Reproductive Toxicology 75 (2018) 56–64

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Effects of diesel exhaust-derived secondary organic aerosol (SOA) on oocytes: Potential risks to meiotic maturation

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ARTICLE INFO

Article history:
Received 2 February 2017
Received in revised form 9 September 2017
Accepted 16 November 2017
Available online 20 November 2017

Keywords:
PM 2.5
Oocyte
Embryo
Diesel exhaust

ABSTRACT

Particulate air pollution (PM 2.5) is a worldwide concern. Growing epidemiological evidence has shown pathophysiological effects of PM 2.5, not only on cardiovascular system but also on reproductive performance. The composition and physicochemical properties of PM 2.5 vary depending on the emission sources, climate conditions, and complex chemical reactions in the air. These factors make it difficult to understand the cause and mechanistic details of the adverse health effects of PM 2.5. Here, we show potential impacts of PM 2.5 on oocyte maturation in mice by utilizing diesel exhaust-derived secondary organic aerosol (SOA), a major component of urban PM 2.5. We found that the SOA destabilized microtubules of mouse oocytes and *p*-benzoquinone is one of the candidates for the microtubule-destabilizing compounds. We propose that some biologically reactive components of PM 2.5 should be prioritized for the regulation of atmospheric quality.

1. Introduction

PM 2.5 consists not only of primary particles from natural (*e.g.*, volcano) and anthropogenic sources (*e.g.*, automobile exhaust, emission from industry, and smoking), but also of *secondarily* generated particles formed by photochemical reactions of volatile organic compounds (VOCs) and gases (NOx, SOx, and ozone) [1]. *Secondary* organic aerosol (SOA) accounts for a large portion of the organic phase of atmospheric particulate matter [2].

Epidemiological studies have revealed an association between ambient air pollution and mortality from respiratory and cardiovascular diseases [3], especially between the concentration of fine particulate matters and respiratory and cardiovascular malfunctions [4]. Adverse health effects of ambient air pollution are not limited to these organs but also reproductive ones. Recent human studies suggested that ambient air pollution is associated to preterm labor (PTL; delivery prior to 37 weeks of pregnancy), low birth weight (LBW; birth weight less than 2500 g), and intrauterine growth retardation (IUGR; birth weight less than that expected for a given gestational age) [5]. Moreover, exposure to PM 2.5 has been shown to significantly associate with PTD [6,7] and IUGR [8,9]. In addition to the effects on fetal development described above, the latest studies also show that PM 2.5 exposure affects the chance for conception [10] and increases a risk of female infertility [11]. Experimental studies using laboratory animal also revealed the embryo toxicity of urban traffic-generated particulate matter [12–14]. These studies suggest a hazard of PM 2.5 on the oocyte/embryo.

However, what components of PM 2.5 (complex mixtures of compounds) contribute to which mechanisms associated with ovotoxicities are not known yet. We used experimentally generated SOA by mixing diesel exhaust with ozone (simulating the photochemical reactions described above), supposing that motor vehicle-derived SOA majorly accounts for man-made, anthropogenic SOA [15]. We report here the first attempt to evaluate the *in vivo* effects on fecundity using the experimentally generated SOA facilitated with whole animal inhalation chambers and the first *in vitro* screen for the potentially hazardous components on the function of oocyte/embryo. Here we show the effects of SOA on oocyte maturation in the ovary and the estimation for the prioritization of SOA components that should be mitigated.

2. Materials and methods

2.1. Chemicals, reagents, and antibodies

3-Isobutyl-1-methylxanthine (IBMX), 9-fluorenone, menadione, *o*-toluic acid, and *p*-benzoquinone were purchased from Sigma (St Louis, MO). Paraformaldehyde (PFA), neutral buffered for-

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https://doi.org/10.1016/j.reprotox.2017.11.006
0890-6238/

malin (NBF), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from WAKO (Osaka, Japan). Alpha-MEM medium, penicillin/streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer were purchased from Gibco/Thermo Fisher Scientific (Grand Island, NY). Mineral oil was purchased from Nacalai Tesque (Kyoto, Japan). Hoechst dye was purchased from Dojin Chemical (Kumamoto, Japan). Fetal bovine serum (FBS) was purchased from HyClone/Thermo Fisher Scientific (Waltham, MA). The following antibodies were used in this study: anti-Tom20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-tubulin (Oncogene Research Products, Boston, MA), anti-γH2AX (Cell Signaling Technology, Danvers, MA), anti-cytochrome C (BD Biosciences, San Jose, CA) and Alexa-conjugated secondary antibodies against IgG from the respective animal species (Molecular Probes/Thermo Fisher Scientific, Eugene, OR).

2.2. Animals, mating, estrus cycle checking, and histology

All animal procedures were performed in accordance with the *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of the National Institute for Environmental Studies (NIES). The scheme of inhalation chamber exposure experiment was described in Fig. S1A. 10 weeks old female C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under the specific pathogen-free controlled environmental condition (temperature, 22 ± 0.5 °C; humidity, 53 ± 5%; lights on 07:00–19:00) with free access to food and water. After the acclimation, 26 female mice were equally divided into two groups ('Clean air' and 'SOA', see below) and were introduced into each chamber 3–5 animals per cage. Inhalation exposure had been conducted from 12 weeks old to 16 weeks old in the high fat diet (F2HFD1: Oriental Yeast, Tokyo, Japan) condition. At the end of inhalation exposure, animals were moved to the conventional condition. Then, 13 female mice in each group were further subdivided into 3 groups (mating immediately, mating after checking for the estrus cycle, and histological analysis). Mating was tested in the proportion of 5 female housed with one male per cage in normal chow (CE-2: CLEA Japan, Tokyo) condition for 2 weeks. The males were then removed and pups were housed in cages with mothers until weaning. For checking the estrus cycle, vaginal smears were collected every day at approximately 4 p.m. and were stained for 1 min with 0.1% methylene blue dissolved in ultra-pure Milli-Q (MQ) water. The standard criteria were adopted for the determination of diestrus (resting period), metestrus, estrus (sexually active period), and proestrus [16]. For checking the ovarian histology, ovaries from female mice at 16 weeks old were collected after pentobarbital anesthesia in the next morning after the completion of inhalation exposure. Ovaries were fixed in 10% NBF at room temperature for 2 days. After washing in PBS for 30 min at room temperature, fixed ovaries were placed in 70% ethanol for 30 min and then stored in fresh 70% ethanol at 4 °C until further processing. Ovaries were then dehydrated, and embedded in paraffin. After the mating test (pregnancy rates, pups numbers, and pups weights), histological analysis was further conducted. Serial sections sagittally sectioned at 3-μm thicknesses were pasted to slide glass at an interval of 70–100 μm to grasp the overall picture of the ovary (follicular maturation, corpus luteum formation, and any signs of tumors or infiltrations). Sections pasted on the slides were stained with hematoxylin and eosin after deparaffination.

2.3. Generation of SOA and collection of particles

SOA was generated as described previously [17]. Animal exposure was conducted in two chambers (SIBATA, Tokyo, Japan), designated as 'Clean air' and 'SOA'. The 'Clean air' chamber was provided with air filtered with High-Efficiency Particulate Air (HEPA) and charcoal filters. In the 'SOA' chamber, diesel exhaust mixed and reacted with 0.6 ppm ozone was introduced. An 81-diesel engine (J08C; Hino Motors, Ltd., Hino, Japan) was used to generate diesel exhaust. The engine was operated at the lower loaded condition (speed, 2000 rpm; torque, 0 Nm) to suppress the generation of soot dust of relatively large size. The engine was operated for five days (from 11:00 pm to 3:00 am) each week for 4 weeks. The following formula mathematically describes the intended level of exposure: 100 $\mu g/m^3$ (a target concentration) × 5/7 (days per week) × 5/24 (exposure hours per day) = 14.9 $\mu g/m^3$. Thus, this level is theoretically comparable to the Japanese environmental standard for PM 2.5; the annual average should not exceed 15 $\mu g/m^3$. The concentration and particle size for SOA used in the present study was 130.9 ± 31.2 $\mu g/m^3$ and 28.3 ± 1.3 nm (mean ± SD), respectively. The ratios of total carbon (TC) to particle mass (mean ± SD) were 0.44 ± 0.25 for the 'Clean air' chamber and 0.76 ± 0.04 for the 'SOA' chamber. The ratios of organic carbon (OC) to TC (mean ± SD) were 0.84 ± 0.07 for the 'Clean air' chamber and 0.63 ± 0.03 for the 'SOA' chamber. The ratios of water-soluble organic carbon (WSOC) to OC (mean ± SD) were 0.40 ± 0.41 for the 'Clean air' chamber and 0.18 ± 0.07 for the 'SOA' chamber.

For *in vitro* experiments, particles collected on Teflon® filters (FD-500; Sumitomo Electric, Osaka, Japan) were submerged and extracted three times in analytical grade methylene chloride by sonication at 4 °C for 20 min. The solvent was evaporated to dryness under nitrogen gas and dissolved in DMSO to prepare a 10 mg/mL solution. The sample was stored at −80 °C until testing and was used at a final concentration of 100 μg/mL for *in vitro* experiments.

2.4. Estimation of compounds structurally related to microtubule destabilizers

The procedures for the selection of candidate compounds, which may perturb tubulin organization (see below), were shown in Fig. S2. Components that were possibly present in the inhalation chamber were selected from the literature [18] and by measurements (Fujitani Y, unpublished data). We extracted the structural features from the predicted components of SOA, 956 registered reference substances, and 22 known colchicine-site ligands [19] (Fig. S2A) using the KAsinhou Tool for Ecotoxicity (KATE) [20], a Quantitative Structure-Activity Relationship (QSAR) modeling system. We first obtained the sub-structural features of the compounds based on the Fragment Identification by Tree Structure (FITS) algorithm [21] by inputting the Simplified Molecular Input Line Entry Specification Syntax (SMILES) of the compounds (Fig. S2B) into KATE. Next, reference substances were ranked in order in each case based on the abundance of sub-structures in common with each colchicine-site ligand. Scores were accordingly assigned using an arbitrary scale as follows: 5 points (colored black), less than tie for fifth; 2 points (colored gray), less than tie for 30th; 1 point (colored light gray), less than tie for 50th. Finally, based on the value of the total score, reference substances were re-ranked in order as depicted in the heatmap (Fig. S2C). The data obtained from reference substances were used for normalizing the estimation scores of the predicted components of SOA (Fig. S2D). This scoring method was used to select the most probable two candidates (Fig. S2E). Since both candidates shared quinone-related structural codes (*e.g.*, the quinones: #5232 and #6042; both aliphatic and aromatic ketone: #4958), we selected two additional candidates focusing not only on the structural complexity (abundance of the number of sub-structural features), but also on specific structural features (Fig. S2E and F). Accordingly four microtubule-destabilizing candidate compounds, menadione, 9-fluorenone, *p*-benzoquinone, and o-toluic acid (Fig. S2G) were chosen for the *in vitro* assay.