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Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on secretion of steroids and STAR, HSD3B and CYP19A1 mRNA expression in chicken ovarian follicles

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HIGHLIGHTS

- TCDD is a negative modulator of steroid secretion by chicken ovarian follicles.
- TCDD inhibits steroidogenesis by influence *STAR*, *HSD3B* and *CYP19A1* mRNA expression.
- Exposure of the laying hen to dioxins may affect growth and maturation of ovarian follicles.

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ABSTRACT

The aim of the study was to investigate the *in vitro* effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on steroid hormone secretion by chicken ovarian follicles and mRNA expression of genes involved in steroids synthesis. In the first *in vitro* experiment, white (WF) and yellowish (YF) follicles and fragments of the theca (TL) and granulosa (GL) layers of the 3 largest yellow preovulatory follicles (F3–F1) were incubated in a medium supplemented with TCDD (0.01–100 nM). In the second experiment, they were incubated in a medium with TCDD (10 nM), ovine LH (10 ng/mL; oLH) or a combination of oLH (10 ng/mL) and TCDD (10 nM). It was found that TCDD decreased estradiol (E2) secretion by WF and the TL of all preovulatory follicles, testosterone (T) secretion by WF, YF, and the TL of F2 and F1 follicles, and progesterone (P4) secretion by the GL of the preovulatory follicles. It also reduced oLH-stimulated E2 and P4 secretion by all examined follicles and T by WF. Real-time qPCR revealed that TCDD affected basal and oLH-stimulated expression of STAR, HSD3B and CYP19A1 mRNAs in all investigated ovarian follicles. In conclusion, the data obtained indicate that TCDD inhibits sex steroids secretion from chicken ovarian follicles. The effects of TCDD depend on its concentration and the stage of follicle maturation, and are associated with modulation of STAR, HSD3B and CYP19A1 mRNAs expression. These results indicate that the exposure of the laying hen to TCDD by influence of ovarian steroidogenesis may impair the selection of white follicles to preovulatory hierarchy and disturb their growth and preovulatory maturation.

1. Introduction

Dioxin-like compounds, which include polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and certain polychlorinated biphenyls, are a group of structurally related chemicals that are produced as unwanted by-products of industrial processes (Kulkarni et al., 2008). The most toxic compound, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), can be produced inadvertently in nature, *e.g.* by bush and forest fires, but also by human activities, such as combustion, chlorine bleaching of pulp and paper, certain types of chemical manufacturing and processing and other industrial processes. Uncontrolled combustion such as burning household trash is expected to become the largest source of dioxin emissions to the environment. As a lipophilic compound resistant to degradation, TCDD is bioaccumulated in animals and humans (Aylward et al., 2005; Giesy et al., 2003). The toxic effects of TCDD are mediated by high affinity binding and activation of the aryl hydrocarbon receptor (AHR) (Beischlag et al., 2008; Karchner et al., 2006). It binds AHR in the cytosol of cells and triggers conformational changes of AHR for nuclear translocation. In the nucleus

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ligand-activated AHR dimerises with AHR nuclear translocator (ARNT) and subsequently as the AHR/ARNT heterodimer binds to the dioxin response elements (DREs), located in the promoter region of several target genes (Lee et al., 2011; Yang et al., 2010; Yasui et al., 2004).

Several lines of evidence indicate that in birds TCDD affects the function of the endocrine system and exerts a negative effect on reproduction (Hrabia et al., 2013; Giesy et al., 2003; Ottinger et al., 2009). Reversible inhibition of egg laying in chickens that were exposed to TCDD during maternal life was observed (Ikeda et al., 2004). *In ovo* injection of TCDD at early stages of embryogenesis increases embryo mortality and decreases hatchability and body weight of hatched chicks (Blankenship et al., 2003; Bruggeman et al., 2003). TCDD affects steroid synthesis and secretion by embryonic chicken gonads (Sechman et al., 2011b), and also inhibits regression of the right ovary and decreases the number of prehierarchical white follicles in adult chickens (Bruggeman et al., 2005). Despite these data on the effects of TCDD on chicken gonadal function during embryogenesis in the available literature there are no data concerning the effects of dioxins on the ovarian steroidogenesis and function of the ovarian follicles in the laying hen.

The chicken ovary provides an exceptional model for study of steroidogenesis process and follicular development. It contains the stroma with primordial follicles, white (1–4 mm; WF) and yellowish (4–8 mm; YF) non-hierarchical follicles and five to eight large, yolk-filled yellow follicles that are arranged in a distinct hierarchy according to size (9–35 mm; F*n*–F1), with the largest (F1) ovulating first, followed by F2, *etc.* The growth and maturation of the ovarian follicle is associated with differentiation of granulosa cells, which occurs before selection of the YF into the preovulatory hierarchy (Johnson and Woods, 2009). Ovarian steroidogenesis occurs both in granulosa and theca layers however, the granulosa produces mainly progesterone (P4) while the theca interna and externa produce testosterone (T) and estradiol (E2), respectively (Bahr et al., 1983; Hrabia et al., 2011; Nitta et al., 1993; Rząsa et al., 2009; Sechman, 2013; Sechman et al., 2011a). During the transition of the YF to a preovulatory hierarchy, the cells of the granulosa layer (which are in the white nonhierarchical follicles steroidogenically inactive), stimulated initially by FSH and next by LH start to express a steroidogenic acute regulatory protein (StAR) and a cytochrome P450 cholesterol side-chain cleavage (P450scc/*CYP11A1*) enzyme and begin to produce P4 predominantly *via* the Δ^4-ketosteroid pathway (Johnson and Bridgham, 2001; Johnson and Woods, 2009; Li and Johnson, 1993; Tilly et al., 1991; Woods and Johnson, 2005). The process of P4 synthesis form pregnenolone (P5) is catalyzed by a 3β-hydroxysteroid dehydrogenase (3β-HSD/*HSD3B*) whose expression steadily increases in granulosa cells of growing hierarchical follicles (Marrone and Sebring, 1989; Nitta et al., 1993). P4 synthesized in the granulosa layer is transported to the theca interna where it is initially converted to testosterone (T) which is eventually metabolized to E2 by cytochrome P450 aromatase (P450arom/*CYP19A1*) in the theca externa cells (Li and Johnson, 1993; Nitta et al., 1991; Sechman, 2013).

The hens' intake of dioxins from various sources leads to an increase in the dioxin content in eggs (De Vries et al., 2006; Ikeda et al., 2004). It seems likely that TCDD transported from blood plasma to the oocyte yolk, acting *via* AhR receptors which expression was found in all compartments of the chicken ovary (Sechman et al., 2011c), may affect the process of ovarian steroidogenesis. Therefore, the present study was designed to evaluate the *in vitro* effect of TCDD on sex steroid secretion by white non-hierarchical and yellow preovulatory follicles of the hen ovary and asses mRNA expression of genes encoding *STAR*, *HSD3B* and *CYP19A1* – the key protein and enzymes involved in steroidogenesis in chicken ovarian follicles.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO), antibiotic–antimycotic solution, bovine serum albumin (BSA), RNALater (Sigma, St. Louis, USA); TCDD (Dr. Ehrenstorfer GmbH, Germany); ovine LH (NIADDK-oLH-26, Dr. A.F. Parlow, National Hormone and Pituitary Program, USA), Eagle'a medium (Laboratory of Sera and Vaccines, Lublin, Poland); TRI-reagent (Molecular Research Center, Inc., Cincinnati, USA), Ribonuclease inhibitor (Fermentas, Vilnius, Lithuania), a High-Capacity cDNA Reverse Transcription Kit, Eukariotic 18S rRNA Endogenous Control, Assay-on-Demand, TaqMan MGB Gene Expression Kits (Applied Biosystems, Foster City, USA), PROG-RIA-CT, TESTO-RIA-CT and E2-RIA-CT DIAsource kits (Belgium). All other reagents were obtained from ICN Biomedicals (USA) or POCH (Poland).

2.2. Animals and tissue collection

The experiments were carried out on *Hy-Line Brown* egg-laying hens at the age of 25–28 weeks and weighing on average 1.96 ± 0.08 kg. Birds were fed *ad libitum* and kept in individual batteries of cages at a neutral temperature (18–20 °C) and under a photoperiodic regime of 14L:10D. The experiments and animal procedures were approved by the Local Animal Ethics Committee in Krakow. Accurate timing of ovulation was determined by checking oviposition every 30 min between 07.00 h and 15.00 h. Ovulation was considered to occur within 15 min after oviposition of the previous egg in the series. Hens were decapitated 2 h after a midsequence ovulation (*i.e.* about 22.5 h before the next ovulation). The white (1–4 mm; WF) and yellowish (4–8 mm; YF) non-hierarchical follicles, and 3 the largest preovulatory follicles (20–36 mm; F3–F1; F3 < F2 < F1) were isolated from the ovary. With respect to the preovulatory follicles, the granulosa layer was separated from the theca one. Before the *in vitro* experiments, the theca and granulosa layers of F3–F1 follicles were divided into 6 (Exp. I) or 4 (Exp. II) equal pieces. The WF and YF follicles were incubated as intact ones; they were divided into 6 (Exp. II) or 4 (Exp. III) equal parts of three WF follicles or one follicle in respect to YF class.

2.3. Effect of increasing doses of TCDD on sex steroid secretion by chicken ovarian follicles (Experiment I)

The *in vitro* experiments were carried out according to the protocol described previously (Sechman et al., 2009, 2011a). Briefly, the WF and YF follicles and fragments of the theca and granulosa layers of the F3–F1 follicles were placed in separate wells in 1 mL Eagle's medium supplemented with 0.05% BSA, 2 μL/mL antibiotic–antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 μg amphoteracin B/mL) as a control medium or with the addition of TCDD at logarithmic scale concentrations of 0.01, 0.1, 1, 10 or 100 nM (*i.e.* 3.21 pg/mL to 32.1 ng/mL). A stock solution of TCDD was dissolved in DMSO and subsequently diluted in Eagle's medium (the final concentration of DMSO in medium was below 0.1%). Incubation was carried out at 39 °C for 6 h in a humidified atmosphere of 95% air and 5% CO_2. Each experiment was carried out in 6 repetitions (*i.e.* one hen was used for each repetition; $n = 6$). Following incubation the medium was collected and stored frozen for further testosterone (T) and estradiol (E2) assay in the case of white follicles and the theca layer of F3–F1 follicles, and progesterone (P4) determination with respect to the medium of F3–F1 granulosa layer. P4 in medium collected from white and yellowish follicles and the theca layer of yellow ones was below detection limit of the RIA method. A similar problem occurred in the case of T and E2 determination in the medium collected