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The aryl hydrocarbon receptor agonist 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) alters early embryonic development in a rat IVF exposure model*

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ABSTRACT

Aryl hydrocarbon receptor (AHR) ligands, including 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD), accelerate reproductive senescence and one proposed target is the early embryo. To discriminate between direct effects on the oocyte and early embryo and those mediated by complex ovarian interactions with TCDD, IVF was carried out in the presence of TCDD (10, 100 nM) and the aryl hydrocarbon antagonist CH-223191 (1 μM) combined factorially. TCDD-induced Cyp1a1 mRNA expression was absent in 2-cell embryos; however morulae exhibit dose-dependent Cyp1a1 expression. TCDD induced accumulation of sperm in the perivitelline space and displacement of blastomere nuclei. At 100 nM TCDD, aberrations in cytokinesis and nuclear positioning were observed 2-cell embryos and morula and these effects were reversed in the presence of CH-223191. Our data suggest that acute exposure to TCDD has direct effects on early development in the rat that permit discrimination of AHR-mediated and AHR-independent mechanisms through which environmental toxicants impair mammalian reproduction.

1. Introduction

The aryl hydrocarbon receptor (AHR) pathway is an evolutionarily conserved, widely expressed orphan receptor pathway activated by many environmental toxicants [1,2]. AHR mediates effects of environmental toxicants on fertility and may also serve as yet undefined role(s) in normal reproduction [3–6]. Activation of the AHR by small coplanar molecules and subsequent signal transduction through the aryl hydrocarbon receptor nuclear translocator (ARNT) protein, heat shock protein 90, and xenobiotic-response elements (XREs) leads to changes in gene transcription in many tissues [7]. The most studied of these response genes include Cyp1a1, Cyp1a2 and Cyp1b1 loci that encode the xenobiotic-metabolizing monooxygenases central to the adaptive metabolic response [8]. Both AHR and Cyp1a1 mRNA are strongly expressed in mouse pre-implantation embryos. Interestingly, it was shown that immediately following fertilization, there is an increase in constitutive CYP1a1 mRNA levels in mouse embryos [9]. AHR ligands (such as dioxins and polychlorinated biphenyls) induce a spectrum of maldevelopmental and toxic responses [10]. It was shown that, in rodents, both acute and chronic exposure to the prototypic AHR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) accelerates reproductive senescence through ovarian targets [11–13]. To discriminate between direct effects on the oocyte versus those mediated by complex ovarian interactions with TCDD, we developed a rat IVF model to examine AHR related functions soon after fertilization and early cleavage. Our past in vivo studies showed that maternal exposure to TCDD disrupts compaction-stage embryonic development [13]. Most previous studies examining maternal dioxin exposure and subsequent fetal health have focused on late gestation, while peri-conceptional and pre-implantation stages of development remain largely unexplored. TCDD affects early embryogenesis during pre-implantation development, but it is still not clear if TCDD induces these reproductive effects during this period in a direct manner [14]. The goal of this study was to evaluate the direct effects of TCDD exposure during fertilization and early embryonic development on embryo quality in vitro.

2. Materials and methods

2.1. In vitro fertilization

2.1.1. Animals

Female Sprague-Dawley rats (age, 28 days) were purchased from Charles River Laboratories (St. Louis, MO, USA) and housed under a 12L:12D photoperiod (0600–1800 h) and controlled temperature (23 ± 2 °C) and humidity. Food (Purina

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Rodent Chow; Ralston Purina Co., St Louis, MO) and water were provided *ad libitum*. Exposure to AHR ligands was limited in these studies to the fertilization period to evaluate fertilization, first cleavage competence, and early embryo morphology. Accordingly, in vitro fertilization (IVF) was carried out in the presence of TCDD (0, 10, 100 nM [15,16]) and the aryl hydrocarbon antagonist CH-223191 (0, 1 μM [17]) combined factorially. TCDD (CAS 1746-01-6; MW: 391.9; purity >99%) was obtained from Cambridge Isotope Laboratories, Inc. (Lenexa, KS, USA) and CH-223191 was obtained from Calbiochem, Inc. (La Jolla, CA, USA). CH-223191 has been demonstrated to specifically antagonize AhR-mediated actions of TCDD in vitro [17]. The following study was approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

2.1.2. Preparation of sperm suspension and capacitation

All IVF procedures were performed as described previously [18]. In our study we used a chemically defined medium for 1-cell rat embryos (mR1ECM) composed of 110 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2{\cdot}6H_2O$, 2.0 mM $CaCl_2{\cdot}2H_2O$, 25.0 mM $NaHCO_3$, 7.5 mM D-glucose, 0.5 mM sodium pyruvate, 13.3 mM sodium lactate, 0.1 mM glutamine, 2% minimal essential medium (MEM) essential amino acid solution (Gibco BRL Grand Island, NY, USA), 1% MEM nonessential amino acid solution (Gibco BRL), 335 mOsm NaCl, 4 mg/ml bovine serum albumin (BSA). Based on previous results [19] and to assure successful fertilization and high rate of development, our mR1ECM medium was supplemented with NaCl (110 nM). Drops of fertilization and culture media (400 μl each) were covered with mineral oil (M-8410; Sigma, MO, USA) in polystyrene culture dishes (60 mm × 15 mm; Falcon 353004; Becton Dickinson, NJ, USA) and equilibrated overnight at 37 °C in 5% CO_2. Sperm obtained from adult Sprague-Dawley males as described previously [18] were diluted (1×10^6 spermatozoa/ml) in pre-equilibrated IVF medium (mRECM1) with or without CH-223191 at 37 °C, in 5% CO_2, 5% O_2 for capacitation to occur.

2.1.3. Collection of cumulus-oocyte complexes, in vitro fertilization and assessment of sperm penetration

After 5 h of sperm capacitation, in vivo matured cumulus oocyte complexes were collected from oviducts of 31-day old female Sprague-Dawley rats ($n = 10$ donors following superovulation with eCG and hCG) [16]. IVF ($n = 6$–10 COCs/treatment group) was undertaken in the presence of the aryl hydrocarbon agonist (TCDD; 0, 10, 100 nM) and antagonist (CH-223191; 0, 1 μM) and incubated at 37 °C, in 5% CO_2, 5% O_2. The dose of aryl hydrocarbon receptor antagonist was chosen based on the studies of Kim et al. [20]. CH-223191 shows high specificity for AHR and in contrast to other known AHR antagonists has no agonist effect on AhR [20]. After 8 h of co-culture with sperm, oocytes were collected, washed, and examined for fertilization under a phase-contrast or differential interference contrast (DIC) optics (Zeiss Axiovert II, 40×, USA). Fertilization rates were calculated as the number of penetrated oocytes (two pronuclei, a sperm tail in the vitellus and emission of the second polar body) divided by the total number of oocytes. First cleavage rates were calculated from the number of 2-cell embryos divided by the number of fertilized embryos for each treatment group. Fertilized oocytes were washed and transferred to media containing appropriate treatments in addition to mRECM1, 300 mOsm NaCl, 4 mg/ml BSA. Cells were cultured for additional 14 h at 37 °C, in 5% CO_2, 5% O_2.

2.1.4. Embryo culture

After 14 h, 2-cell embryos ($n = 6$/group) were transferred to mRECM1 rat ≥2-cell media. Embryos from each treatment group were either frozen for analysis of Cyp1a1 gene expression, or fixed for fluorescence microscopy analysis of DNA and f-actin. 96 h after insemination, multi-cellular embryos were transferred to mRECM1 rat ≥2-cell media supplemented with 10% FBS. Additional samples were frozen for gene expression analysis or fluorescence microscopy of embryos at 96 h and 120 h after insemination.

As controls for IVF, we performed natural mating experiments to obtain embryos processed as described previously [13,21,22]. Female Sprague-Dawley rats (21 days, $n = 5$/group) were purchased and housed as described in Section 2.1.1. Proven males were introduced on evening of proestrus following superovulation with pregnant mare serum gonadotropin (PMSG) and hCG [13,16]. Mating was confirmed by the presence of sperm on vaginal cytology the following morning. Pre-implantation embryos were collected in EmbryoMax® FHM HEPES buffered medium (Chemicon, CA, USA) pre-warmed to 37 °C by flushing oviducts and uteri on 1 day after mating. Embryos were pooled and cultured as in experiment 1 (10–20 embryos per treatment group) in medium supplemented with TCDD (0, 100 nM) and aryl hydrocarbon antagonist CH-223191 (0, 1 μM) combined factorially for additional 72 h. Exposure to AHR ligands was limited in these studies to the culture period to evaluate first cleavage competence, and embryo morphology. Embryos were counted at the 2-cell stages and fixed for fluorescence analysis at the 8–16 cell stages.

2.1.5. Fluorescence microscopy

Pre-implantation embryos from both IVF and natural mating experiments were processed for chromatin (DNA, Hoechst 33258, Invitrogen, USA) and f-actin (Alexa 568 phalloidin, Invitrogen, USA) staining and analyzed by fluorescence microscopy as previously described [13]. Embryos were fixed for 30 min in 4% PFA at 37 °C and stored at 4 °C in wash solution comprising PBS supplemented with 2% BSA, 2% skim milk powder, 2% normal goat serum, 100 mM glycine, 0.01% Triton X-100 and 0.2% sodium azide until processing for microscopy. Pre-implantation embryos were extracted for 30 min at room temperature in 0.1% Triton X-100 and incubated overnight at 4 °C in wash solution. DNA was stained with Hoechst 33258 (1 μg/ml in wash solution) for 30 min and cytoskeletal integrity was analyzed by staining f-actin with rhodamine-labeled phalloidin (1 unit/ml in wash solution; Molecular Probes, Invitrogen, USA) for 30 min. Pre-implantation embryos were mounted under coverslips without compression in medium containing 50% glycerol and 25 mg/ml sodium azide. Embryos were analyzed by whole mount fluorescence microscopy (Zeiss Instruments, Axiovert II, 40× oil immersion, N.A. 0.95).

2.1.6. Real-time PCR

Quantitative real-time PCR was used to assess changes in Cyp1a1 gene expression in 2-cell and morula stage in vitro fertilized embryos as a marker of toxicant action. The induction of Cyp1a1 mRNA has long been used as an indicator of AHR activation in response to both endogenous and exogenous ligands [10,22]. RNA was isolated from embryos using the RNAqueous-Micro kit (Ambion, TX, USA). A single round of linear amplification was performed using the MessageAmp II aRNA kit (Ambion, TX, USA). Amplified embryo RNA and universal reference RNA (Stratagene, CA, USA) were reverse transcribed to cDNA using Arrayscript Reverse Transcriptase (Ambion, TX, USA) and assayed in duplicate for β-actin (housekeeping gene) and Cyp1a1 gene expression using specific primer and probe sets and TaqMan chemistry [22–24]. Real-time PCR was performed in a 25 μl final reaction volume using TaqMan Gene Expression Assay and TaqMan® 2× Universal PCR Master Mix (Applied Biosystems, USA). The final concentration of each real-time PCR reaction component was: 1× TaqMan Universal PCR Master Mix, 250 nM for the probe, and 900 nM for each primer. Real-time PCR was carried out in the 7300 real-time PCR System (Applied Biosystems, USA). The non-template control (NTC) samples were included in each run. Additionally to exclude the amplification of contaminating genomic DNA, reactions without reverse transcriptase were performed. Assays were pre-validated by Applied Biosystems and used FAM as a reporter dye. Ct values were calculated for each endpoint and corrected for β-actin gene expression. Relative gene expression was calculated using a universal rat cDNA standard curve.

2.2. Statistical analysis

Data were tested to confirm normality and homogeneity of variances by the Kolmogorov–Smirnov normality test and Bartlett's test respectively. Data were then analyzed by ANOVA followed by Fisher's test as a post hoc test for comparison of means. The nonparametric Mann–Whitney *U* test was used to identify difference among treatment groups for endpoints that did not meet the above criteria. *P*-values of less than 0.05 were considered significant.

3. Results

3.1. Fertilization rate and 1st cleavage

Both fertilization rate and 1st cleavage rate did not differ among IVF treatment groups (Table 1). Similarly, in embryos derived from natural mating first cleavage rate and general morphology were not affected by TCDD treatment (Table 2). Additionally, percentage of 2-cell embryos from control group fertilized in vitro (86%) is comparable with embryos fertilized in vivo (Tables 1 and 2). Thus, at the level of fertilization efficiency and ability to progress through the first embryonic cell cycle, TCDD had no effect. In contrast, analysis of zygotes by DIC optics (Fig. 1), revealed two striking differences between TCDD exposed and control groups. Control oocytes had one sperm in the perivitelline space, while those fertilized in the presence of TCDD had multiple sperm. In addition, 2 cell embryos

Table 1
The effect of TCDD on fertilization and 1st cleavage rates following IVF.

Treatment	% of oocytes examined	
	Penetrated[a]	2-cell stage[b]
Control	65.9 ± 5.4	86.0 ± 5.2
1 μM CH-223191	55.9 ± 17.7	100.0 ± 9.4
10 nM TCDD	59.3 ± 7.9	83.3 ± 0.0
10 nM TCDD, 1 μM CH-223191	56.0 ± 8.5	99.3 ± 14.8
100 nM TCDD	65.1 ± 10.4	78.1 ± 11.2
100 nM TCDD, 1 μM CH-223191	57.1 ± 11.8	80.2 ± 11.2

[a] Calculated as the number of oocytes with 2 pronuclei and sperm tail in the vitellus divided by the total number of oocytes observed per group ($n = 40$–60/group).
[b] Percentage is the portion that developed to the 2-cell stage from those that were considered fertilized after 22 h of culture.