicate that the clinical variability of FASD is related to three distinct window periods of exposure: preconception, preimplantation, gastrulation (Haycock & Ramsay, 2009) and organogenesis. We have shown previously that ethanol exposure for 1 month before gestation induced, in immature hybrid female mice, deleterious effects on oocytes (Cebral et al., 1998a), fertilization (Cebral et al., 1997) and preimplantation embryo development (Cebral et al., 1999, 2000, 2001). However, evidences of embryo growth alterations were reported when preimplantation mouse embryos were *in vitro* exposed to ethanol (Kowalczyk et al., 1996; Stachecki et al., 1994a). We postulate that short-term alcohol ingestion before gestation and during peri-implantation is able to produce embryo differentiation and growth alterations around the time of implantation.

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During preimplantation, the mouse embryo develops into the blastocyst, which consists of two types of cells. The outer cells surround the fluid cavity of blastocoel and constitute the trophoectoderm (TE), whereas the inner cell mass (ICM) is a group of cells attached to the inside of the TE. TE cells are involved in implantation (Sasaki, 2010). After hatching, interactions with the uterus (from stages E4 to E6.5) involve blastocyst attachment and trophoblast outgrowth. The mural TE cells stop dividing, continue to replicate DNA to become polyploidy and differentiate into invasive trophoblast giant cells (TGCs). From the polar TE emerge the extraembryonic ectoderm and the ecoplacental cone (Bevilacqua & Abrahamsohn, 1988). The polar TE continues proliferating and gives rise to all the remaining trophoblast cell types of the placenta (Simmons et al., 2007). After tight apposition between the luminal epithelium and the apical surface of TE, TGCs attach to the basal membrane and completes their differentiation into the invasive phenotype. At the apposition and adhesion phases, the pinopodes of the apical uterine epithelium surface interdigitize with microvilli on the apical TE surface of the blastocyst. Finally, invasion starts with penetration of the trophoblast through the uterine epithelium (Staun-Ram & Shalev, 2005).

The trophoblast exerts a crucial role in implantation and placentation; impaired trophoblast invasion has been implicated in several complications of pregnancy, such us unexplained miscarriage (Khong et al., 1987), pre-eclampsia and intrauterine growth restriction (Khong et al., 1986). Because we recently showed that perigestational ethanol 10% exposure 17 days preceding gestation and up to day 10 of gestation produced, in outbred CF-1 adult females, reduced embryo viability, delayed embryo differentiation and growth, increased frequency of neural tube defects (Coll et al., 2011) and impaired the arachidonic acid metabolism (Cebral et al., 2007), we hypothesized that organogenic embryo-trophoblast alterations are the result of abnormal peri-implantational embryogenesis induced by alcohol exposure preceding gestation and up to day 4 and/or 5 of gestation.

The implantation process has been widely studied *in vitro*. Because blastocysts incubated on Petri dishes adhere and outgrowth reflecting their invasive capacity during the early stages of implantation *in utero* (Yelian et al., 1995), the *in vitro* implantation system was useful to enable evaluation of effects produced by *in vivo* exposure on cellular differentiation, trophoblast expansion and cell migration of outgrowing embryos.

Our aims were to examine the effects of short-term periconceptional ethanol exposure on CD-1 embryo-trophoblast differentiation and growth at pre- (day 4 of gestation) and implantation (day 5 of gestation) and evaluate potential ethanol effects on invasiveness during *in vitro* implantation.

Methods

Animals

Two-month-old sexually mature outbred CD-1 mice from the colony of the Breeding Laboratory of the Biomedical Science Institute of São Paulo University (São Paulo, Brazil) were housed in groups of 3–5 mice in separate same-sex communal cages. They were kept on a 12-hour light/dark cycle at controlled room temperature ($25 \pm 2\,^{\circ}C$) and were fed commercial mouse chow (Nuvilab CR-1; Nutrivital, Curitiba, Brazil) *ad libitum* and tap water. CD-1 female mice had an average body weight between 29 and 30 g at the outset of ethanol treatment.

Experimental design: alcohol administration

All procedures were performed in accord with the guidelines of the National Institutes of Health (Bethesda, MD) and animal experimentation ethics adopted by the Brazilian Society of Laboratory Animal Science and was approved by the ethics committee on animal experimentation.

Females were orally exposed to 10% (w/v) ethanol in drinking water *ad libitum* (treated females; TFs) previous to and during early gestation, and the ethanol solution was replaced every 2 days. After 15 days of exposure, they were caged overnight with a fertile nontreated CD-1 male (1:1). Positive mating was confirmed by observation of a vaginal plug on the following morning, which was considered day 1 of gestation. If the vaginal plug was not observed, the female was caged again with 10% ethanol-drinking water and, on afternoon of the same day, was again mated overnight. Finally, females without a vaginal plug were discarded and not included in the experimental groups. With this experimental protocol, three mating times were made in which the ethanol-deprivation intervals were 14 hours. Mated CD-1 females were housed again with 10% ethanol in drinking water and ethanol administration continued up to day 4 (TFs; number of pregnant females used: 6) or 5 of gestation (number of pregnant females: 23). Control females (CFs) for day 4 (number of pregnant females: 5) and control females for day 5 (number of pregnant females: 23) of gestation received ethanol-free drinking water *ad libitum*. Control and ethanol-treated female mice were weighed at the beginning, throughout and at the end of treatment. The amount of daily liquid intake was determined by volume differences between offered and remaining volumes. The amount of food consumed by female mice was determined by daily offered chow minus remaining food minus the amount spilled. Daily caloric intake was estimated by the caloric value of the diet used (3976 kcal/kg). From these data, daily patterns of caloric intake and percentage of ethanol-derived calories were determined. On the afternoon of day 4 (2:00 p.m.–4:00 p.m.) and on the morning of day 5 of gestation (10:00 a.m.–12:00 p.m.), control and treated mice were killed by cervical dislocation to study embryo trophoblast *in vivo* and *in vitro* development.

Embryo collection

Females were laparotomized to externalize uterine horns, uteri were removed into phosphate-buffered saline (PBS), 0.3% bovine serum albumin (BSA) and cut at the uterotubal junction and cervix. Embryos were collected in a Petri dish by flushing uterine horns with 0.5 mL/horn of prewarmed PBS (Sigma-Aldrich Chemical Co., St Louis, MO) supplemented with 10% fetal bovine serum (FBS) using a 25-gauge needle attached to a syringe. Embryos were recovered using a flame-pulled glass Pasteur pipette attached to a mouth piece, and debris was removed by repeated passage of embryos through

warm (37 °C) wash drops of PBS-FBS. Embryos were transferred into drops of Dulbecco's modified Eagle's medium (DMEM)/10% FBS (50 µL) under oil in plastic Petri dishes and were counted under a stereomicroscope, observed and photographed with an inverted phase-contrast microscope to evaluate differentiation, growth, morphology, cell division and trophoblast invasiveness capacity on days 4 and/or 5 of gestation.

In vivo embryo differentiation on day 4 and 5 of gestation

Total isolated embryos from each female were observed by inverted phase-contrast microscopy to be classified into their differentiation status. Preimplantation embryo differentiation stages on day 4 of gestation included morulae (uncompacted and/or compacted), early (with initial cavity), expanded (with expanded cavity) and hatched (zona pellucida extruded) blastocysts. Collected hatched/implantative embryos from day 5 of gestation were classified into differentiation status based on the morphologic aspect as type 1, type 2 or type 3 embryos. The presence of blastocoelic cavity (expanded or not), initial expansion of trofoectoderm cells (trophoectoderm cell processes) and differentiation of TGCs were observed (Table 1). Accordingly, the most developed type of embryo was type 3. The number of the embryo types over the total number of recovered embryos from each group was recorded and expressed as a percentage.

In vivo embryo growth on day 4 and 5 of gestation

To study embryo growth on day 4 of gestation, the nuclear number, quality and mitotic index (MI) were assessed by the air-drying technique described by Tarkowski (1966) on recovered morulae and blastocysts. Briefly, embryos were swollen in drops of 1% sodium citrate for 20 minutes, transferred to clean slides and fixed for 30 seconds by dropping methanol/acetic acid (3:1, v/v) onto embryos. With this, the cytoplasm was dispersed, leaving only the fixed nuclear chromatin. Then, slides were stained with Giemsa solution (1:10, v/v) for 10 minutes, washed in distilled water and air-dried as observed under light microscopy. Total nuclei per embryo were quantified and are expressed as the mean number of nuclei per embryo ± standard deviation (SD). The number of interphase nuclei was quantified over the total number of nuclei. Morphological appearance of interphase and pyknotic nuclei per embryo were observed and recorded. The MI was calculated by quantification of nuclei in prophase, metaphase, anaphase and telophase over the total nuclear number per embryo.

To analyze embryo-trophoblast growth on day 5 of gestation, embryonic area size (μm^2) was measured in images from an inverted phase-contrast microscope by tracing the perimeter of embryos with the Image-Pro© Plus software program (Media Cybernetics, Inc., Rockville, MD). All area data were divided into three area ranges, and, finally, embryos were classified into three growth categories: (1) small embryos, with a range area size between 4500 and 7400 μm^2; (2) medium embryos, with a 7401–10,300-μm^2 embryo area size and (3) large embryos, with a 10,301–13,200-μm^2 embryo area size. In each embryo growth category, mean area size per embryo and SD were calculated.

Culture of day 5 embryos

On the morning of day 5 of gestation, embryos from 7 CFs ($n = 50$) and 8 TFs ($n = 45$) were cultured in glass coverslips placed into wells of 24-well culture plates (3–5 embryos/well) and with 500 µL of DMEM (Sigma-Aldrich), supplemented with 10% FBS, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 1% minimum essential medium nonessential amino acids, 0.4 µg/mL of insulin, 520 µg/mL of L(+) lactic acid, 56 µg/mL of pyruvic acid, and 4 mg/mL of BSA. The embryo culture was performed under standard conditions (36.5 °C in a humid atmosphere of 5% CO_2 in air) for 72 hours.

Determination of embryo differentiation stages during *in vitro* culture

Embryos were observed every 24 hours of culture up to 72 hours and photographed with an inverted phase-contrast microscope to identify *in vitro* embryo stages of differentiation according to morphological appearance. The grade of trophoblast cell development and the presence of differentiated TGCs were noted. Finally, three embryo differentiation types for 24, 48 and 72 hours of culture were established and are summarized in Table 1. Frequencies (percentage) of embryo types for each of the culture times were calculated.

Embryo-trophoblast growth/expansion during *in vitro* culture by morphometry

During *in vitro* culture times, day 5 embryonic growth was evaluated by measurement of embryo-trophoblast expansion

Table 1. Morphological embryo types of differentiation.

Culture times	Embryo type 1	Embryo type 2	Embryo type 3
T 0 hours	With expanded blastocoelic cavity Without trophoblast processes	With reduced blastocoelic cavity With incipient trophoblast processes	Without visible blastocoelic cavity With low trophoblast processes
T 24 hours	No or little trophoblast processes Without TGCs	Low trophoblast processes With some TGCs	Low-to-moderate trophoblast processes With TGCs
T 48 hours	Without or low trophoblast processes With TGCs	Low-to-moderate trophoblast processes With TGCs	Moderate trophoblast processes With TGCs
T 72 hours	Moderate trophoblast processes With TGCs	Moderate-to-high trophoblast processes With TGCs	High trophoblast processes With TGCs

Embryos were observed from the beginning of the culture (T 0 hours, day 5 of gestation) to 72-hour culture every 24 hours and were classified as type 1, 2 and 3 of differentiation according to cavity, trophoblast cell expansion and differentiation of TGCs.

(area sizes [μm^2]; Image-Pro Plus; Media Cybernetics) in images from an inverted phase-contrast microscope. Three embryo growth categories were established according to area ranges for each of the culture times: for 24-hour culture, small (5400–22,930 μm^2), medium (22,931–40,460 μm^2) and large embryos (40,461–58,000 μm^2) and for 48-hour culture, small (5500–26,600 μm^2), medium (26,601–47,700 μm^2) and large embryos (47,701–69,000 μm^2). Finally, means of area and ± SD were calculated for each category of embryo size.

Determination of embryo morphology on days 4 and 5 of gestation

In vivo recovered and cultured embryos were examined and photographed by inverted phase-contrast microscopy and the following abnormalities were recognized: cytoplasmic or perivitelline fragmentation; intracellular vacuoles; no intact blastomeres (with signs of necrosis or lysis); blastomeres of unequal size; persistent uncompacted blastomeres in compacted morulae; swelling of cells or appearance of intracellular vacuoles; abnormal cavitation (two or more blastoceles) and vesicles in the trophoectoderm and small inner cell mass. The number of morphologically abnormal embryos over the total number of embryos was quantified.

Statistical analysis

Reported mean values ± SD of control and ethanol-treated groups were compared by one-way analysis of variance and the Student's *t*-test. Values of frequency (percentages) were evaluated by Fisher's exact test. For statistical analysis, we used GraphPad Instat software (v2.05 a; GraphPad Software Inc., San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

Results

Weight, food and liquid consumption in CD-1 mice

There were no weight differences between the groups either before or after ethanol exposure. At the beginning of the ethanol administration period, control females weighed a mean (±SD) of 30.4 ± 2.4 g and ethanol-treated females weighed 29.2 ± 4.7 g. By the end of the periconceptional period, weights were 31.07 ± 2.1 g for controls and 29.5 ± 1.8 g for the ethanol-treated group. During the ethanol exposure period, ethanol-treated mice drank a mean (± SD) of 177.4 ± 30.7 mL/kg/day (5.2 ± 0.9 mL/mouse/day) of ethanol. This represents an ethanol-derived calorie intake of approximately 125.9 ± 21.7 kcal/kg/day, which constituted 13.6% of total daily calorie intake. There were no differences in the daily pattern of food intake between the two groups. Total calorie intake in control and ethanol-treated groups was similar: 958.2 ± 219.8 and 926.3 ± 265.0 kcal/kg/day, respectively.

Effects of periconceptional ethanol exposure on CD-1 embryo differentiation, morphology, growth and nuclear quality on day 4 of gestation

First, we analyzed embryo development on day 4 of gestation. To determine the gestational performance of CD-1 female mice, the total number of recovered embryos was evaluated and quantified. In both control and ethanol-exposed females, the mean number of total recovered embryos (± SD) was similar in control and ethanol-exposed groups (CF: 11.2 ± 3.1, $N = 5$ females, $n = 56$ embryos; TF: 10.5 ± 2.2, $N = 5$; $n = 64$ embryos).

We analyzed embryo differentiation to ascertain whether periconceptional ethanol exposure was able to induce alterations in the timing of embryogenesis. The embryonic stages of differentiation found on this day of gestation in both control and ethanol-treated females were compacted morulae (Theiler Scale [TS] = 3, fifth cell cycle; Theiler, 1989), early blastocysts (embryos with 32 cells or more, with evident cavity or nascent blastocele, TS = 4, sixth cell cycle), fully expanded blastocysts (TS = 4.5, seventh cell cycle) and hatched blastocysts (TS = 5, eight cell cycle; implantative with visible blastocoelic cavity and without pellucida zone; Figure 1A).

The frequency of embryo stages of differentiation reached on day 4 of gestation is shown in Figure 2(A). In control CD-1 mice, the major percentage of embryos was found in the expanded blastocyst (60.7%), but compacted morulae as well as early and hatched blastocysts were also noted. Instead, in ethanol-exposed females, percentages of expanded and hatched blastocysts were significantly lower than in controls ($p < 0.05$), and there was an increased percentage of morulae, compared to controls ($p < 0.01$; Figure 2A).

The morphology of preimplantational embryos was evaluated. Figure 3 shows the features of abnormal embryo characteristics, recorded as abnormal compactation and cavitation, lysis and/or fragmentation in the inner mass cells or in trophoblast cells. Ethanol-exposed females had a significantly increased percentage of morphologically abnormal embryos, compared to controls (12.6 vs. 1.78%, respectively; $p < 0.05$).

Growth of morulae (Figure 4A) and blastocysts (Figure 4B) was evaluated by quantification of interphasic plus mitotic nuclear number (Figure 4C, D) per embryo. Morulae and expanded blastocysts from control and ethanol-exposed females had similar mean numbers of nuclei/embryo, but early blastocysts had a significantly reduced mean number of nuclei/embryo, compared to controls ($p < 0.05$; Figure 5A). To evaluate cell division in each type of embryo, the MI was calculated. The early blastocysts from the ethanol-exposed females presented significantly reduced MI ($p < 0.05$), but expanded blastocysts had significantly increased MI, compared to controls ($p < 0.01$; Figure 5B). The quality of interphasic nuclei in morulae, early and expanded blastocysts from both groups was evaluated by observing the presence of nucleus with micronucleus, nuclei with budding or lobes (preapoptotic) and pyknotic/condensed nuclei (apoptotic). The pyknotic/condensed nucleus was the only nuclear type found in embryos from control and ethanol-exposed females (Figure 4E, F). Early blastocysts from ethanol-exposed females presented a significantly increased percentage of pyknotic nuclei, compared to the same embryo type from controls ($p < 0.05$; Table 2).

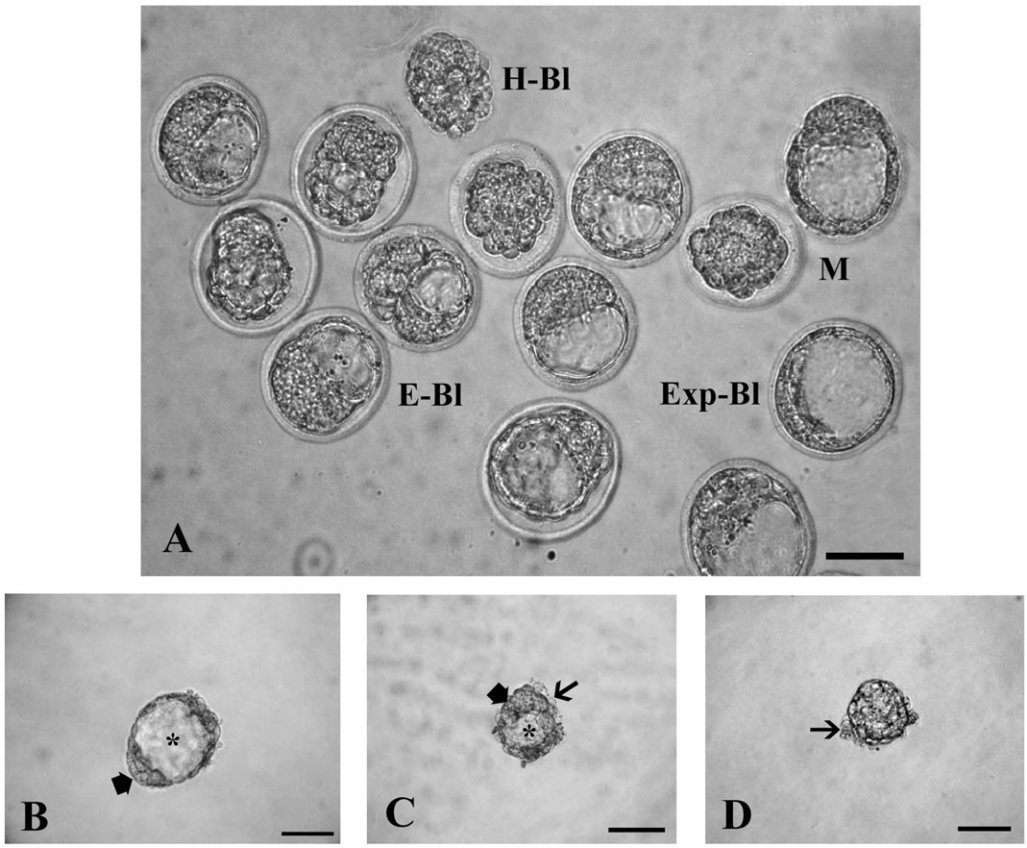

Figure 1. *In vivo* embryo differentiation on days 4 and 5 of gestation in CD-1 control and ethanol-exposed females. On days 4 and 5 of gestation, the stages of embryo differentiation were evaluated according to a morphologic criterion. (A) Embryonic differentiation stages recovered on day 4 of gestation. M, morulae; E-Bl, early blastocyst; Exp-Bl, expanded blastocyst; H-Bl, hatched blastocysts. (B–D) *In vivo* embryo differentiation on day 5 of gestation. (B) Embryo type 1: early implantative hatched blastocyst with expanded cavity and without trophoblast processes. (C) Embryo type 2: implantative hatched blastocyst with small cavity and early and incipient trophoblast processes. (D) Embryo type 3: late implantative hatched blastocyst with quite reduced or no cavity and median trophoblast processes. ➡, inner cell mass; *, blastocoel cavity; →, trophoblast processes. Scale bar: 50 μm.

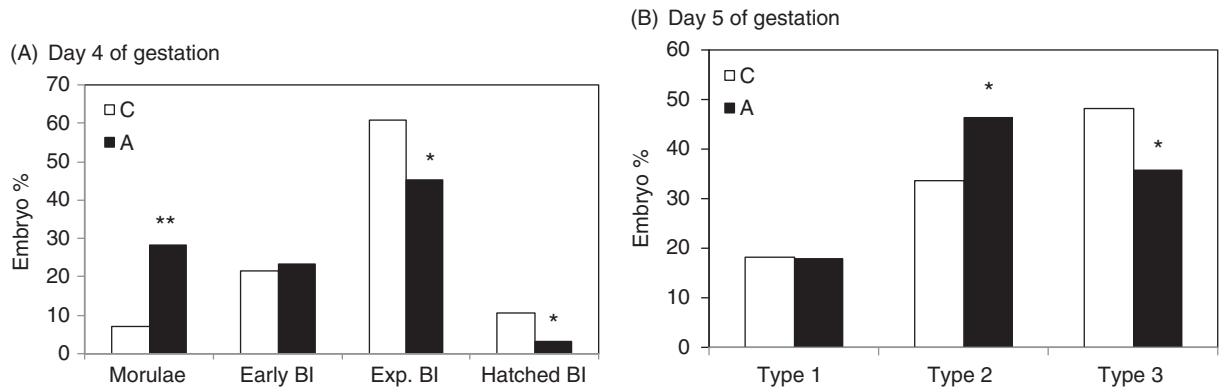

Figure 2. Embryo differentiation on days 4 and 5 of gestation. Embryos were collected on days 4 and 5 of gestation, classified and quantified as percentage of embryo type (number over the total number of recovered embryos). (A) Distribution of morulae and blastocysts in control ($n = 56$ embryos) and ethanol-exposed females ($n = 64$ embryos) on day 4 of gestation. (B) Embryo types 1, 2 and 3 from control ($n = 116$ embryos) and ethanol-exposed ($n = 84$ embryos) females on the morning of day 5 of gestation. Bl, blastocysts; Exp, expanded; C, control group (white bars); A, ethanol-exposed group (black bars). *$p < 0.05$; **$p < 0.01$ versus control group; chi-squared test.

Effects of periconceptional ethanol exposure on CD-1 day 5 *in vivo* development

The total number of recovered embryos was recorded in both control and ethanol-exposed females. The mean embryo number ($\pm$SD) of ethanol-exposed females was significantly reduced, compared to controls (CF: 5.04 ± 2.2, $N = 23$ females; TF: 3.7 ± 1.8, $N = 23$ females; $p < 0.05$).

On day 5 of gestation, the embryonic stages of differentiation found in both control and ethanol-treated females were assessed as hatched embryo types 1, 2 and 3 (Figure 1B–D). Embryo type 1 presented a fully expanded

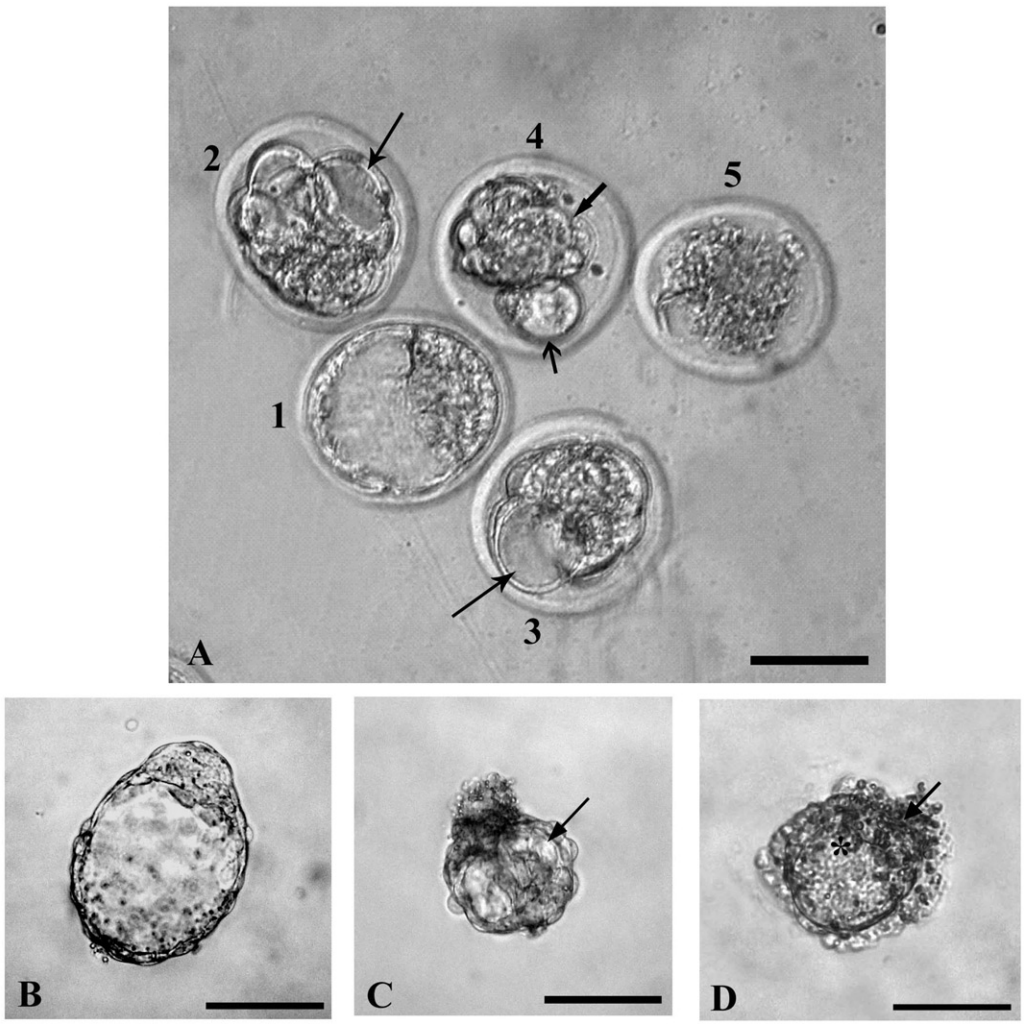

Figure 3. Embryo morphology on days 4 and 5 of gestation. At preimplantation and implantation gestational period, morphology of the different stages of embryo differentiation was evaluated. (A) Micrograph shows the morphology of the blastocyst on day 4 of gestation. 1, normal blastocyst; 2–3, abnormal cavitation; 4, altered compactation in morula; 5, embryo fully fragmented presenting severe cellular lysis. (B–D) Morphology on day 5 of gestation. (B) Normal day 5 embryo. (C) Abnormal embryo with partitioned double cavitation. (D) Abnormal embryo with altered trophoblast cell processes (fragmented and lisated trophoblast). →, abnormal cavitation; →, protruded blastomeres/cells; ➡, fragmentation; *, lysis and/or severe fragmentation. Scale bar: 50 μm.

blastocoelic cavity (Figure 1B), embryo type 2 had a reduced blastocoelic cavity and incipient trophoblast processes protruding from the embryo (Figure 1C) and embryo type 3 did not present a visible blastocoelic cavity and had moderately expanded trophoblast processes concordant to the attaching embryonic condition (Figure 1D; Table 1, T0 hours). Whereas control females presented the highest percentage of the most advanced embryo stage (type 3), ethanol-exposed females had a significantly increased percentage of embryo type 2 ($p < 0.05$) and a reduced percentage of embryo type 3, compared to controls ($p < 0.05$; Figure 2B).

Morphology of the embryo types was analyzed (Figure 3). Compared to a day 5 normal embryo (Figure 3B), abnormal embryos presented a partitioned blastocelic cavity (Figure 3C) and/or fragmentation in trophoblast or in the inner cell mass (Figure 3D). The percentage of abnormal day 5 embryos in ethanol-exposed females was significantly increased, compared to controls ($p < 0.05$; data not shown).

Day 5 *in vivo* embryo growth was assessed by morphometric analysis of the embryo size. Mean areas of small, medium and large ethanol-treated derived embryos were similar to those of controls, showing that growth of embryos, in terms of embryo-trophoblast expansion, was not modified by ethanol exposure.

In vitro embryo development after day 5 *in vivo* periconceptional ethanol exposure

We studied *in vitro* embryo differentiation by observation of morphological aspect of trophoblast development throughout 72-hour culture. The most advanced embryo type found at each culture time was embryo type 3. Taking into account the TS for *in vivo* development, the characteristics of embryo type 3 development throughout culture time were as follows: 0 hours of culture (TS 5, *in vivo* stage E5 of development): zona-free blastocyst with incipient to low trophoblast processes (TPs); 24 hours of culture (TS 6, E5.5): attaching-contacting blastocysts with low-to-moderate TPs and few TGCs and 48 hours of culture (TS 6.5, E6): contacting-invasive embryo with moderate-polarized trophoblast and some TGCs. Embryo outgrowth developed as a monolayer of

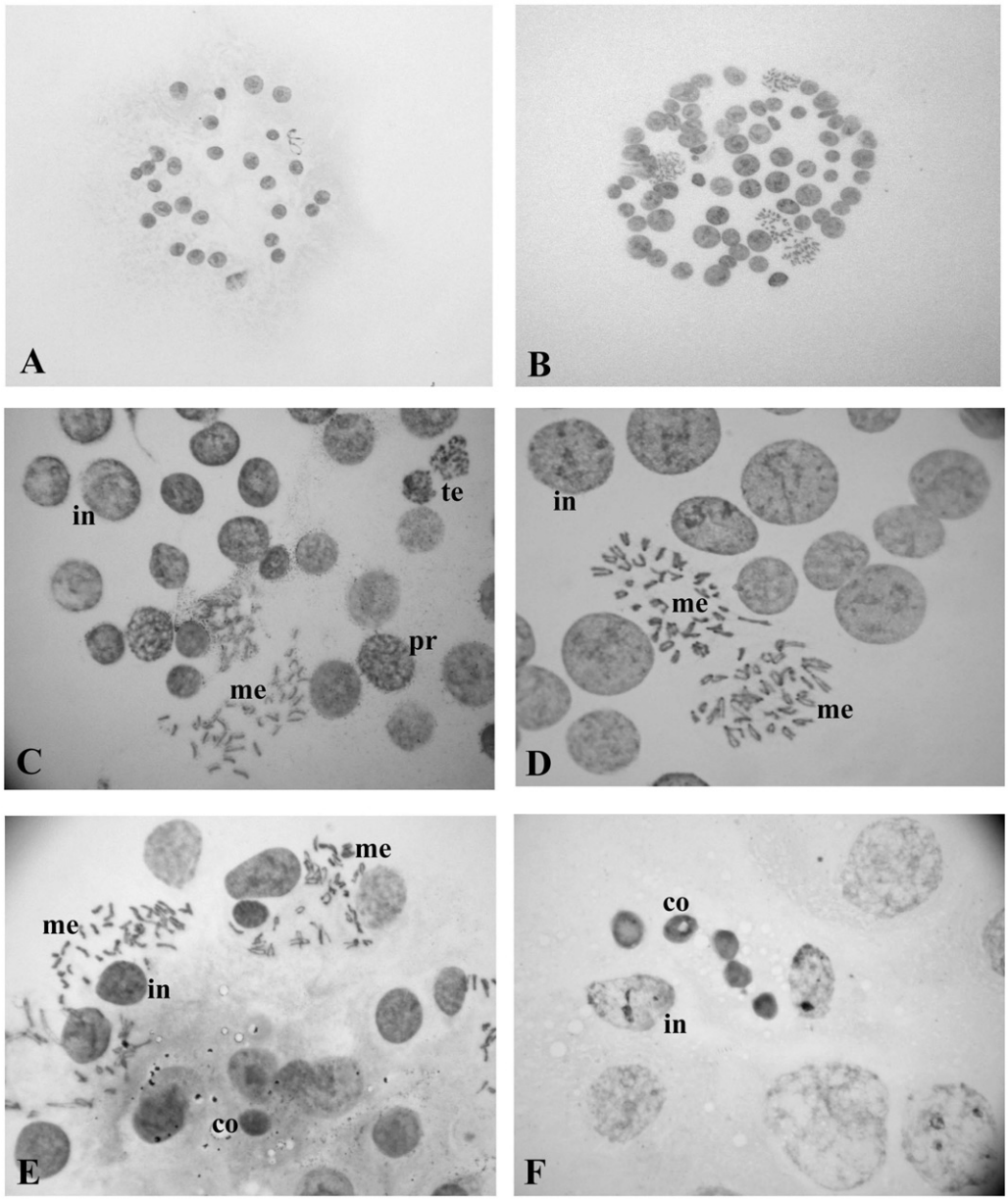

Figure 4. Embryo nuclear quality and cell division on day 4 of gestation. Embryo growth (total number of nuclei per embryo), quality of interphase nuclei and MI by quantification of phases of cell division was evaluated by the Tarkowski method on preimplantation day 4 recovered embryos. (A) Morulae (10×). (B) Blastocysts (10×). (C) Nuclei from control-derived embryos: interphase nucleus (in); prophase (pr); telophase (te) and metaphase (me) (40×). (D) Two normal metaphases (me) and normal interphase nuclei from control-derived embryos (60×). (E) Nuclei from ethanol-exposed derived embryos: abnormal interphase nuclei and pyknotic/condensed nuclei (co) (reduced size nucleus with high stain intensity; 40×). (F) Severe abnormal interphase and condensed nuclei in ethanol-exposed derived embryos (60 ×).

spreading trophoblast cells surrounding a protruding aggregate of undifferentiated inner cell mass. With 72 hours of culture (TS 7, E7), the embryo implanted with fully expanded outgrowth of trophoblast and fully differentiated TGC (Figure 6).

To analyze the frequency of viable *in vitro* embryo differentiation, we first evaluated embryo morphology. Figure 7 shows abnormal embryos at 24, 48 and 72 hours of culture. The main severe deleterious characteristics were the presence of lyses cells and high fragmentation, usually found in the inner mass cells. These embryos were assessed as nonviable and were not considered for *in vitro* differentiation quantification. Then, according to image embryo analysis, the frequency of the different embryo types was assessed throughout 72 hours of culture and the percentages of embryo type distribution are shown in Figure 8. Whereas, in control females, embryo type 1 percentage increased to a maximum between 24 and 48 hours of culture, in ethanol-exposed females, this embryo type had a constantly lower pattern of development up to 48 hours ($p < 0.05$ vs. control), and at 72 hours of culture, it was significantly diminished (Figure 8A).

Embryo type 2 percentages from control females had a range oscillating between 13 and 40% over culture times. Embryo type 2 percentage derived from ethanol-exposed females had a similar pattern, but, at each culture time, was significantly increased, compared to controls ($p < 0.05$; Figure 8B).

The development pattern of embryo type 3 from control females was constant up to 24 hours, significantly declined at 48 hours (48.5 vs. 26.6%; $p<0.05$) and at 72 hours significantly increased ($p<0.001$). Embryo type 3 percentage from ethanol-exposed females was constant and similar up to 24 hours, but was significantly diminished at 48 hours of culture (43.3 vs. 18.1%; $p<0.05$). At 72 hours, embryo type 3 percentage was significantly reduced versus controls ($p<0.05$), although it was not significantly different, compared to ethanol-derived embryo type 3, at 48 hours (Figure 8C).

Finally, the dynamics of embryo-trophoblast growth/expansion was measured as a parameter that can indicate trophoblast invasiveness capacity. Three embryo-range area sizes during each culture time in control and ethanol-exposed females were found (Figure 9). The mean area of small embryos from control and ethanol-exposed groups increased similarly from 0 to 24 hours in each group's embryos. However, at 48 hours of culture, the small embryos from ethanol-exposed females had a significantly increased mean area, as compared to control group embryos ($p<0.05$; Figure 9A). This increase represented approximately 33.8% of the control value. The medium embryos from control and ethanol-exposed females had similarly increased area up to 24 hours of culture, but this area significantly increased at 48 hours in treated embryos ($p<0.05$). At this time, the ethanol-exposed derived medium embryos presented a 12% increment over controls (Figure 9B). The large embryo from controls had an increment of area between 0 and 24 hours of culture ($p<0.05$) and a similar value between 24 and 48 hours. The large embryo from ethanol-exposed females showed an increased area between 0 and 48 hours of culture ($p<0.05$); however, we found a significantly reduced area at 24 hours of culture ($p<0.05$), compared to the area of control embryos, with a decrease of 21.5% versus controls (Figure 9C).

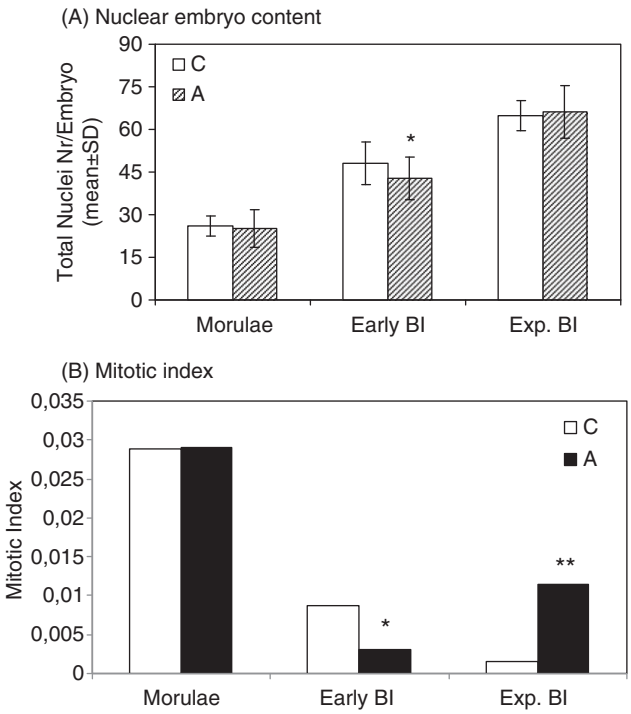

Figure 5. Embryo growth on day 4 of gestation. Preimplantation embryos were prepared for the Tarkowski method and the mean number of nuclei per embryo types (± SD) (A), and the MI (number of mitotic nuclei over the total nuclear of embryos) (B) was calculated for morulae, early and expanded blastocysts derived from C (control) and A (ethanol-treated) females. *$p<0.05$; **$p<0.01$ versus control group, chi-squared test. Bl, blastocysts; Exp, expanded.

Discussion

The present results show that short-term ethanol exposure of outbred CD-1 female mice preceding conception and up to days 4 and 5 of gestation altered peri-implantational embryo growth, morphology, differentiation and trophoblast invasiveness. Moderate concentrations of ethanol intake produced embryo implantational loss at day 5 of gestation beyond day 4 of gestation (preimplantation stage) when embryo fragmentation was not altered, suggesting that embryonic loss may occur after day 4, around day 5 of gestation (18–20-hour time difference). Previously, we found that preconceptional and chronically 10% ethanol exposed hybrid females had high oocyte fragmentation (Cebral et al., 1998a) and also that a regimen of alcohol ingestion was adverse for preimplantation development because of embryo cleaving arrest and high fragmentation and lysis (Cebral et al., 2000). Other researchers suggested that developing embryos that did not reach a stage either arrested or became fragmented, showing that fragmentation can occur in unfertilized ova that undergo parthenogenetic activation (Takase et al., 1995). We think that the present ethanol exposure severely delayed embryos and produced increased arrested morulae at day 4 of gestation, which became fragmented and lost between days 4 and 5 of gestation. Moreover, if ethanol exposure can rapidly affect

Table 2. Nuclear quality in control and ethanol-exposed embryos on day 4 of gestation.

	Control group			Ethanol group		
	Morulae	Early blast.	Exp. blast.	Morulae	Early blast.	Exp. blast.
Total embryo nr	4	12	20	15	15	25
Total nuclear nr	104	575	1296	378	650	1650
Total interphase nuclear nr	101	570	1294	367	648	1631
Pyknotic nuclear nr	4	4	1	15	15	0
Pyknotic nuclear (%)	3.8 (%)	0.7 (%)	0.077 (%)	3.9 (%)	2.3 (%)[a]	0 (%)

Embryos from control ($n=5$) and ethanol-exposed ($n=5$) groups were dissected on day 4 of gestation, classified as morulae, early and expanded blastocysts (Exp. blast.) and the Tarkowski method was applied to evaluated nuclear types and quality. The only abnormal type of nuclei found was pyknotic and was recorded as percentage over the total number (nr) of nuclei.

[a]$p<0.05$ versus control embryos; chi-squared test.

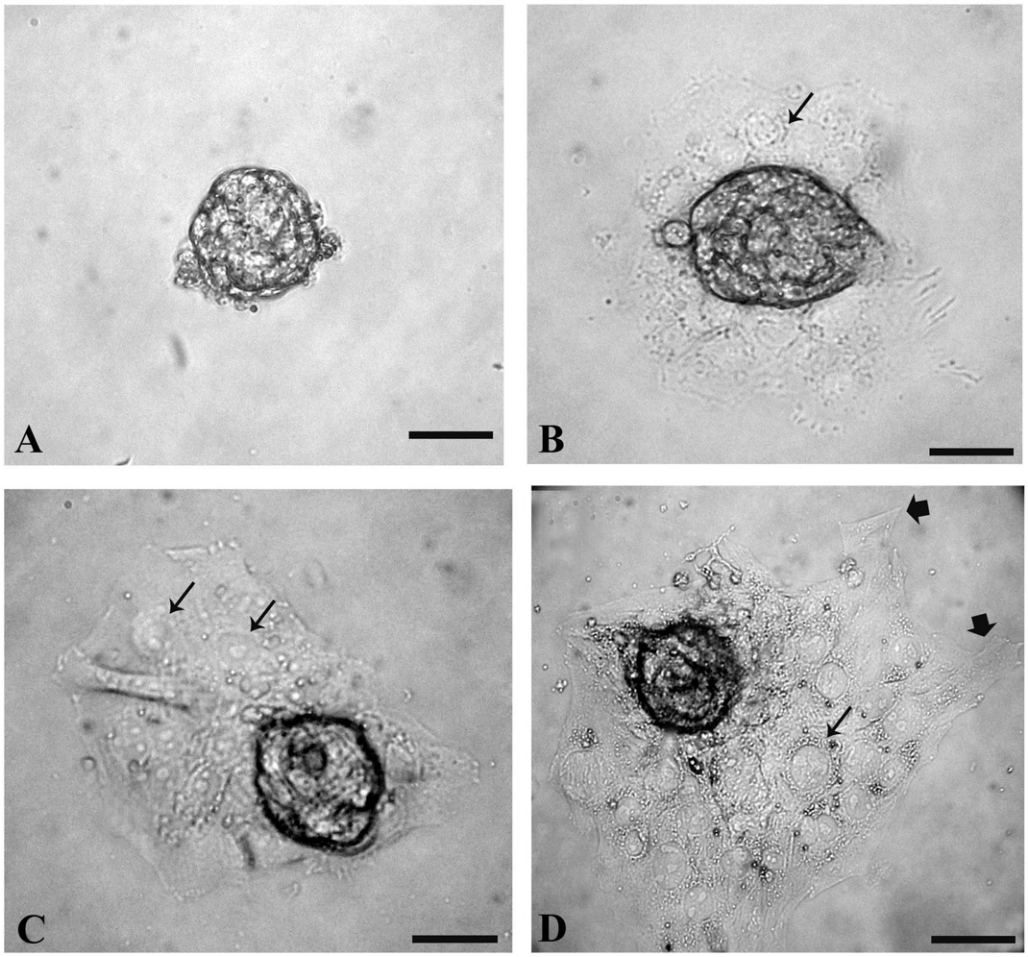

Figure 6. *In vitro* phases of day 5 embryo differentiation. Embryos collected on day 5 of gestation were cultured up to 72 hours and the types of *in vitro* embryo differentiation were analyzed, taking into account the classification of the TS. Micrographs show the representative type 3 embryo found at each culture time. (A) Hatched embryo at 0 hours of culture (TS 5, *in vivo* stage E5 of development). (B) Embryo type 3 at 24 hours of culture (TS 6; E5.5): embryo without cavity; with low-to-moderate TPs and few TGCs. (C) Embryo type 3 at 48 hours of culture (TS 6.5, E6): embryo without cavity, with moderate expansion of TPs and some TGCs. Note embryo outgrowth development as a monolayer of spreading trophoblast cells surrounding a protruding aggregate of undifferentiated inner cell mass. (D) Embryo type 3 at 72 hours of culture (TS 7, E7): embryo without cavity, with high expansion and cytoplasmic extensions of TPs and fully developed TGC. →, TGC; ➡, cytoplasmic extensions of TPs. Scale bar: 50 μm.

some intracellular signals, for example, cyclic adenosine monophosphate (Tournaye et al., 1993), embryo fragmentation/apoptosis would be expected to be instantaneous. On the other hand, postzygotic nuclear and chromosomal abnormalities and loss of developmental synchrony between embryos and the maternal environment can be the cause of developmental arrest and fragmentation that lead to reduced embryo number on day 5 of gestation.

On days 4 and 5 of gestation, ethanol-exposed females had similar patterns of delayed embryonic differentiation because diminished expanded/hatched blastocysts and advanced embryo type 3 were observed. Moreover, the decreased percentage of day 4 expanded blastocysts was similar to the percentage of day 5 embryo type 3 (12 and 14%). Direct ethanol exposure of preimplantation embryos was demonstrated to have both inhibitory and stimulatory effects on development of preimplantation embryos, depending on stage of development and ethanol concentration (Leach et al., 1993; Stachecki et al., 1994b). However, recently, we showed delayed differentiation of early embryo organogenesis after periconceptional ethanol exposure in CF-1 mice (Coll et al., 2011). Also, retarded differentiation and impaired embryo growth were found at all embryo preimplantational stages when hybrid adult females were chronically treated with 10% ethanol for 1 month preceding gestation (Cebral et al., 1999, 2000). In view of our previous results and others (Kowalczyk et al., 1996), the present findings raise the possibility that delayed embryo differentiation was the result of activation of oocytes and parthenogenetic development. It was found that parthenogenetically activated single-pronuclear haploid mouse embryos with a slow cleavage rate during preimplantation developed mainly to the morula stage (Kaufman, 1990). On the other hand, this ethanol-induced embryo arrest may be related to nuclear abnormalities and chromosomal malsegregation resulting from anomalous cell-cycle checkpoints around ovulation or during early stages of cleavage (Hardy et al., 1993). Moreover, the parthenogenetic activation induced by ethanol consumption was associated with anomalous chromosome segregation and interference with cell division (Kaufman, 1997). If a high incidence of nuclear and chromosomal abnormalities was induced by ethanol exposure, the embryos could not reach advanced blastocyst stages and became arrested near days 4 and 5 of gestation.

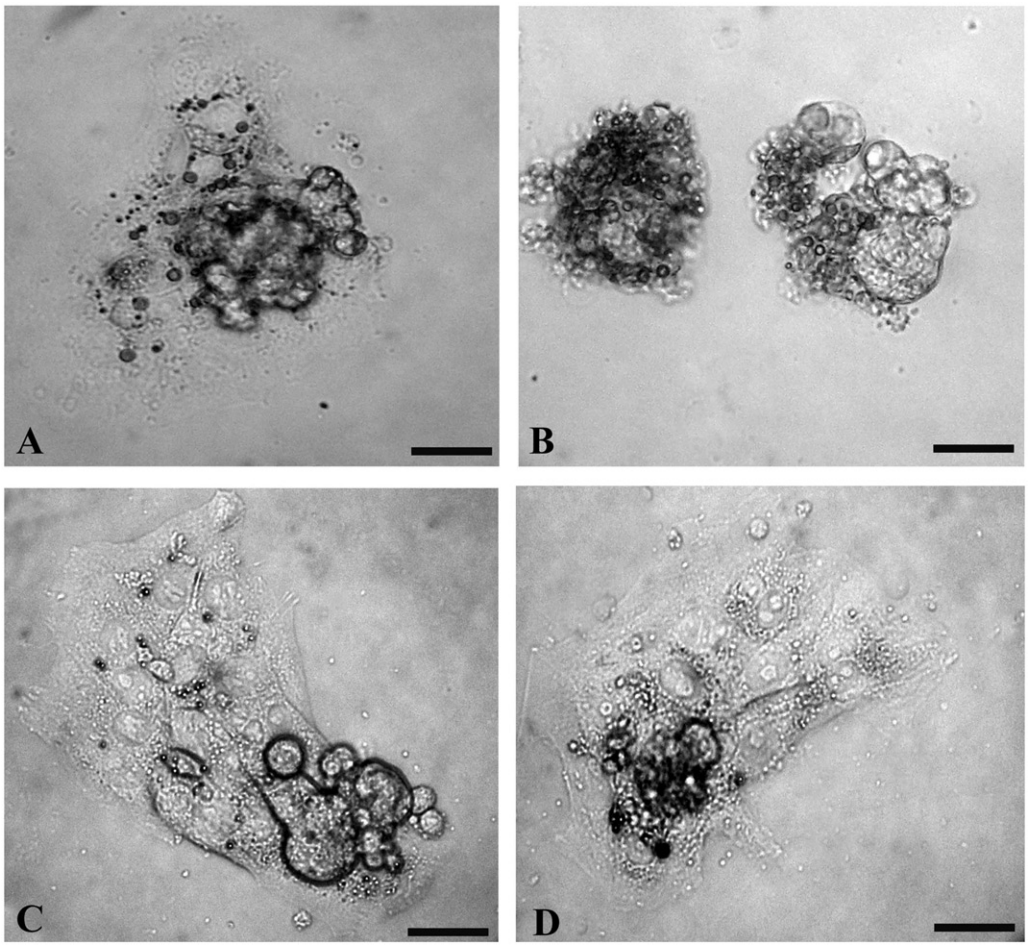

Figure 7. Morphology of embryo types during *in vitro* culture. At culture times, the embryonic abnormalities observed were underdeveloped trophoblast cells and undifferentiated cell mass with fragmentation, necrosis and/or lysis. (A) 24 hours; (B and C) 48 hours; (D) 72 hours. Scale bar: 50 μm.

The present ethanol exposure affects differentiation of morulae to blastocysts and/or perhaps blastocoel expansion at the TS 4.5 stage, although on day 5 of gestation (TS 6, E5.5), all embryos recovered from ethanol-treated females were hatched. Transition from morula to blastocyst is dependent on the functioning of Na^+/K^+ adenosine triphosphatase (ATPase), which is implicated in the development of the blastocoel cavity. Because this pump is the major consumer of adenosine triphosphate (ATP) during blastocoele formation, reduced cellular ATP levels will inhibit the pump and blastocoele fluid accumulation (Brison & Leese, 1994). Direct exposure to ethanol produces inhibition of placental Na^+/K^+ ATPase activity (Swann, 1990), which suggests that the present perigestational ethanol exposure had a deleterious effect on the metabolism of glucose to lactate, Na^+/K^+ ATPase activity and blastocoele fluid accumulation. Many other altered mechanisms could be operating in abnormal blastocyst formation. We have shown that chronic administration of low levels of ethanol reduced synthesis of prostaglandin E (PGE) by blastocysts developed *in vivo* (Cebral et al., 1998b), suggesting that the present poor blastocyst differentiation may also probably be related to impaired levels of embryonic PGE. Prostaglandins are implicated in blastocyst expansion, participate in the hatching of blastocysts and are involved in the initiation of implantation (Kennedy, 1977). Also, other embryonic intracellular pathways can be affected by ethanol exposure. Direct ethanol exposure induces a transient increase in $[Ca^{2+}]$ concentration, the major transductional signal that regulates cellular growth and differentiation (Shiina et al., 1993) that produces elevated cavitation rates (Stachecki et al., 1994b). Ethanol can initiate a signal-transduction cascade that involves inositol phosphates, diacylglycerol and activation of protein kinase C and release of intracellular calcium, producing an effect on regulation of development of mouse embryos (Stachecki et al., 1994b). Periconceptional ethanol exposure up to day 4 or 5 of gestation could affect these key developmental regulatory mechanisms of differentiation.

We found blastocysts with fragmentation and lyses into the blastocele cavity and/or around the inner cell mass and abnormal cavitation. We think that morphologically abnormal peri-implantative embryos with decompacted blastomeres and fragmentation in the perivitelline space were the retarded ones. Parthenogenetic embryos can cleave at early stages, but later parthenogenetic morulae and blastocysts may become morphologically abnormal, showing small cytoplasmic fragments extruded into the perivitelline space. The retarded embryos around day 5 of gestation probably derived from activated oocytes, were disorganized and had ectoderm vesicles and degenerating polar trophoectoderm.

We assessed embryo growth on day 4 of gestation by quantifying the number of nuclei per embryo, assuming that

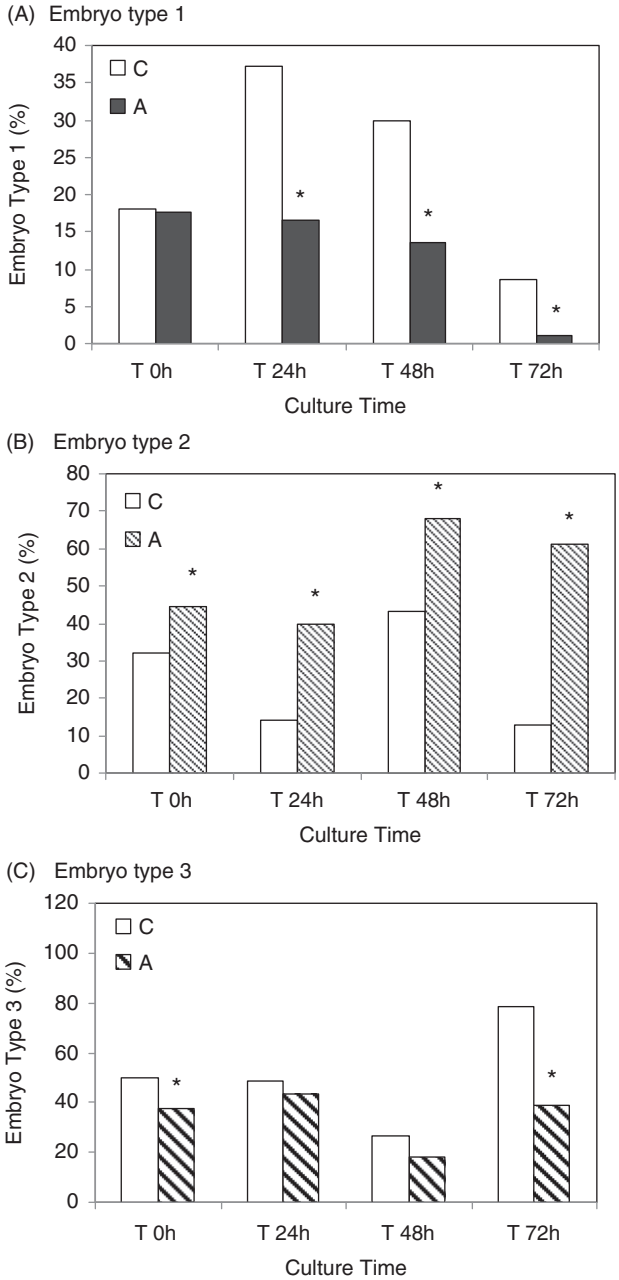

Figure 8. Distribution of *in vitro* embryo differentiation in control and ethanol-exposed females. The number of type 1 (A), type 2 (B) and type 3 (C) embryos over the embryos cultured up to 72 hours was recorded and expressed as percentages. C, control females; A, ethanol-exposed females. $*p < 0.05$ versus control group, chi-squared test.

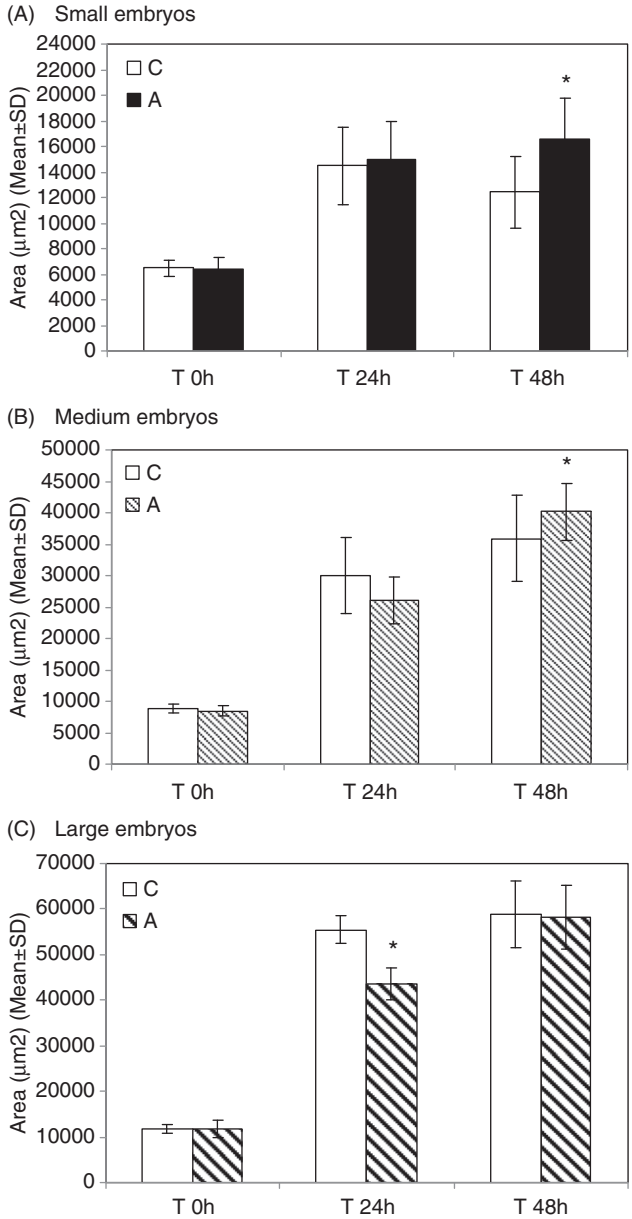

Figure 9. *In vitro* embryo-trophoblast growth/expansion in control and ethanol-exposed females. According to three interval areas of embryo-trophoblast growth (expansion), embryos were classified as small, medium and large embryos at each culture time (T0, T24 and T48 hours). Graphs show the mean areas (μm^2) of trophoblast (± SD) for each of the embryos in control (C) and ethanol-exposed females (A). $*p < 0.05$ versus CF, Student's *t*-test.

the present ethanol paradigm did not induce multinucleation and that the number of nuclei is equal to cell number. The reduced nuclear number in early blastocysts from ethanol-exposed derived females indicates decreased embryonic size, low growth and/or delayed or arrested mitotic cycle, suggesting a reduction of embryonic cell proliferation between the fifth and sixth cell cycle. However, the reduced embryo nuclear number could also be result of increased embryonic apoptosis, because the increased pyknotic nuclei in early blastocysts may indicate that ethanol could induce an imbalance in the proliferation/apoptosis ratio. This process can be the consequence of abnormal mitoses in first cleavages that generate cells with abnormal chromosomal complements, which, at the blastocyst stage, if chromosomal damage is not corrected, leads to blastocyst-cell mitosis arrest and induction of apoptosis (Musacchio & Hardwick, 2002). Moreover, even if mitosis and gene activation do not fail, impaired cytokinesis through cytoskeletal and spindle malfunction may cause arrest and cellular division block because of lack of sufficient cells to produce a functional ICM. On the other hand, the reduced cell number of embryos reaching the blastocyst stage can be involved in defective further embryo differentiation, because a critical cell number is needed for normal ICM formation and differentiation. In this regard, the cleavage rate can decrease because of slow division and cell death in the ICM. Moreover, ethanol-derived blastocysts with small numbers of cells found on day 4 may reach less differentiated embryo stages on day 5 of gestation, which, with a probable

low cell number, would be less viable and therefore their number severely diminished through the culture times.

Although the expanded blastocysts did not have a reduced number of cells, the increased MI could be the result of a possible elevated trophoectodermal proliferation, and, together with increased apoptosis in ICM, no modification of the nuclear number of expanded blastocysts may result. However, an imbalance between TE proliferation and ICM apoptosis may manifest as increased abnormal advanced implantative stages on day 5 of gestation and during culture, because, particularly, fragmentation and lysis in ICM can support our hypothesis of apoptosis induction in the ICM of expanded blastocysts on day 4 of gestation. In relation to the implications of altered MI, it is unclear whether there is a link between altered trophoectodermal cell cycle and cavitation and extent of blastocoel expansion. Although it was reported that damage in ICM does not seem to affect hatching and outgrowth of trophoblast, it has been suggested that these events are independent of the regulation of cell cycles within a certain limit of cell number reached in the embryo. For cavitation and expansion, if the trophoectoderm must stop its cell mitotic division to start differentiation into TGCs to control blastocoel formation, then a potentially increased MI in TE of ethanol-derived embryos would imply poor expansion and hatching and delayed blastocyst differentiation. Moreover, differential functional demarcations and sensitivity of ICM and TE cells (Antezak & Van Blerkom, 1997) to detrimental ethanol effect are probably the reasons behind the present results.

In our study of development during implantation, the pronounced fall of embryo type 1 at the final phase of implantation (72-hour culture) in control females suggests spontaneous early pregnancy loss during implantation resulting from poor development of these types of embryos. In ethanol-exposed females, a higher quantity of type 1 embryos was lost in earlier stages than in controls and did not survive beyond the end of the culture time, indicating an increased risk of early pregnancy loss during implantation. However, the high number of type 2 embryos at all implantation stages suggests that this embryo population retained the highest survival rate up to the final phases of implantation (TS 7, E7). Moreover, the reduced quantities of type 3 embryos in ethanol-exposed females with 72 hours of culture, compared to frequency of controls, not only confirms retardation and/or deregulation of differentiation near implantation, but also suggests that periconceptional alcohol consumption may cause embryo retarded differentiation at early postimplantation phases.

To ascertain the embryo growth, together with implantative/invasive capacity of embryos during implantation, the embryo-trophoblast area of expansion was evaluated. Three populations of embryo growth, according to the expansion area range, were examined. The small embryos from ethanol-treated females presented a higher growth or expansion than that of controls at 48 hours, suggesting that an increased expansion-rescue response is able to occur around 48 hours to allow implantation of this type of embryo. The medium embryo population from ethanol-treated females also showed similarly higher embryo-trophoblast expansion area, compared to the controls, at 48 hours. Because endoreduplication in TGCs and the associated cell hypertrophy allow tissue growth (Hu & Cross, 2010), it is possible that the present high degree of trophoblast expansion of embryos derived from the ethanol-treated females resulted from an increase of TGC ploidy status. This TGC hypertrophy may allow the ethanol-exposed embryos to acquire major invasiveness in less time. It was shown that blastocysts exposed to ethanol in culture to 0.1, 0.2, 0.4 or 1.0% (w/v) ethanol for either 5 minutes or 24 hours had greater outgrowth area than did stage-matched controls (Stachecki et al., 1994a). Moreover, culture of one- or two-cell embryos for 24 hours in medium containing 0.1% ethanol induced a precocious onset of trophoblast differentiation (Leach et al., 1993). These ethanol effects in preimplantation embryos seem contradictory. In regard to this question, the ethanol effects on embryos depend not only on the ethanol concentration, stage of development and time of exposure, but also on the direct or indirect (maternal blood alcohol concentration and metabolism) ethanol exposure. Thus, depending on the threshold of alcohol concentration used, the ethanol may have a positive or negative effect on the development of preimplantation embryos. In the present study, we showed that 10% ethanol ingestion preceding gestation and up to the early phases of peri-implantation is able to produce deleterious embryo effects on differentiation and embryo-trophoblast growth.

Stimulation of preimplantation embryo development by increasing embryo $[Ca^{2+}]_I$ and perturbation of second-messenger molecules is possible under specific concentrations of ethanol (Stachecki et al., 1994a). However, the toxic ethanol effects on early stages of embryo development depend on the dose and may be related to how preimplantation embryos metabolize ethanol. In this regard, the absence of the primary ethanol-metabolizing enzymes (class I alcohol dehydrogenase [ADH] and aldehyde dehydrogenase [ALDH]) during preimplantation development suggests toxic acetaldehyde embryo effects during maternal alcohol consumption (Rout & Armant, 2002; Kalmus & Buckenmaier, 1989). Two-cell-stage mouse embryos are approximately 16-fold more sensitive to acetaldehyde toxicity, compared to ethanol (Lau et al., 1991). Initially, the mitochondrial ALDH of oocytes and zygotes can oxidize cellular acetaldehyde, but once embryogenesis begins, maternal expression of the acetaldehyde-oxidizing enzyme is not maintained, presenting a potential danger of cellular damage to embryos by aldehydes during maternal alcohol consumption. Rout and Armant (2002) reveal that ADH and ALDH messenger RNA species are differentially transcribed during preimplantation mammalian development. The absence of class I ADH transcripts may favor embryo tolerance of exposure to ethanol during moderate maternal drinking. However, the absence of acetaldehyde-metabolizing enzyme during later stages of preimplantation development may increase the deleterious effects of systemic acetaldehyde accumulating during heavy alcohol consumption (Hard et al., 2001).

Overall, the present results suggest that periconceptional alcohol exposure induces delayed embryo differentiation and stimulation of trophoblast expansion to increase their invasiveness during implantation. At implantation end stage (TS 7), whereas control females had mostly embryo type 3 with probably the highest implantation capacity, the

ethanol-treated females had a high quantity of embryo type 2 and probably with a major level of invasiveness. As suggested in other pathologies (Pringle & Roberts, 2007), impaired trophoblast expansion/invasion regulation during implantation may occur in the ethanol-exposed females, which can be implicated in early increased miscarriage and/or intrauterine growth restriction after periconceptional alcohol consumption.

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Declaration of interest

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