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# Metal embryotoxicity from urban particles in Sao Paulo city: An experimental study in chicken embryos ☆

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ABSTRACT

Chicken eggs were inoculated with suspensions of ambient air particles ($\leq 10$ μm, $PM_{10}$) from Sao Paulo city in 3, 0.3 or 0.03 μg doses on one of the four early days of embryo development. On the eleventh day of development alterations were observed on embryos inoculated with $PM_{10}$ 3 μg on the third day. Particles analysis showed high content of metals. Hence, embryos were also inoculated with $PM_{10}$ (3 μg) combined with metal chelating EDTA. $PM_{10}$ (3 μg) embryos presented underdevelopment (stage $29.44 \pm 11.4$) compared to vehicle and positive controls (stage $36.44 \pm 0.51$ Saline and stage $31.20 \pm 9.7$ Cyclophosphamide, $p \leq 0.05$); higher (47%) mortality rate (23% Saline and 42% Cyclophosphamide) and low (68%) viability (100% Saline and 70% Cyclophosphamide, $p = 0.04$). Effects were attenuated when embryos received $PM_{10}$+EDTA (stage $33.63 \pm 0.94$, 18.9% mortality rate and 82% viability). $PM_{10}$ from Sao Paulo city is embryotoxic and metal may be implicated in the toxic mechanism.



## 1. Introduction

Epidemiological studies have shown the possible impact of airborne particulate matter (PM) on fetal health (Glinianaia et al., 2004; Srám et al., 2005; Ritz and Wilhelm, 2008), including low birth weight, intrauterine growth retardation, preterm birth, stillbirth and organ malformation. These findings suggest that embryonic development may be a critical phase for the action of ambient particle toxicity. In the city of Sao Paulo, Brazil, evidence of adverse fetal outcomes associated to the $PM_{10}$ (airborne particulate matter $\leq 10$ μm) prenatal exposure has been reported in terms of low birth weight and stillbirth (Gouveia et al., 2004; Pereira et al., 1998).

The biological process that regulates embryo–fetal development is complex, involving environmental factors as well as physiological and behavioral phenomena. Because of differences in age, reproductive status and individual exposure to the complex mixture of airborne contaminants, studies focusing on the reproductive effects of air pollution in humans may encounter difficulties controlling confounding variables. For this reason, controlled animal studies are important to inform research gaps on the mechanism of embryotoxicity of air pollutants (Woodruff et al., 2009).

Instead of using mammalian assays which involve the complex mother–placental–fetus interaction, the chicken embryo is a versatile experimental system (Stern, 2005) that provides a controlled toxicological screening of possible injuries caused by pollutants during prenatal exposure (Binková et al., 1999, 2003; Alexander and Tuan, 2003; Ji et al., 2002). The embryotoxic approach of the Chicken Embryo Screening Test (CHEST) has a standard practice of *in ovo* exposure during critical periods of development in a self-contained entity. The early four days of chicken embryogenesis are a time of particular vulnerability, and disruptions occurring during this critical period may result in embryonic death, abnormal neuronal processing and organ malformation (Jelínek et al., 1985; DeWitt et al., 2005).

Ambient particles in Sao Paulo are derived predominantly from vehicles: an observation confirmed by chemical analysis and

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receptor modeling (CETESB, 2002; Mauad et al., 2008; Ynoue and Andrade, 2004; Castanho and Artaxo, 2001). It is composed of a high content of metals, such as arsenic (As), zinc (Zn), lead (Pb), vanadium (V) and nickel (Ni) (Carvalho-Oliveira et al., 2005a).

The present study was designed to address the toxic effects of urban particles in Sao Paulo on chicken embryos and the role of metals in the physiopathological pathway. We hypothesized that the CHEST approach would help characterize the role of urban ambient particles in embryotoxicity.

## 2. Materials and methods

The Review Board for Human and Experimental Studies of the Clinical Hospital, School of Medicine of the University of Sao Paulo (CAPPesq-HC-FMUSP), approved this study under research number 811/03.

### 2.1. $PM_{10}$ monitoring and characterization

In order to reflect the ambient air quality of São Paulo (PM monitoring), daily measurements of ambient levels of $PM_{10}$ made by (FH62 I-n Beta Attenuation Monitor; Graseby Andersen, Smyrna, GA) of Sao Paulo State Basic Sanitation, Technology and Environmental Protection Agency (CETESB, Sao Paulo, Brazil), were obtained online (http://www.cetesb.sp.gov.br/Ar/ar_dados_historicos.asp).

Samples of $PM_{10}$ of Sao Paulo city were collected nearby the Cerqueira César Monitoring Station to conduct specific laboratorial procedures designed for this study, including the $PM_{10}$ chemical analysis of trace elements and the $PM_{10}$ *in ovo* exposure, as follows.

### 2.2. $PM_{10}$ collection

From January to July 2003, particle samples were collected at the rooftop level (25 m above ground) of the School of Medicine of the University of Sao Paulo in downtown Sao Paulo, Brazil (23°33′19.38″S 46°40′12.42″W). The building of the School of Medicine is situated 20 m next to the road and 150 m next to an intersection with heavy traffic. Particles were collected on 5 polycarbonate filters using a low volume air sampler (10 L/min) equipped with a cut-off device for particles below 10 μm of diameter ($PM_{10}$) (Carvalho-Oliveira et al., 2005a, Mauad et al., 2008). Filters were weighed (MX5 Automated-S microbalance, Mettler Toledo, Switzerland) prior and after $PM_{10}$ sampling in order to determine the mass collected over 24 h of exposure.

### 2.3. $PM_{10}$ measurements

The PM samples collected in polycarbonate filters were stored in petri dishes before chemical analysis of trace elemental composition. In special concern to metals, the polycarbonate filters were used directly as targets for Energy Dispersive X-rays Fluorescence (EDXRF) spectrometry (EDX 700-HS, Shimadzu Corporation Analytical Instruments Division, 152 Kyoto, Japan) using a low power Rh-target tube at a voltage of 5–50 kV and a current of 1–1000 μA. The characteristic X-rays were detected by a Si(Li) detector. The EDX-RF spectra were collected for 240 s on the 10 $mm^2$ surface area of the samples in a vacuum. The measured sample intensities were converted to element concentrations (ng $cm^{-2}$) by calibration with the NIST Standard SRM 2783-Air Particulate on Filter Media (National Institute of Standards, Gaithersburg, MD, USA). Carbon was used as a mass balance. The same procedure was performed on non-exposed (blank) polycarbonate filters from the same stock.

### 2.4. Filter extract

For the toxicological studies, $PM_{10}$ aqueous suspensions were prepared. After EDX-RF analysis, the same PM filters were used to prepare the inoculation solutions. For this purpose, the PM filters were submerged in sterile 0.9% sodium chloride solution pH = 5.0 (saline AZB1307, Baxter Hospitalar Ltda, Brazil), and particles were extracted via agitation in an ultrasound water bath for 20 min (Carvalho-Oliveira et al., 2005a; Macchione et al., 1999). Filters were dry-weighed (MX5 Automated-S microbalance, Mettler Toledo, Switzerland) before and after re-suspension to determine the mass of the extracted PM. The concentration of particles in the aqueous suspension was established as 10 μm/mL, and posterior test dilutions were made in 3, 0.3 and 0.03 μg of PM/mL.

In all test solutions, the metals concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) (ICP-MS ELAN DRC II Perkin Elmer, at the Laboratório de Toxicologia e Essencialidade de Metais, Faculdade de Ciências Farmacêuticas, University of Sao Paulo, Brazil). Briefly, extracts of particulate matter in aqueous form were digested in closed vessels with a microwave oven decomposition system (Milestone START D) according to the following procedure: samples (0.25 g) were accurately weighed in a PFA digestion vessel, and then 5 mL of nitric acid 20 v/v and 2 mL of 30% (v/v) $H_2O_2$ were added. The bomb was placed inside the microwave oven, and the decomposition was carried out according to the method proposed by Sucharova and Suchara (2006). After that, the digestate was left to cool, and then Milli-Q water was added to make the total volume 25 mL. Then, rhodium was added as an internal standard to achieve a final concentration of 10 μg $L^{-1}$. All operations were performed on a clean bench. Multielement stock solutions containing 1000 mg $L^{-1}$ of each element were obtained from Perkin-Elmer (PerkinElmer, Norwalk, CT, USA). Analytical calibration standards were prepared daily over the range of 0–20 μg $L^{-1}$ for all elements by suitable serial dilutions of the multielement stock solutions in 4% (v/v) $HNO_3$.

### 2.5. Toxicological assays

A total of 408 fertilized eggs (avg. wt. 50 g) of the Plymouth Red chicken strain were obtained from a local commercial hatchery.

The study was conducted in two experiments.

The first experiment checked the most sensitive period of early chicken embryogenesis to particle suspensions. For this purpose, 264 eggs were used. Each egg was only once inoculated with one of the three different doses (3, 0.3 and 0.03 μg) of $PM_{10}$ on one of the four early days of embryo development (1st, 2nd, 3rd or 4th day of incubation), creating 12 experimental groups (three doses x four periods of development; $n = 22$ for each PM group). Prior to inoculation, all eggs were weighed and then candled, so the air sac could be traced and the shell checked for cracks. Cracked eggs were discarded prior to experimentation. The *in ovo* injections (0.3 mL for all test solutions) were performed by wiping the shell with 70% ethanol, poking a small hole in the center of the air sac with a sterile needle (21G11/4) and inserting the solutions directly over the highly permeable air sac membrane with a hypodermic syringe (1 mL). The injection hole was then sealed with paraffin (Henshel et al., 1997; DeWitt et al., 2005).

In the second experiment, we tested the possible role of soluble metals in embryotoxicity. Therefore, six groups of embryos were used to test the toxicity of the 3 μg dose of $PM_{10}$ administered on day three. One group of embryos received the same particle solution plus the metal chelating agent EDTA (ethylenediaminetetraacetic acid, 1.125 mg/0.3 ml/50 g egg weight). At this step, 144 eggs were evaluated.

For both experiments, we used incubation control of non-inoculated eggs (the Intact group), vehicle controls of blank filters sonicated with either pure saline (the Saline group) or saline with EDTA (the Saline+EDTA group) and a positive control (Cyclophosphamide, 5 μg/ml of saline; Gilani & Chatzinoff, 1983).

The pH of the vehicle used in all test solution (0.9% sterile saline) was originally 5.0. All test solutions had their pH determined (pHmeter Q400AS, Quimis, Brazil). No pH adjustment was made to prepare the inoculation solutions.

Eggs were horizontally incubated in an automatic rotating incubator (2 h intervals; Scotbrood, Brazil) at 37.8 ± 0.2 °C and a relative humidity of 60 ± 5% for 11 days, using the first 24 h of incubation as the reference day.

All eggs were opened on the eleventh day of incubation. Mortality was recorded, and living embryos were euthanized in an ether chamber. Each embryo was weighed and submitted to phenotypical analysis for developmental staging based on the normal stages in development of a chicken embryo (H–H developmental stages; Hamburger and Hamilton, 1951). The Hamburger and Hamilton stages (H–H) is a series of 46 chronologically defined steps of the morphological characteristics that encompass the normal chicken development, starting from the laid fertilized egg and ending with a newly hatched chicken. It is universally used in chicken embryology and provides detailed developmental chronology of the developing chicken to be accurately staged both at embryonic and fetal stages.

After staging, embryos were individually fixed in labeled containers filled with a buffered formalin solution (pH = 7.0) for 48 h.

Alterations in the following parameters indicated embryotoxicity:

1) Embryo weight (g);
2) Embryo mortality (%), characterized by the presence of degenerated neovascularization at the embryonic pole or the presence of a dead embryo;
3) Embryo developmental stage based on phenotypical features established by the H–H normal developmental stages, and;
4) Embryo viability (%) of each experimental group, established only for living embryos with an H–H developmental stage over 32 in the second experiment.

### 2.6. Statistical analysis

Results were evaluated by means of descriptive analyzes. Statistics were performed using the following tests: Univariate General Linear Model (embryo development) or a one-way ANOVA followed by post hoc Bonferroni (embryo weight) and LSD tests (embryo development and embryo mortality). Embryo