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Detection of lindane and 7,12-dimethylbenz[a]anthracene toxicity at low concentrations in a three-dimensional ovarian follicle culture system

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ABSTRACT

Exposure to environmental toxicants that target ovarian follicles can have long-lasting effects on women's reproductive health and health of the offspring. Experiments in rodents have contributed knowledge about the effects of individual toxicants on ovarian follicles. However, little is known about the effects of mixtures of toxicants on ovarian follicular health. We studied the combined effects of low, physiologically- and environmentally-relevant concentrations of toxicants on murine secondary ovarian follicles cultured in an encapsulated three-dimensional (3D) system. Exposure to lindane and 7,12-dimenthylbenz(a)anthracene (DMBA) led to decreased follicle survival, impaired development and compromised maturation in a concentration-dependent manner. DMBA showed a greater toxicity to cultured follicles compared to lindane. The mixtures of lindane and DMBA did not produce a synergistic toxic effect on follicles. Rather, ovarian follicles exposed to the mixtures showed survival and growth patterns similar to the follicles exposed to the same concentrations of individual toxicants. Our findings regarding follicle toxicity at such low concentrations help informing what might be overlooked when regulating environmental toxicants. The proposed 3D culture system allowed studying the effects of mixtures of environmental toxicants in a physiological setting, providing much needed information on how simultaneous exposure to multiple toxicants affects complex and sensitive biological structures, such as ovarian follicles.

1. Introduction

Contamination of the environment with organic pollutants has emerged as a significant public health concern due to the pervasive nature of these xenobiotics. Such contaminants could target the ovaries, thus exerting long-lasting adverse effects on women's reproductive health. Females are born with finite numbers of follicles, which sustain the ovarian endocrine function and fertility [1]. Ovotoxic xenobiotic exposure can deplete the non-renewable follicular reserve, resulting in primary ovarian insufficiency (POI) [2–4]. Noticeably, follicles at all stages of development can be impacted by xenobiotic exposure, leading to deleterious effects on follicle development, oocyte competence, ovulation and hormone production [5]. Identifying the hazards of those xenobiotic substances to the reproductive health of women is imperative, as their toxicology can present severe symptoms and effects, even at very low concentrations [6].

Lindane (gamma-hexachlorocyclohexane), a persistent organic pollutant used as a pesticide/insecticide, causes impairments of female fertility by altering ovarian follicle development and function in women, laboratory and farm animals [7]. Lindane inhibits intercellular communication by affecting gap junctions [8,9], and suppresses follicle-stimulating hormone (FSH) and transforming growth factor beta 1 (TGF β1)-stimulated steroidogenesis in rats [10]. Additionally, lindane can abolish oocyte-directed follicle organizing activity in vitro [11]. Banned from agricultural uses [12], lindane is still used as a pharmacological treatment of scabies and lice. Because of its widespread use, lipophilic nature, and resistance to biodegradation, low concentrations of lindane can persist in lipid-rich human tissues [13]. Yet, its toxicity to ovarian follicles, especially at low concentrations and in a 3D culture environment has not been established.

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7,12-dimethylbenz[a]anthracene (DMBA) is a polycyclic aromatic hydrocarbon (PAH). DMBA is found in burning of organic matter incineration [14], and acts as a immunosuppressor and a carcinogen [15]. DMBA exposure in cultured neonatal rat ovaries induces DNA double strand breaks and subsequent DNA repair response [16], and depletes ovarian follicles at all developmental stages [3,16–18]. In individually cultured preovulatory rat follicles (600–800 μm), DMBA induces granulosa cells and theca cells apoptosis and that the onset of apoptosis is preceded by an increase in reactive oxygen species (ROS) [19]. However, DMBA toxicity, especially in combination with other toxicants, has not been established in vitro in individually cultured mouse secondary follicles.

Environmental xenobiotics, such as lindane and DMBA, present a special case of environmental impacts as they have been massively produced. Those chemicals are most interesting because they provide a manner on which to base more specific epidemiological studies, as the area where they are used, or produced, can be monitored for subsequent phenomena indicating altered fertility patterns in a prognostic manner [6]. The essential problem of environmental ovotoxicants is to determine their range of effects. This can be extremely difficult since it can vary according to doses even at the picomolar concentrations [20]. Additionally, epidemiological data on outcomes of human exposure to environmental toxicants are limited. Alternatively, the use of animals in laboratories is particularly useful. However, those in vivo assays could be labor intensive, time consuming and costly, and therefore are limited in throughput. In contrast, in vitro assays allow for greater flexibility and higher throughput to capture the complexity of varied exposure scenarios in a rapid and relatively inexpensive manner.

Here we describe the application of encapsulated in vitro follicle growth (eIVFG) system, composed of fibrin-alginate interpenetrating networks (FA-IPNs), for investigation of toxicants' effects on mouse secondary follicles [21]. The eIVFG system recapitulates key events of mammalian follicle development in physiological, in-vivo-like conditions and serves as a high-throughput tool to screen for toxic effects of multiple compounds [22]. Encapsulated in the FA-IPN system, follicles are individually cultured in a 96-well plate, which allows study of low-concentration effects of toxicants with high sensitivity in individual follicles and oocytes. Moreover, people are exposed to a cocktail of xenobiotics and yet, limited scientific information is available on potential toxic effects of mixtures of environmental pollutants on human reproductive health. Therefore, here we investigated the effects of exposure to lindane and DMBA individually and to lindane-DMBA mixtures at physiological- and environmentally-relevant concentrations on ovarian follicle survival, growth, and maturation.

2. Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Media formulations were purchased from Thermo Fisher Scientific (Carlsbad, CA). Nalgin MV-120 sodium alginate (Carrageenan, Xanthan, Alginate, Ingredients Solution, Inc., Waldo, ME, USA) and the fibrinogen-thrombin kits (Baxter Healthcare, BioScience Division, Westlake Village, CA) were used for fibrin-alginate gel formation.

2.1. Animals

Mice were purchased (Harlan, Indianapolis, IN), and housed in ventilated cages in a temperature and light controlled environment (12L:12D), and provided with food and water. Animals were treated in accordance with the guidelines and regulations set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the established Institutional Animal Use and Care (IACUC) protocol at the University of Michigan. Experiments with animals were performed in strict accordance with the protocols approved by the University of Michigan IACUC. All animals used in this study were euthanized by overdose of isoflurane via inhalation followed by decapitation.

2.2. Ovarian follicle isolation, encapsulation and culture

Secondary follicles (120–135 μm) were mechanically isolated from 12 to 14 day-old F1 hybrids (C57BL/6NHsd inbred × CBA/JCrHsd) as previously described [22,23]. Only follicles with intact morphology were selected for encapsulation and culture. Selected follicles were encapsulated individually in FA-IPN as previously described [22]. Encapsulated follicles were placed in 96-well plates, with each well containing 100 μL growth medium: 50% αMEM Glutamax® and 50% F-12 Glutamax® supplemented with 1 mg/mL bovine fetuin, 3 mg/mL bovine serum albumin (BSA), 0.1% insulin-transferrin-selenium (ITS), and 10 mIU/mL urofollictropin (Bravelle® 75 IU urofollitropin for injection, Ferring Pharmaceuticals, Saint-Prex, Switzerland). For all experiments, follicles were cultured at 37 °C with 5% CO_2. Follicles were imaged every 2 days using an inverted Leica DMI 3000 microscope with 20X objectives (Leica Microsystems, Bufflo Grove, IL). After imaging, half of the growth medium (50 μL) was replaced with pre-equilibrated, fresh medium.

Follicles were considered dead if they showed dark granulosa cell layers, and/or if the oocyte was visibly fragmented. Survival curves were created using Graphpad Prism 7 software by recording the numbers of dead follicles on specific days of cultures. Dead follicles were excluded retrospectively in growth curves. Diameters of live follicle were obtained by averaging 2 perpendicular measurements from basement membrane to basement membrane of individual follicles in ImageJ software (National Institute of Health, Bethesda, MD). Growth curves of survived follicles were then obtained by plotting the average follicle diameters over time.

2.3. In vitro exposure of toxicants to follicles

Cultured follicles were exposed in vitro to either lindane or DMBA or both toxicants on Days 0, 2, and 4 of eIVFG. Lindane and DMBA were first dissolved in dimethyl sulfoxide (DMSO) and then diluted with growth media to reach desired concentrations. The final concentrations of DMSO were kept at 0.05% for all toxicant treatment conditions, which was consistent with previous publications [22,24]. If treated, toxicants of the same concentration were also added to the fresh media on Days 2 and 4 to keep the toxicant concentration constant. Starting on Day 6, fresh media without toxicant was used when changing media.

2.4. In vitro follicle maturation (IVFM)

In vitro follicle maturation was performed on day 12 of eIVFG as previously described [22]. Briefly, preovulatory follicles were harvested from the remaining alginate by dissolving the gels with 10 IU/mL alginate lyase after removing all the culture media in the wells. Harvested follicles were subsequently transferred to maturation media (αMEM Glutamax® supplemented with 10% FBS, 5 ng/mL epithelial growth factor (EGF), 1.5 IU/mL human chorionic gonadotropin (hCG), and 10 mIU/mL urofollictropin) for up to 20 h of incubation. Oocytes were then denuded from surrounding cumulus cells using 0.3% hyaluronidase. Oocytes were considered as metaphase II (MII), if a polar body was present in the perivitelline space. If the nucleus was clearly visible, oocytes were considered arrested at prophase I in the germinal vesicle stage