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Disruption of oocyte maturation by selected environmental chemicals in zebrafish

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ABSTRACT

Oocyte maturation can be a target of endocrine disruption by environmental chemicals capable of acting as hormone mimics, receptor blockers, and/or enzyme inhibitors. Six environmental chemicals (genistein, endosulfan, malathion, iprodione, carbaryl, and glyphosate) were selected to determine their ability to interfere with oocyte maturation in zebrafish. The translucent oocytes undergoing germinal vesicle (nucleus) breakdown (GVBD) were counted and expressed as a ratio of oocytes undergoing GVBD and total oocytes exposed. The GVBD increased significantly in oocytes exposed to 10 IU/ml to 100 IU/ml human chorionic gonadotropin (hCG). The lowest effective concentration of genistein that inhibited hCG-induced GVBD was 30 μM, while endosulfan inhibited it at 0.03 μM concentration. In addition, malathion inhibited hCG-induced GVBD at the lowest concentration of 60 μM. These inhibitory effects were likely due to the chemicals acting as estrogen mimics, induction of estrogen receptors, or increase in aromatase activity resulting in enhanced estrogen action. Fungicide iprodione, possibly acting as a progestin mimic, promoted hCG-induced GVBD at the lowest concentration of 20 μM, while the weed killer glyphosate inhibited hCG-induced GVBD starting at the 50 μM concentration. These results demonstrate the feasibility of using fully grown zebrafish oocytes arrested at the prophase I stage in an *in vitro* incubation system to evaluate the effects of a variety of environmental chemicals on oocyte maturation.

1. Introduction

Environmental chemicals or contaminants such as pesticides, drugs, chemical wastes from industries, or personal care products may act as endocrine disrupting chemicals (EDCs) which interfere with the normal function of the endocrine system. Many of these contaminants act as estrogen or anti-androgen mimics which accumulate in surface waters and pose great risk to aquatic organisms, mostly by impacting reproductive function (Kelce et al., 1994; Rahman et al., 2009; Soto et al., 1991). Most often, EDCs exert their effect on reproduction along the hypothalamus-pituitary-gonadal (HPG) axis by mimicking or inhibiting steroid hormones, interfering with hormone synthesis and/or secretion, or affecting receptor-mediated pathways. The major components of HPG axis in all vertebrates are remarkably similar, including the basic mechanisms of oocyte growth and maturation (Charlier et al., 2012).

Oogenesis, the formation of female gametes (oocytes) in mammals and teleost fish follow a similar pattern. Oogenesis in mammals begins around birth, but oogonia are arrested at the diplotene stage of prophase-I (Brooker, 2009). There is a rise in estrogen as well as progesterone levels preceding the resumption of meiosis in the oocytes that is triggered by a pre-ovulatory surge of luteinizing hormone (LH) from the pituitary gland (Molina and Ashman, 2013; Sun et al., 2009). By the end of the follicular phase, these arrested oogonia contain a large nucleus called the germinal vesicle (GV). However, a decrease in estrogen and a concomitant increase in progesterone levels help the follicular phase enter the oocyte maturation phase (Molina and Ashman, 2013). Likewise in teleost fish, oogenesis is arrested in prophase-I during the secondary oocyte growth phase and oocyte maturation is activated by a progestin, maturation inducing hormone (MIH) produced in response to the LH-surge (Lubzens et al., 2010; Nagahama and Yamashita, 2008). In fish ovaries, 17β estradiol (E_2) is produced during oocyte growth, while the MIH is produced during oocyte maturation. Thus, a dramatic shift from E_2 to MIH occurs in fish ovarian follicles immediately prior to oocyte maturation (Kobayashi et al., 1988; Scott et al., 1983).

There are five stages of oocyte development in zebrafish (Lubzens et al., 2010; Selman et al., 1993). The primary growth (stage I) and

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cortical alveolus (stage II) are both hormone independent, while vitellogenesis occurs during stage III in which a large amount of yolk proteins are produced in the liver in response to increasing estrogen levels and are then stored in the growing oocyte (Lubzens et al., 2010). In zebrafish, the ovarian form of cytochrome P450 aromatase, converts testosterone to 17β estradiol (Villeneuve et al., 2013), which plays a crucial role in oocyte growth, particularly during vitellogenesis (Nagahama, 1994; Nagahama and Yamashita, 2008). At stage IV, meiosis reinitiates, the germinal vesicle migrates toward the oocyte periphery, and germinal vesicle breakdown (GVBD) occurs under the influence of a progestin, MIH (Nagahama, 1997; Nagahama and Yamashita, 2008). The LH induces 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), the MIH in zebrafish oocytes, which stimulates the synthesis of maturation promoting factor (MPF) (Nagahama, 1997; Nagahama and Yamashita, 2008). Phosphorylation of MPF leads to the GVBD and oocyte maturation (Kondo et al., 1997; Nagahama, 1997). Finally, during stage V oocytes become mature eggs arrested at metaphase II; they are ready for fertilization, a process regulated by genomic mechanisms (Pinter and Thomas, 1999; Selman et al., 1993).

During zebrafish oocyte maturation, the MIH and its membrane receptor are the two major components that play important roles in the oocyte maturation in zebrafish (Hanna and Zhu, 2009; Hanna et al., 2010; Nagahama, 1997). Any environmental chemicals affecting these components are likely to impact the oocyte maturation. For example, natural isoflavone genistein has been shown to mimic the actions of estrogen in mouse by inhibiting oocyte maturation, *in vivo* and *in vitro*, and fertilization and embryonic development (Chan, 2009; Jung et al., 1993). This effect of genistein appears to be mediated through the estrogen receptor pathways (Wang et al., 1996). Other chemicals may mimic the actions of progestins such as the fungicide prochloraz, which is also known to stimulate oocyte maturation by inhibiting cytochrome P450 aromatase in mammals and fish (Blystone et al., 2007; Kan et al., 1985), thereby reducing estrogen production and promoting progestin action. The organochlorine pesticides, methoxychlor, lindane, and dieldrin are capable of inhibiting oocyte maturation in mammals and in marine invertebrates (Picard et al., 2003) by acting as progestin receptor antagonists. The organochlorine pesticide endosulfan and an organophosphorus insecticide malathion have also been shown to inhibit LH-induced oocyte maturation *in vitro* in the common carp, *Cyprinus carpio* (Haider and Imbaraj, 1988).

Three concentrations of each selected chemical were tested to assess their effects on zebrafish oocyte maturation. The concentrations for each chemical were determined based on those used previously in similar *in vitro* experiments in fish and mammals (Haider and Imbaraj, 1988; Jung et al., 1993; Monod et al., 2004), levels that cause embryo toxicity or deformity in zebrafish (Moon et al., 2016; Sassi-Messai et al., 2009), and their known concentrations in the environment. Genistein, a weak estrogen, occurs naturally in plants and has been used to treat breast cancer at 25 and 250 mg genistein/kg diets (Lamartiniere, 2000). It has been found at 111 nM concentration in air-dried wood pulp, and at concentrations of 48.5 nM in untreated and 38.8 nM in treated pulp mill effluent (Kiparissis et al., 2001). Malathion is mostly used to control mosquitoes, and has been detected in surface water, for example at concentrations as high as 27.2 nM to 266.4 nM in Iran (Baghfalaki et al., 2013) and 7.2 nM to 90.3 nM in India (Sankararamakrishnan et al., 2004). The insecticide endosulfan is linked to various health hazards in wildlife and humans and was scheduled to be phased out by US Environmental Protection Agency by the end of 2016 after it was already banned in many other countries (Miller, 2010). The acute toxicity of endosulfan on zebrafish embryos has been observed at concentrations reaching 3.69 μM (Moon et al., 2016). Carbaryl is an insecticide, which is known to inhibit cholinesterase at the range of concentrations from 49.7 nM to 49.7 μM (Ferrari et al., 2004), and has been reported to induce developmental defects in zebrafish embryos at 99.4 μM concentration (Schock et al., 2012). Iprodione has been detected in surface water up to 200 μM (US-EPA, 2007) and glyphosate at 17.7 μM to 218.8 μM concentrations in water samples from soil filtration assays and 1.2 μM to 8.9 μM in water samples collected in close proximity of soils with soybean cultivation (Peruzzo et al., 2008). Zebrafish embryo deformities have been reported after exposure to glyphosate at 147.9 μM to 443.6 μM concentrations (Sulukan et al., 2017). Although concentrations of some of the chemicals tested in this study are at the higher end of environmentally relevant concentrations, their continued use in at least certain areas of the world and environmental persistence justifies an examination of the lowest effective concentration of these chemicals.

This study provides an evaluation of the reproductive toxicity of six selected chemicals, a phytoestrogen (genistein), an organochlorine pesticide (endosulfan), an organophosphorus insecticide (malathion), a fungicide (iprodione), an insecticide (carbaryl), and a commonly used weed killer (glyphosate) by examining GVBD as a marker of oocyte maturation in zebrafish oocytes. Although some of these chemicals have been shown to alter oocyte maturation in other model organisms, the present study in zebrafish tested the potential reproductive toxicants to select one or more chemicals for future *in vivo* exposure experiments in this species. We hypothesize that environmental chemicals that mimic estrogens or progestin hormones will inhibit oocyte maturation or induce oocyte maturation, respectively, and the other selected reproductive toxicants may impair oocyte maturation by mechanisms other than interference with estrogen or progestin receptors. This study provides data on the effects of selected chemicals with potentially different mechanisms of action on gonadotropin-induced GVBD as a marker of oocyte maturation, a critical stage of gamete maturation relevant to reproductive success.

2. Materials and methods

2.1. Chemicals

Progestin hormone (17α, 20β-DHP), human chorionic gonadotropin (hCG), iprodione (purity: 99.5%; CAS # 36734–19-7), carbaryl (purity: 99.9%; CAS # 63–25-2), endosulfan (purity: 99.4%; CAS # 115–29-7), genistein (purity: ≥98%; CAS # 446–72-0), malathion (purity: 98.7%; CAS # 121–75-5), glyphosate (purity: 99.7%; CAS # 1071-83-6), and 60% Leibovitz L-15 medium (L-15) were purchased from Sigma-Aldrich, USA. The GIBCO antibiotic-antimycotic solution and other routine laboratory chemicals were purchased from Thermo Fisher Scientific, USA.

2.2. Animal care

Adult zebrafish were purchased from a commercial vendor (The Wards). They were maintained in glass aquaria in the science building (Animal Care Facility) at approximately 25 °C with 14 L: 10D photoperiod. They were acclimated at least for 2 weeks before using female fish as egg donors for the experiments. The fish were handled and experiments conducted according to the national and institutional guidelines after the animal use protocol was approved by the Institutional Animal Care and Use Committee. Aquaria were kept covered with lids to avoid fish from jumping out. They were fed a diet of dry freshwater flakes at least once a day. Fish were checked daily for any sign of disease and sick fish were immediately removed from aquaria using a net. Water quality was tested at least once a week to maintain optimal conditions for the growth and survival of zebrafish (Reed and Jennings, 2011).

2.3. Germinal vesicle breakdown assay

Ovaries were obtained from gravid females and the ovarian fragments were placed in a culture dish, and washed with 60% Leibovitz L-15 medium (L-15). Ovarian follicles were observed carefully under a microscope and their diameters were measured with an ocular