In vitro fertilization, embryo development, and cell lineage segregation after pre- and/or postnatal exposure of female mice to ambient fine particulate matter

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Objective: To evaluate effects of pre- and/or postnatal exposure to ambient fine particulate matter on fertilization, embryo development, and cell lineage segregation in preimplantation blastocysts using the IVF mouse model.

Design: Animal model.

Setting: Academic institution.

Animal(s): Six-week-old, superovulated mice.

Intervention(s): Pre- and postnatal exposure to filtered air (FA-FA), filtered-ambient air (FA-AA), or ambient air (AA-AA) in exposure chambers 24 hours a day for 9 weeks.

Main Outcome Measure(s): Gestation length, litter size, sex ratio, ovarian response to superovulation, fertilization rate, embryo development, blastocyst and hatching rates, total cell count, and proportion of cell allocation to inner-cell mass (ICM) and trophectoderm (TE).

Result(s): Gestation length, litter size and birth weight, live-birth index, and sex ratio were similar among exposure groups. Ovarian response was not affected by the exposure protocol. A multivariate effect for pre- and/or postnatal exposure to ambient fine particulate matter on IVF, embryo development, and blastocyst differential staining was found. Cell counts in ICM and ICM/TE ratios in blastocysts produced in the FA-FA protocol were significantly higher than in blastocysts produced in the FA-AA and AA-AA protocols. No difference in total cell count was observed among groups.

Conclusion(s): Our study suggests that exposure to ambient fine particulate matter may negatively affect female reproductive health by disrupting the lineage specification at the blastocyst stage without interfering in early development of the mouse embryo. (Fertil Steril® 2008; ■ ■ ■ ■. ©2008 by American Society for Reproductive Medicine.)

Key Words: Fine particulate matter, traffic emissions, ambient air, in vitro fertilization, embryo development, blastocyst, inner-cell mass, trophectoderm, lineage specification, mouse

The ubiquitous exposure to traffic emissions present in the ambient air of large metropolitan areas is likely to have a negative impact on human health. Several studies have shown that an episodic short-term increase of ambient air pollution level, a common event in many urban environments, is associated with increased morbidity and mortality from cardio-pulmonary causes (1–3). Although the effects of air pollution on respiratory and cardiovascular systems are evident, the influence of air contamination on reproduction is not yet well established. The difficulty of substantiating a clear correlation between traffic emissions and fertility impairment could be related to the multitude of factors involved not only in reproductive competence, which include age, hormonal and nutritional status, patency of the reproductive tract, gamete reserve and production, heredity, genetic polymorphism, and behavior, but also to the methodological challenges found in the laboratory in measuring and determining this correlation.

In recent years, considerable data from epidemiological and experimental studies have implied environmental factors as contributors to human infertility. Air pollution has been shown to be associated with decreased fertility and poor outcome such as intrauterine growth retardation, intrauterine mortality, preterm birth, low birth weight, and birth defects (4–8). Very few experimental or clinical studies evaluating the possible effects of the exposure to the main contaminants present in urban ambient air, including carbon monoxide (CO), sulfur dioxide (SO2), nitrogen dioxide (NO2), benzene, formaldehyde, polycyclic hydrocarbons, and suspended particulate matter (PM), on female reproductive health have been conducted (6, 9, 10).

Evaluation of preimplantation embryos in an IVF and embryo culture system allows observation of the unique events...
of reproductive and developmental processes that cannot be followed in vivo. The possibility of assessing aspects of embryo development in vitro such as cell division rate, morphology and quality (regularity, symmetry, fragmentation, multinucleation), rate of progression to the blastocyst stage, and rate and pattern of the hatching process, and identification of the first cell lineage segregation in the blastocyst could represent a relevant model for evaluating the reproductive effects of exposure (acute or chronic) to ambient air pollution. Nevertheless, observations concerning these effects are limited to studies involving chemical air contamination with different product groups such as anesthetic gases, refrigerants, hydrocarbons, and aromatic compounds in IVF laboratories and their direct impact on early embryo development and pregnancy rates (11, 12).

To our knowledge, no studies addressing the impact of exposure to ambient fine PM (PM diameter ≤2.5 μm, PM$_{2.5}$) predominantly derived from traffic emissions on pre-implantation embryo development have been reported. Therefore, the purpose of this study was to evaluate the potential effects of pre- and/or postnatal exposure to ambient PM$_{2.5}$ on fertilization, embryo development, and cell lineage segregation in preimplantation blastocysts using the IVF mouse model.

MATERIALS AND METHODS

Animals

Random-bred Swiss albino mice (School of Medicine, University of São Paulo, São Paulo, Brazil) were used for the study. Mice were housed in polycarbonate cages with wood shavings on the floor in a room under controlled environmental conditions of 12:12 photoperiod, relative humidity of 50% ± 10%, and temperature of 24°C ± 1°C for the breeding protocol and under ambient conditions (temperature, humidity, and photoperiod) 24 hours a day during the entire experiment for pre- and postnatal exposure to filtered air (FA) and/or ambient air (AA) protocols. Cage cleaning and bedding changes were done inside the experimental environment every other day. Animals received a supply of balanced commercial food (Nuvital-Nutrients Ltd., Colombo, Paraná, Brazil) and fresh water ad libitum. Protocols used in the present study were approved by the Ethics Committee of the School of Medicine, University of São Paulo, and followed the Principles of Laboratory Animal Care published by the National Institutes of Health.

Exposure Setting and Inhalation Chambers

A detailed description of the exposure system used in this study was published in previous studies from our laboratory (6, 13). The exposure chambers were set up in the gardens of the School of Medicine, University of São Paulo, in downtown São Paulo, Brazil, near heavily trafficked streets. The system’s concept is based on the principle of creating a PM level gradient by filtering AA sampled from the surroundings. The exposure chambers, assembled side-by-side, are cylindrical aluminum structures measuring 2.0 m in diameter and 2.15 m in height, covered by plastic ultraviolet film. The air intake and distribution occur in the base of the cylinder for uniform dispersion throughout the chamber. AA is forced into the chamber by large fans and exits at the top through a wide opening. Both chambers received AA at 20 m$^3$/minute, making this essentially a normobaric system: the pressure inside did not exceed that of the outside by more than 3 cm H$_2$O. In the FA chamber, three stages of filters in hermetically sealed boxes are lined up immediately after the fans. The first-stage filter (Model TB; Purafil, São Paulo, São Paulo, Brazil) eliminates large particles, and the second (Model JFL-90; Purafil) and third (HEPA filter; Purafil) trap fine particles. Gaseous pollutants were not retained by the filtering system, thus concentrations of NO$_2$, SO$_2$, and CO were assumed to be similar in both chambers.

Ambient Fine PM Monitoring

Inside the exposure chambers, the 24-hour concentration of PM$_{2.5}$ was determined gravimetrically (14) by collecting particles from polycarbonate filters using Harvard impactors (Air Diagnostics, Harrison, ME) at a flow rate of 10 liters per minute (LPM) every other day. The results were expressed in units of micrograms per cubic meter. The mean (±SD) concentrations of PM$_{2.5}$ for the FA and AA exposure chambers were 6.77 ± 2.03 μg/m$^3$ and 28.93 ± 7.72 μg/m$^3$ during the prenatal period and 7.03 ± 1.87 μg/m$^3$ and 28.97 ± 14.22 μg/m$^3$ during the postnatal period. Pre- and postnatal PM$_{2.5}$ mean concentrations for each exposure chamber showed no differences.

Experimental Design

Natural cycling 6- to 8-week-old virgin females (n = 60) were placed with proven 10-week-old breeder males (n = 20) to mate naturally in the proportion of three females to each male. Mating was confirmed the following morning by the presence of a vaginal plug (gestation day 0 = day of postcoital plug), and the females were separated from the males. For prenatal exposure, the pregnant mice (n = 35) were randomly placed into either FA (n = 23) or AA (n = 12) chambers, four per cage. As soon as females displayed visual evidence of pregnancy they were checked daily until parturition. Within the first 24 hours after parturition, gestation length, litter size, litter birth weight, live-birth index (number of live offspring/number of offspring delivered), and sex of the offspring (determined by anogenital distance) were recorded. At this time, 11 females and offspring from the FA chamber were randomly selected and transferred to the AA exposure chamber (postnatal exposure). The remaining 24 females and offspring were kept in their original location. After weaning, the animals were separated, and only females born under experimental conditions continued in the chambers, four per cage. The study is made up of three different experimental groups: [1] pre- and postnatal exposure to FA (FA-FA group, n = 40); [2] prenatal exposure to FA and postnatal exposure to AA (FA-AA group, n = 36);
[3] pre- and postnatal exposure to AA (AA-AA group, n = 36). After 6 weeks of postnatal exposure, all females were superovulated as described below and killed by cervical dislocation immediately before oocyte collection.

**IVF and Embryo Culture**

IVF and embryo culture were performed according to procedures described elsewhere (15). Briefly, different culture media were used for each IVF step: [1] for collection of oocytes from oviducts and isolation of the cauda epididymis and vas deferens (collection medium), modified human tubal fluid (mHTF no. 90126; Irvine Scientific, Santa Ana, CA) pre-warmed to 37°C was used; [2] for sperm capacitation and IVF (insemination medium), HTF (no. 90125; Irvine Scientific) containing 10% serum substitute supplement (no. 99193; Irvine Scientific) was used; [3] for embryo culture, potassium-enriched simplex optimized medium (KSOM) with glucose and amino acids (no. MR-107; Specialty Media, Chemicon, Phillipsburg, NJ) supplemented with 5 mg/mL of human serum albumin (solution no. 10064; Vitrolife AB, Gothenburg, Sweden) was used. Insemination and embryo culture media were overlaid with embryo-tested mineral oil (Oil for Embryo Culture no. 9305; Irvine Scientific) and equilibrated overnight in a humidified atmosphere of 5% CO2 in air at 37°C. The same batch of each culture medium was used in all experiments. For each exposure group, oocytes were obtained from 6-week-old mice using IP injection of 10 IU of equine chorionic gonadotropin (eCG, Folligon, 1,000 IU; Intervet International, Sao Paulo, Sao Paulo, Brazil) followed 48 hours later by IP injection of 10 IU of hCG (Choragon, 5,000 IU; Ferring, Sao Paulo, Sao Paulo, Brazil). Females were killed 14–16 hours after hCG administration. Ovulated oocytes were released from the ampullar oviduct and placed into insemination medium. To obtain sperm, proven 10-week-old breeders were killed 12–13 hours after the females were injected with hCG, and the cauda epididymis and vas deferens were removed from each testis and placed in insemination medium. Spermatozoa were gently squeezed out of the epididymis using a watchmaker’s forceps, and residual caudal tissue was discarded. Capacitation was allowed to proceed for 60–90 minutes at 37°C in 5% CO2. IVF and embryo culture were performed in a HEPA-VOC filtration system incubator (Series II Water Jacketed Incubator, model 3110; Thermo Fisher Scientific, Waltham, MA). Sperm and oocytes were incubated for 4–6 hours. Fertilized oocytes from each female were allotted in groups of 10 and cultured in 20 μL drops of embryo culture medium for 120 hours.

**Embryo Evaluation**

Examination was done on the heated stage (~37°C) of an inverted microscope at ×200 magnification (Eclipse TE2000-U with Hoffman modulation contrast; Nikon, Melville, NY) 6, 96, and 120 hours after insemination (days 1, 4, and 5, respectively). Fertilization assessment was carried out 6–8 hours after insemination. Oocytes presenting two pronuclei and two polar bodies were considered normally fertilized. On days 4 and 5, embryo morphology, developmental progress, number of embryos reaching blastocyst stage (the blastocoel occupies at least 80% of total embryo volume), and number of blastocysts that at least started hatching were recorded. Blastocyst morphology classification was based on criteria described by Gardner and Lane (16). The hatching status of blastocysts identified on days 4 and 5 was graded on a 4-point scale from 0 to 3, where 0 = zona enclosed, 1 = initially (herniation of zona pellucida by less than 50% of the embryo), 2 = partially (herniation of zona pellucida by at least 50% of the embryo), and 3 = completely hatched blastocysts. A weighted hatching score representing the sum of the hatching status grade of every blastocyst divided by the total number of blastocysts was calculated for evaluations at 96 and 120 hours postinsemination (Score96 and Score120, respectively) for each female. The present system awards a total possible score of 3 for each evaluation day. A discriminatory scoring system (D Score) was then developed to identify the developmental potential between evaluations of the cohort of blastocysts from each female. The D Score was weighted by the equation:

\[
D\text{ Score} = \frac{[(\text{Score120}/3) + (\text{Score96}/3)] - (\text{Score120} - \text{Score96})/6}
\]

The rationale behind this system was to balance the hatching status and the developmental potential of blastocysts to facilitate the separation of females with cohorts of blastocysts presenting similar hatching status but different developmental potential. This scoring system produces a maximum score of \([(3/3) + (3/3)] - [(3 - 3)/6] = 2\).

**Differential Inner-Cell Mass and Trophoderm Cell Counts**

By day 5 of culture (120 hours after insemination), differential cell counts of the inner-cell mass (ICM) and trophoderm (TE) of three blastocysts produced in vitro randomly selected from each female mouse were determined by a modification in a method described by Thouas et al. (17). Intact blastocysts (groups of three to five) were incubated for approximately 10 seconds (or until TE visibly change color) in 500 µL of HTF medium containing 50 μg/mL propidium iodide (Chemicon) and 1% Triton X-100 (TX-100; Sigma, St. Louis). The blastocysts were then transferred to fixative solution containing 25 μg/mL of bisbenzimid (H33258; Hoechst AG, Frankfurt, Germany) and 100% ethanol (ethanol PA no. 1.09983.1000; Merck KGaA, Darmstadt, Germany) and stored overnight at 4°C. Fixed and stained blastocysts were then transferred into glycerol, individually placed on glass microscope slides under coverslips, and visualized for cell counting. Counting was performed from digital photographs of images obtained on an epifluorescence microscope (Eclipse 90i; Nikon, Melville, NY) fitted with a mercury lamp and excitation filters (460 nm for blue and red fluorescence and 560 nm for red only). An observer blinded to experimental protocols performed all cell counts.
Statistical Analysis

The experimental unit was an individual female mouse for the assessment of the reproductive success and the morphological development of zygotes produced by IVF according to the pre- and postnatal exposure to FA and/or AA. Gestation length, litter size, litter birth weight, live-born index (number of live offspring/number of offspring delivered), and sex ratio (male/female + male) variables were analyzed using two-sample Hotelling’s $T^2$ test assuming homogeneity of variance by the Bartlett-Box test. The genetic variability of the mice used in this study determined different patterns of ovarian response to superovulation, which were identified and grouped through the K-means cluster analysis method performed on the number of oocytes retrieved and inseminated as poor (centroids, $25.4 \pm 10.7$ and $15.8 \pm 13.6$, respectively), moderate (centroids, $61.5 \pm 11.1$ and $56.6 \pm 10.0$, respectively), or high (centroids, $105.5 \pm 19.8$ and $89.9 \pm 16.8$, respectively) responders. Thus ovarian response was analyzed as a categorical independent variable to evaluate its effect on each experimental group. Categorical data regarding ovarian response to superovulation in each experimental group were evaluated using a $\chi^2$-test. For the assessment of the morphological development of zygotes in vitro, the experimental unit was the mean number of zygotes per female mouse. However, only the drops of culture medium containing all 10 zygotes were included in the analysis. The dependent variables for this assessment included number of zygotes, fertilization rate, number of blastocysts and number of blastocysts that at least started hatching at 96 and 120 hours after insemination, blastocyst formation and hatching rates at 96 and 120 hours after insemination, D Score, total cell count, and number of ICM and TE cells of intact blastocysts that had developed from zygotes 120 hours after insemination. All percentage data were subjected to arcsine transformation. The effects of pre- and/or postnatal ambient $PM_{2.5}$ exposure and ovarian response on each dependent variable were evaluated with multivariate analysis of variance (MANOVA). The Tukey Significant Difference (HSD) post hoc test was used for all comparisons of individual dependent variables between groups. The distribution of the cell populations was displayed using notched box plots. A computer-generated list of random numbers was used whenever randomization was necessary. Data are presented as mean ± SD. The data were analyzed using the Statistical Package for the Social Sciences, version 13.0 (SPSS Inc., Chicago).

Ovarian Response to Superovulation after Pre- and/or Postnatal Exposure to AA

Ovarian response to superovulation was absent in 20.0%, 16.7%, and 22.2% of the mice in groups FA-FA, FA-AA, and AA-AA, respectively. The number of females presenting low, moderate, or high response to superovulation in the three groups were as follows: in the FA-FA group, 8, 13, and 11; in the AA-AA group, 6, 18, and 6; and in the AA-AA group, 8, 10, and 10, respectively, showing that the distribution of the different patterns of ovarian response was not affected by the pre- and/or postnatal exposure to AA ($\chi^2 = 4.507, P = .608$).

IVF, Embryo Development, and Differential Nuclear Staining

Means (±SD) for all in vitro embryonic development variables according to ovarian response pattern for each exposure group are reported in Table 1.

Effects of the Ovarian Response

A multivariate effect for ovarian response on IVF and embryo development and on blastocyst cell segregation was found (Pillai’s trace = 0.60; $F = 2.23; P = .001$; power = 0.998). The univariate analysis of the effect of the ovarian response on each parameter of IVF and embryo development to blastocyst stage is shown in Table 2. The ovarian response pattern revealed a significant effect on fertilization and fertilization rate, on the zygotes that reached blastocyst stage by 96 and 120 hours postinsemination, and on the hatching process of blastocysts by days 4 and 5 postinsemination but did not reveal any significant difference among the patterns in the discriminatory score. Post hoc comparison of the embryonic developmental parameters by ovarian response pattern showed significant differences between the patterns. A significant impairment of IVF, embryo development up to blastocyst stage, and hatching process at the level of $P < .05$ was observed in the high-response subgroup when compared with the moderate- and poor-response subgroups (Table 3). MANOVA revealed no significant effects on total cell count, ICM cells, TE cells, or ICM/TE ratio by ovarian response pattern, as shown in Table 2.

Effects of the Exposure to AA

A multivariate effect for pre- and/or postnatal exposure to AA on IVF and embryo development and on blastocyst cell segregation was found (Pillai’s trace = 0.62; $F = 2.40; P = .001$; power = 0.999). The univariate analysis of the effect of the exposure protocol on each parameter of IVF and embryo development to blastocyst stage is shown in Table 2. No significant differences except for the blastocyst rate at 120 hours postinsemination were found for any exposure protocol in the univariate analysis. Post hoc comparison of the blastocyst rate by day 5 of culture showed a significantly lower result for the FA-FA group when compared with the AA-AA and AA-AA groups (Table 4). Figure 1 shows the distribution of the total number of cells, cells in the ICM and TE, and the ratio...
TABLE 1
Effects of pre- and postnatal exposure of female mice to FA and/or AA on embryo development and lineage specification according to ovarian response.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>FA-FA exposure</th>
<th>FA-AA exposure</th>
<th>AA-AA Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poor responder</td>
<td>Moderate responder</td>
<td>High responder</td>
</tr>
<tr>
<td>Zygote</td>
<td>9.64 ± 0.69</td>
<td>9.16 ± 0.81</td>
<td>8.92 ± 0.75</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>0.96 ± 0.07</td>
<td>0.92 ± 0.08</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>Blastocyst (96 hours)</td>
<td>7.21 ± 3.13</td>
<td>7.11 ± 1.96</td>
<td>6.24 ± 2.40</td>
</tr>
<tr>
<td>Blastocyst rate (96 hours)</td>
<td>0.73 ± 0.32</td>
<td>0.77 ± 0.19</td>
<td>0.69 ± 0.26</td>
</tr>
<tr>
<td>Hatching (96 hours)</td>
<td>4.62 ± 2.59</td>
<td>4.03 ± 2.21</td>
<td>2.79 ± 1.68</td>
</tr>
<tr>
<td>Blastocyst (120 hours)</td>
<td>8.33 ± 2.99</td>
<td>8.34 ± 1.12</td>
<td>7.36 ± 1.96</td>
</tr>
<tr>
<td>Blastocyst rate (120 hours)</td>
<td>0.85 ± 0.29</td>
<td>0.91 ± 0.10</td>
<td>0.82 ± 0.19</td>
</tr>
<tr>
<td>Hatching (120 hours)</td>
<td>7.80 ± 2.82</td>
<td>7.51 ± 1.39</td>
<td>6.36 ± 2.07</td>
</tr>
<tr>
<td>D Score</td>
<td>0.63 ± 0.23</td>
<td>0.61 ± 0.22</td>
<td>0.50 ± 0.16</td>
</tr>
<tr>
<td>Total cell count</td>
<td>118.0 ± 15.5</td>
<td>122.7 ± 11.5</td>
<td>120.3 ± 11.4</td>
</tr>
<tr>
<td>ICM cells</td>
<td>29.3 ± 5.5</td>
<td>30.7 ± 8.1</td>
<td>29.9 ± 4.8</td>
</tr>
<tr>
<td>TE cells</td>
<td>88.7 ± 11.2</td>
<td>92.0 ± 9.6</td>
<td>90.4 ± 10.6</td>
</tr>
<tr>
<td>ICM/TE ratio</td>
<td>0.33 ± 0.04</td>
<td>0.34 ± 0.09</td>
<td>0.33 ± 0.06</td>
</tr>
</tbody>
</table>

Note: Values are means ± SD. For explanation of the discriminatory score (D Score), see text.

of ICM to TE cells of blastocysts originated from oocytes fertilized in vitro obtained from females exposed pre- and/or postnatally to filtered and/or AA. The univariate analysis of the effect of the exposure protocol on each parameter of blastocyst cell segregation is shown in Table 2. Allocation of ICM

### TABLE 2

Multivariate general linear model analysis: individual independent variables’ effects and full-factorial interaction between variables.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Group</th>
<th>Response</th>
<th>Group × response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Zygote</td>
<td>0.196</td>
<td>.823</td>
<td>5.461</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>0.135</td>
<td>.874</td>
<td>8.311</td>
</tr>
<tr>
<td>Blastocyst (96 hours)</td>
<td>1.505</td>
<td>.228</td>
<td>4.599</td>
</tr>
<tr>
<td>Blastocyst rate (96 hours)</td>
<td>2.297</td>
<td>.107</td>
<td>5.084</td>
</tr>
<tr>
<td>Hatching (96 hours)</td>
<td>1.280</td>
<td>.284</td>
<td>4.065</td>
</tr>
<tr>
<td>Blastocyst (120 hours)</td>
<td>1.382</td>
<td>.257</td>
<td>5.848</td>
</tr>
<tr>
<td>Blastocyst rate (120 hours)</td>
<td>4.074</td>
<td>.021</td>
<td>8.792</td>
</tr>
<tr>
<td>Hatching (120 hours)</td>
<td>1.110</td>
<td>.335</td>
<td>6.540</td>
</tr>
<tr>
<td>D Score</td>
<td>1.078</td>
<td>.345</td>
<td>1.745</td>
</tr>
<tr>
<td>Total cell count</td>
<td>1.516</td>
<td>.226</td>
<td>2.023</td>
</tr>
<tr>
<td>ICM cells</td>
<td>9.421</td>
<td>.000</td>
<td>0.041</td>
</tr>
<tr>
<td>TE cells</td>
<td>6.100</td>
<td>.003</td>
<td>2.845</td>
</tr>
<tr>
<td>ICM/TE ratio</td>
<td>13.967</td>
<td>.000</td>
<td>1.008</td>
</tr>
</tbody>
</table>


and TE cells for each exposure protocol is shown in Figure 2. The exposure protocol revealed a significant effect on ICM cell count, TE cell count, and ICM/TE ratio but no significant difference among the exposure protocols in total cell count. Post hoc comparison of the blastocyst cell segregation

### TABLE 3

Effects of ovarian response pattern to superovulation on the IVF and embryo development of Swiss albino mice.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Subset 1</th>
<th>Subset 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Response</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Zygote</td>
<td>Low</td>
<td>9.44 ± 0.76</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>9.36 ± 0.66</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>Low</td>
<td>0.94 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>Blastocyst (96 hours)</td>
<td>Low</td>
<td>8.00 ± 2.09</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>7.60 ± 1.63</td>
</tr>
<tr>
<td>Blastocyst rate (96 hours)</td>
<td>Low</td>
<td>0.81 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>0.84 ± 0.22</td>
</tr>
<tr>
<td>Hatching (96 hours)</td>
<td>Low</td>
<td>4.08 ± 1.99</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>3.30 ± 1.91</td>
</tr>
<tr>
<td>Blastocyst (120 hours)</td>
<td>Low</td>
<td>8.88 ± 1.90</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>8.68 ± 0.91</td>
</tr>
<tr>
<td>Blastocyst rate (120 hours)</td>
<td>Low</td>
<td>0.93 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>Hatching (120 hours)</td>
<td>Low</td>
<td>8.22 ± 1.80</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>7.88 ± 1.16</td>
</tr>
</tbody>
</table>

Note: P<.05, subset 1 vs. subset 2 (Tukey post hoc HSD test, after MANOVA). Ovarian response to superovulation with mean values of dependent variables showing no difference between them were grouped in homogeneous subsets.

parameters by the exposure protocol showed significant differences between groups. The cell counts in the ICM and the ICM/TE ratios in blastocysts produced in the FA-FA protocol were significantly higher \((P < 0.05)\) than in blastocysts produced in the FA-AA and AA-AA protocols. However, cell counts in the TE in blastocysts produced in FA-FA protocol were significantly lower \((P < 0.05)\) than in blastocysts produced in the FA-AA and AA-AA protocols (Table 4).

Table 2 shows that no significant ovarian response versus exposure protocol interaction on IVF and embryo development was observed \((Pillai’s trace = 0.45; F = 0.70; P = .940; power = 0.831)\).

### DISCUSSION

The experimental data provide robust evidence that the exposure to PM\(_{2.5}\) predominantly derived from traffic emissions and present in urban AA disrupts the allocation pattern of the first two cell lineages at the blastocyst stage, as evidenced by the reduction in ICM cell number and ICM/TE ratio, without interfering in the early development of mouse embryos. The total cell number of blastocysts was not affected by the exposure, indicating that cell segregation was deviated to TE cell lineage. On the basis of the similar pattern of lineage specification at the blastocyst stage observed in embryos from mice pre- and/or postnatally exposed to PM\(_{2.5}\), we speculate that the prenatal exposure to AA does not contribute to the disruption of lineage specification in blastocysts, although it has a negative impact on preimplantation embryo development as evidenced by the significant impairment of IVF and embryo development observed in the high-response group when compared with the moderate- and poor-response subgroups. Cell lineage specification at the blastocyst stage, rather than early embryo development, can be proposed as a biomarker for monitoring the effects of PM\(_{2.5}\) exposure on female reproductive health in experimental studies.

Despite the considerable amount of scientific knowledge focused on the correlation between exposure to urban air pollution and human health, few studies addressed the specific question whether urban air pollution can have a significant impact on female reproductive function \((6, 18, 19)\). A previous study evaluated the effects of chronic exposure to air pollution in a large urban center on the fertility of Bal-b/c female mice \((6)\). The analysis of the reproductive endpoints revealed a significant decrease in the number of viable fetuses, an increase in the number of implantation failures, and a slight decline in the male-to-female ratio at birth in the exposed group. The number of pregnancies and fetal deaths and the fetal and placental weights showed no differences between groups. The investigators concluded that although their study design did not allow identification of the factors involved in the pathogenesis of the fertility reduction promoted by exposure to air pollution, the findings demonstrated that female reproductive health is negatively impacted by air pollutants. Even though the reproductive outcome of pregnant mice exposed to PM\(_{2.5}\) was not a primary endpoint of our study, the results showed that gestation length, litter size, litter birth weight, live-birth index, and sex ratio did not differ significantly between exposure groups, which is in partial disagreement with the previous study. The differences observed could be attributed to our study design, in which a distinct mouse strain and different exposure period were employed.

### TABLE 4

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Subset 1</th>
<th>Subset 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposure</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Blastocyst rate (120 hours)</td>
<td>FA-AA</td>
<td>0.92 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>AA-AA</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>ICM</td>
<td>FA-AA</td>
<td>24.45 ± 5.58</td>
</tr>
<tr>
<td></td>
<td>AA-AA</td>
<td>24.08 ± 4.79</td>
</tr>
<tr>
<td>TE</td>
<td>FA-AA</td>
<td>102.60 ± 10.82</td>
</tr>
<tr>
<td></td>
<td>AA-AA</td>
<td>95.43 ± 12.28</td>
</tr>
<tr>
<td>ICM/TE ratio</td>
<td>FA-AA</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>AA-AA</td>
<td>0.26 ± 0.06</td>
</tr>
</tbody>
</table>

*Note: \(P < .05\), subset 1 vs. subset 2 (Tukey post hoc HSD test, after MANOVA). Exposure protocols with mean values of dependent variables showing no difference between them were grouped in homogeneous subsets.*
A previous study from our laboratory pointed out the possible effects of chronic exposure to air pollution on male gametes (20). Although a lower reproductive performance in female mice exposed to air pollution was observed in other studies (6, 21, 22), they were unable to differentiate the role of female gametes from other possible causes (uterine environment, for instance) in the pathogenesis of the observed adverse pregnancy outcomes. Thus, the current experiment was specifically designed to explore the role of abnormalities of female gametes on the reproductive dysfunction elicited by exposure to ambient particles. We found that a lineage specification defect in blastocysts, rather than impairment of the fertilization process or early embryo development, may provide a clue to further clarify the reason why adverse reproductive outcome is observed in mice exposed to PM2.5 predominantly derived from traffic emissions present in AA. Although the blastocyst rate by day 5 of culture showed significantly higher results for mice exposed either pre- and/or postnaturally to PM2.5, the mean number of zygotes that reached the blastocyst stage at 120 hours was similar among the exposure protocols. This observation is difficult to interpret but could possibly reflect the lower number of zygotes.
that reached the blastocyst stage by day 5 of culture in the poor-responder group of mice exposed to FA.

The formation of the blastocyst results from the functional differentiation of embryonic cells into two distinct lineages: the ICM cells that bring about all embryonic tissues and part of the extraembryonic membranes and the TE cells that contribute mainly to the formation of the fetal placenta (23). Both cell lineages and their ratios have a fundamental role in embryo survival and fetal viability, and the blastocyst has the tools necessary to control the specification of those cell lineages within a relatively narrow range (24). Experimental and clinical studies showed a positive relationship between ICM cell number and morphology and the rate of embryo implantation (25–27). There is convincing evidence that an imbalance in blastocyst cell lineage differentiation may compromise the subsequent postimplantation developmental potential of the embryo (24). Additionally, because of differences in cell positioning and metabolic requirements, ICM and TE cell lines have differential susceptibility to embryotoxic agents, and in most instances ICM cells appear less resistant to disruption than TE cells (28).

In our results pre- and/or postnatal exposure to ambient PM$_{2.5}$ (predominantly derived from traffic emissions) resulted in a significant decrease in ICM cell count (by $\sim$20%) and ICM/TE ratio (by $\sim$25%). Several studies evaluating the impact of ICM cell population on postimplantation growth in vivo showed that a marked reduction in the number of ICM cells resulted in lower implantation rates, higher rates of embryo resorption, and lower fetal weight, confirming that the absolute number of ICM cells is essential for normal postimplantation embryo development and pregnancy viability (29–31). In murine ET experiments, Tam (29) showed that a reduction by $\sim$30% in the number of cells in the ICM cell lineage of blastocysts transferred to pseudopregnant mice was associated with a higher risk of early pregnancy loss and lower fetal developmental potential. Similarly, Lane and Garder (30), while studying the effect of ammonium in the culture medium on the regulation of embryo physiology, observed that increased ammonium levels in the culture media significantly reduced the total number of blastocyst cells, specifically the ICM cell number and ICM/TE ratio, impairing the ability of transferred blastocysts to establish a viable pregnancy. On the basis of this finding, we could hypothesize that the significant decrease in the ICM cell population as a result of unbalanced allocation of cells during specification of the blastocyst lineages could represent the biological mechanism whereby ambient PM$_{2.5}$ might influence pregnancy viability. However, the pathway by which PM$_{2.5}$ predominantly derived from traffic emissions decreases the number of ICM cells and increases the number of TE cells is still unknown and needs clarification.

The decision to analyze the effects of pre- and/or postnatal exposure to ambient PM$_{2.5}$ on the ovarian response was based on clinical studies showing that smoking may accelerate ovarian follicular depletion, leading to a significant reduction in the ovarian reserve and poorer response to ovarian

![Differential staining of blastocysts obtained from Swiss albino female mice exposed pre- and postnatally to FA and/or AA: (A) FA-FA group, (B) FA-AA group, and (C) AA-AA group. Blue and pink colors indicate ICM and TE cells, respectively.](image-url)
stimulation at an earlier age (32–34). Even though the mechanisms involved in the detrimental reproductive effect of smoking were not the same as those from the exposure to air pollution, they may share similar pathways. Our data showed that the distribution of the different patterns of ovarian response to superovulation was not affected by pre- and/or postnatal exposure of female mice to ambient PM$_{2.5}$. This finding suggests either that the exposure to PM$_{2.5}$ may not affect ovarian reserve or that, if affected, it may have no clinical significance in young female mice.

The strengths of our study were the use of an experimental design that enabled us to create an exposure protocol representative of a real-world situation present in any large urban center and the observation that lineage specification at the blastocyst stage, despite the genetic variability of the mouse strain selected and consequent unevenness of ovarian response, was the only developmental parameter affected by exposure to air pollution, suggesting it might be a helpful biomarker for monitoring the effects of PM$_{2.5}$ exposure on female reproductive health in experimental studies. The study design has some limitations that should be addressed. IVF by itself can negatively affect the numbers of ICM and TE cells in blastocysts produced from ova fertilized in vitro when compared with those fertilized in vivo (35). However, the inclusion of another control group (in which blastocysts were produced after in vitro fertilization) to rule out a possible effect of the IVF procedure seemed unnecessary since previous studies have shown that the medium used for the embryo culture protocol in this experiment (KSOM with glucose and amino acids) allowed the in vitro development of blastocysts from the zygote stage with ICM and TE cell counts similar to those produced in vivo (36, 37). Since gaseous pollutants were not retained by the filtering system, the concentrations of NO$_2$, SO$_2$, and CO were assumed to be similar in both chambers, so reproductive toxicity should not be attributed solely to PM$_{2.5}$ because its physical and chemical interaction with gaseous pollutants could modify this effect. However, epidemiological studies evaluating the role of ambient O$_3$, NO$_2$, SO$_2$, and CO as confounders of ambient PM$_{2.5}$ health effects showed that the use of multiple pollutant models in these studies may not be suitable and that the health effects attributed to ambient gases may actually be the result of exposure to ambient PM$_{2.5}$, a reasonable surrogate for personal PM$_{2.5}$ exposure (38, 39). Although we found biological evidence that suggests the mechanism through which air pollution might negatively influence pregnancy viability, we did not further investigate this assumption by transferring blastocysts produced in each exposure protocol to pseudopregnant mice. Further studies evaluating the effect of exposure to AA on in vitro preimplantation and in vivo postimplantation embryo development are required to adequately address this question.

In conclusion, to our knowledge this study provides the first evidence that exposure to PM$_{2.5}$ predominantly derived from traffic emissions and present in the AA of a large urban center may negatively affect murine reproductive health by disrupting the allocation pattern of the first two cell lineages at the blastocyst stage. On the basis of this observation and of the fact that a significant reduction of the ICM cell population is incompatible with a normal embryo development after implantation, our results suggest that the differential sensitivity of ICM and TE cells to air pollution may contribute to the poorer reproductive outcome. Differential cell staining at the blastocyst stage can be proposed as a biomarker for monitoring the effects of air pollution on female reproductive health in experimental studies. The extrapolation of these observations to the clinical arena has important implications for the reproductive health status of women living in large urban areas where they are subject to long-term, repeated exposure to PM$_{2.5}$.

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