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Dimethadione embryotoxicity in the rat is neither correlated with maternal systemic drug concentrations nor embryonic tissue levels

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ABSTRACT

Pregnant rats treated with dimethadione (DMO), the N-demethylated metabolite of the anticonvulsant trimethadione, produce offspring having a 74% incidence of congenital heart defects (CHD); however, the incidence of CHD has high inter-litter variability (40–100%) that presents a challenge when studying the initiating events prior to the presentation of an abnormal phenotype. We hypothesized that the variability in CHD incidence was the result of differences in maternal systemic concentrations or embryonic tissue concentrations of DMO. To test this hypothesis, dams were administered 300 mg/kg DMO every 12 h from the evening of gestational day (GD) 8 until the morning of GD 11 (six total doses). Maternal serum levels of DMO were assessed on GD 11, 12, 13, 14, 15, 18 and 21. Embryonic tissue concentrations of DMO were assessed on GD 11, 12, 13 and 14. In a separate cohort of GD 12 embryos, DMO concentrations and parameters of growth and development were assessed to determine if tissue levels of DMO were correlated with these endpoints. Embryos were exposed directly to different concentrations of DMO with whole embryo culture (WEC) and their growth and development assessed. Key findings were that neither maternal systemic concentrations nor tissue concentrations of DMO identified embryos that were sensitive or resistant to DMO *in vivo*. Direct exposure of embryos to DMO via WEC also failed to show correlations between embryonic concentrations of DMO with developmental outcomes *in vitro*. We conclude that neither maternal serum nor embryonic tissue concentrations of DMO predict embryonic outcome.

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1. Introduction

Congenital heart defects are the most common class of anomaly, accounting for almost one third of all birth defects (Dolk et al., 2011). Of these, the most common is the interventricular septation defect (VSD) (Rosamond et al., 2008; Bernier et al., 2010), occurring in an estimated four out of every thousand live births (Hoffman and Kaplan, 2002). Despite their commonality, very little is known about the etiology of VSD. It is estimated that 10–30% of congenital heart defects are the result of known genetic mutations (Hoffman, 1990), with the remainder being linked to a variety of environmental factors including maternal health, nutritional status, pharmaceutical therapy, recreational drug use, and exposures to industrial chemicals and pesticides (Jenkins et al., 2007). With the increasing incidence of gestational exposures to chemicals or pharmaceutical therapies, it has been suggested that the prevalence of congenital heart defects will rise (van der Linde et al., 2011). This has prompted us to investigate the mechanisms by which chemicals induce VSD.

To understand how chemicals disrupt *in utero* heart development, our strategy has been to identify chemicals that are particularly potent inducers of congenital heart defects (CHD). One such chemical that has been used by a number of independent investigators is the anticonvulsant trimethadione, which depending upon the strain of rat studied and the dosing regimen used, induces 48–100% incidence of VSD (Clark and Takao, 1990; Solomon et al., 1997; Fleeman et al., 2004). Trimethadione was removed from the market due in part to the 87% combined incidence of fetal loss and congenital malformations in infants after *in utero* exposure (Feldman et al., 1977). We have further developed this rat model of chemical-induced VSD by administering instead, dimethadione (DMO), the N-methylated active metabolite and the proximate teratogen of the anticonvulsant trimethadione (Butler, 1953; Chamberlin et al., 1965; Buttar et al., 1978; Wells et al., 1989). In our hands, on a study-to-study basis, DMO induces an average incidence of between 68% and 74% in offspring (Weston et al., 2011; Rodger et al., 2014). Unlike trimethadione, which reportedly induces only membranous VSD in rat (Fleeman et al., 2004), DMO induces

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both muscular and membranous VSD (Weston et al., 2011), more reflective of the clinical presentation of VSD. Taken together, these data suggest that studies using DMO may yield important translatable knowledge about how *in utero* chemical exposures disrupt heart development.

Despite the mean 74% incidence of VSD after *in utero* exposure to DMO, the inter-litter incidences of VSD ranged from 40% to 100% (Weston et al., 2011). In our model, dams are dosed with DMO on GD 8–11, the time at which the initial damage to the developing heart primordium occurs; however, the VSD phenotype does not present until GD 16, the time at which the ventricular septum closes (Purssell et al., 2012). Thus, it is not possible to *a priori* identify those litters or embryos that are destined to develop VSD with morphological endpoints, and this is further complicated by the high degree of inter-litter variability in the incidence of VSD. Thus, to elucidate the early cellular events that lead to chemical-induced CHD, there is a need to accurately identify litters or embryos that will develop VSD prior to the closure of the septum on GD 16.

The vulnerability of some litters to DMO exposure, suggests maternal factors that modulate systemic DMO concentrations (metabolism or excretion) may explain the high inter-litter variability in the incidence of VSD. Thus, one goal of these studies is to characterize maternal serum and embryonic tissue concentrations of DMO using our previously described DMO dosing regimen (Weston et al., 2011; Purssell et al., 2012; Aasa et al., 2014; Rodger et al., 2014). One hypothesis is that maternal serum or embryonic tissue concentrations of DMO can be used to identify embryos that are sensitive or the embryotoxic effects of DMO.

An alternate approach to study the early events that lead to chemical-induced VSD is to use whole embryo culture (WEC) as originally described by New (1978). In this technique, whole embryos are removed from the uterus and cultured *in vitro* under very tightly controlled conditions, thereby allowing the investigation of direct effects of a chemical on the embryo removed from confounding maternal influences, such variability in DMO clearance (New, 1978; Webster et al., 1997). As a step toward using WEC to elucidate the early effects of DMO on heart development, the second goal of this paper was to determine the *in vitro* culture concentrations of DMO that would recapitulate the vivo maternal exposures, bearing in mind that WEC allows for a 44 h exposure to a constant level of DMO, whereas during the dosing window, the *in vivo* exposures are sinusoidal with an overall escalating trend for 72 h in duration.

In summary, the major goals of this paper were to: (1) describe the maternal and embryo/fetal concentrations of DMO in our previously reported chemically-induced VSD model (Weston et al., 2011; Purssell et al., 2012; Aasa et al., 2014; Rodger et al., 2014), and to (2) determine whether tissue concentrations of DMO were correlated with altered developmental outcomes.

2. Methods

2.1. Animals and housing

Time-mated female Sprague-Dawley rats [Crl:CD (SD)IGS BR] were obtained from Charles River Laboratory (Kingston, NY) and individually housed in polycarbonate shoebox cages with. Food (PMI Certified Rodent Chow 5002, PMI Feeds, Inc., Richmond, IN) and municipal tap water softened and further treated by reverse osmosis and ultraviolet sterilization provided *ad libitum*. Room conditions were set at 12-h light/dark cycle (lights on/off; 0700 h/1900 h), relative humidity of 50 ± 20%, and temperature of 70 F ± 5 °F (22 ± 3 °C). All procedures underwent veterinary review and were approved either by Pfizer's or Queen's University's Institutional Animal Care and Use Committee (IACUC).

2.2. Test article and treatment regimen

Dimethadione (5,5-dimethyl-2,4-oxazolidinedione CAS# 695-53-4), purchased from Sigma Aldrich (St. Louis, MO) was dissolved in de-ionized H_2O and made fresh prior to oral administration by gavage using 10 ml/kg body weight of a 30 mg/ml stock. Control animals were administered de-ionized water at 10 ml/kg body weight. For WEC experiments DMO was dissolved in PBS and administered at no more than 50 μl/ml of culture media.

Animals were dosed with 300 mg/kg DMO beginning on the evening of GD 8 (morning of plug = 0) and every 12 h thereafter until the morning of GD 11 (total of six doses). This is the maximum maternally tolerated dose, and this dosing regimen induces a 74% incidence of VSD (Weston et al., 2011). *In vitro* concentrations of DMO were set to approximate the area under the curve (AUC) of the *in vivo* exposure (AUC_{GD8-12} 67,376 μg*h/ml) or the highest, the mean and the lowest serum concentrations of dams on GD 11 after *in vivo* administration of DMO. The concentrations were, respectively, 1900 μg/ml, 1600 μg/ml or 1300 μg/ml.

2.3. Experimental design and conduct

All animals were observed at least twice daily for clinical signs, and body weights and food consumption were measured daily. Dams were euthanized by exsanguination and carbon dioxide inhalation. Serum was prepared from the harvested blood and retained for analysis of DMO. Embryos/fetuses were harvested on GD 11, 12, 13 14, 15, 18 and 21 and visible malformations noted and photographed. Embryos were then rinsed in PBS, placed in pre-weighed tubes and the excess fluid removed prior to freezing and future analysis of DMO concentrations.

3. WEC methods

Embryo culture was conducted based on methods originally described by New (New, 1978) and subsequently modified (Thomson et al., 2011). Briefly, male and female Sprague Dawley rats were on a 12-h lights on/off cycle of 1100 h/2300 h because this permitted the harvest of rat embryos with 4–8 somites between 0800 h and 1000 h in the morning. At approximately 1600 h, a vaginal impedance monitor (Fine Instruments Model EC-40, Vancouver, BC) was used to determine the proestrus phase of the estrous cycle (>5.0 mOhm by vaginal impedance). Females determined to be in proestrus were then housed overnight with a male breeder. At 0700 h the following morning, the male and female were separated; this was designated as GD 0. On the morning of GD 10, pregnant females were anesthetized by carbon dioxide asphyxiation and bled *via* cardiac puncture using an 18 gauge needle and syringe.

3.1. Embryo culture medium preparation

Embryos were grown in 90% heat inactivated rat serum diluted with Tyrode's balanced salt solution (Sigma Aldrich, T2397) and penicillin/streptomycin (Sigma Aldrich, P0781) added to a final concentration of 50 U/50 mg/ml.

3.2. Embryo harvesting and culture conditions

Briefly, embryos were harvested from dams on the morning of GD 10 and had between 4 and 6 somites. At the initiation of culture (1100 h ± 1 h) a gassing mixture of 5% O_2, 5% CO_2, balance N_2 was administered to culture bottles for 2–3 min. After a period of acclimation (2 h), DMO was added through the rubber stopper with a Hamilton syringe (details below). At between 1600 h–1700 h, all bottles were gassed (2–3 min) with 20% O_2, 5% CO_2 balance N_2. Thirty hours after the initiation of culture (1600 h–1700 h on the second day), embryos were gassed (2–3 min) with 50% O_2, 5% CO_2, balanced N_2. Culture bottles were rotated at 30 rpm using a New Brunswick Scientific roller drum (Model TC-7) located within a 37 °C incubator. Cultures were terminated approximately 44 h after the initiation of culture.

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