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Transcriptional profiling of porcine granulosa cells exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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HIGHLIGHTS

- TCDD effect on granulosal gene expression using NGS was studied for the first time.
- TCDD affected the expression of 141 genes of porcine granulosa cells.
- TCDD effect on gene expression profile varied depending on incubation times.

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a toxic man-made chemical compound contaminating the environment. An exposure of living organisms to TCDD may result in numerous disorders, including reproductive pathologies. The aim of the current study was to examine the effects of TCDD on the transcriptome of porcine granulosa cell line AVG-16. By employing next-generation sequencing (NGS) we aimed to identify genes potentially involved in the mechanism of TCDD action and toxicity in porcine granulosa cells. The AVG-16 cells were treated with TCDD (100 nM) for 3, 12 or 24 h, and afterwards total cellular RNA was isolated and sequenced. In TCDD-treated cells we identified 141 differentially expressed genes (DEGs; $p_{adjusted} < 0.05$ and log2 fold change ≥ 1.0). The DEGs were assigned to GO term, covering biological processes, molecular functions and cellular components. Due to the large number of genes with altered expression, in the current study we analyzed only the genes involved in follicular growth, development and functioning. The obtained results showed that TCDD may affect ovarian follicle fate by influencing granulosa cell cycle, proliferation and DNA repair. The demonstrated over-time changes in the quantity and quality of genes being affected by TCDD treatment showed that the effects of TCDD on granulosa cells changed dramatically between 3-, 12- and 24-h of cell culture. This finding indicate that timing of gene expression measurement is critical for drawing correct conclusions on detailed relationships between the TCDD-affected genes and resulting intracellular processes. These conclusions have to be confirmed and extended by research involving proteomic and functional studies.

1. Introduction

Numerous man-made chemicals, including polychlorinated dibenzodioxins (PCDDs, dioxins) are produced and released into the environment. Dioxins are by-products of herbicide, fungicide, chlorine and paper industry as well as of color metal production and recycling. Also, an incineration of hospital and industrial wastes, burning in house furnaces, car traffic and cigarette smoking

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are significant sources of dioxins. As a result, PCDDs are present in air, soil, water sediments as well as in plant and animal organisms (Larsen, 2006). They are soluble in lipids and highly resistant to degradation. Hence, dioxins show a high potential for retention in human and animal body, especially in fat tissue. The half-life of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic dioxin congener, ranges in humans from 7 to 10 years, while in an environment from 25 to 100 years (Nicolopoulou-Stamati and Pistos, 2001; Larsen, 2006). The toxicity of TCDD and its biological effects depend on animal species, strain, sex, age and target tissue as well as the dioxin dose and the route of administration (Henck et al., 1981; Pohjanvirta et al., 1993; Enan et al., 1996; Kransler et al., 2007; Silkworth et al., 2008).

The main mechanism of dioxin action and toxicity involves the aryl hydrocarbon receptor (AhR) pathway. The AhR is a ligand-activated transcription factor, belonging to the bHLH-PAS (basic helix-loop-helix/PER-ARNT-SIM) protein family (Hahn, 1998; Whitlock, 1999; Gu et al., 2000). In cytosol, unliganded AhR forms an inactive complex with chaperone proteins (Denison et al., 2002; Pocar et al., 2005; Ikuta et al., 2009). Binding of a ligand is followed by a translocation of the ligand-AhR complex to the nucleus, dissociation from chaperones and dimerization with AhR nuclear translocator (ARNT). The ligand-AhR-ARNT complex binds to specific DNA sequence – dioxin response element – and induces the expression of a variety of genes, including phase I and II drug metabolizing enzymes (e.g., cytochrome P450 enzyme family 1: CYP1A1; CYP1A2, CYP1B1 as well as glutathione S-transferase and NADPH-quinone-oxidoreductase) (Rowlands and Gustafsson, 1997; Nebert et al., 2004; Barouki et al., 2007). Next, the AhR is degraded *via* the 26S proteasome pathway (Pollenz, 2002).

Due to the high dioxin biostability and its ability to bioaccumulate, an exposure of living organisms to dioxins is chronic and widespread. It results in a variety of harmful short- and long-term effects, including a wasting syndrome, neurological, immune and digestive system dysfunctions, cancer as well as reproductive defects (endometriosis, teratogenesis, abortion and diminished fertility) and endocrine disruption (Heimler et al., 1998b; Piekło et al., 2000; Grochowalski et al., 2001; Petroff et al., 2001; Matsumura, 2003; Moran et al., 2003; Nebert et al., 2004; Mandal, 2005; Jablonska et al., 2011b).

Granulosa cells play a fundamental role in the proper growth, development and functioning of ovarian follicles (Albertini et al., 2001). They protect and nurture the oocyte as well as produce steroid hormones to support oocyte maturation and to ensure an optimal environment for fertilization, implantation and embryo development. Disruption of granulosal steroidogenesis may lead to follicular dysfunction and atresia as well as may affect functions of the entire female reproductive tract. It was found that TCDD affected progesterone (P_4) and estradiol (E_2) production by granulosa and theca interna cells in pigs (Piekło et al., 2000; Grochowalski et al., 2001; Jablonska et al., 2011b), rats (Heimler et al., 1998b) and humans (Moran et al., 2003). Moreover, the expression of dioxin receptor - AhR was demonstrated both in porcine granulosa cells (Jablonska and Ciereszko, 2013) as well as in whole porcine ovaries (Jablonska et al., 2011a). Although, numerous studies of TCDD effects on gene expression in ovarian cells were performed in laboratory and farm animals as well as in humans and fish, these studies involved examinations of a limited number of genes. Here, we aimed, for the first time, to identify genes involved in the mechanism of TCDD action and toxicity in granulosa cells with the use of next-generation sequencing (NGS), a modern and high-throughput technique. Such a complex approach should help to reveal the full spectrum of possible pathways contributing to TCDD action. The obtained data will improve our understanding of the nature of TCDD toxicity and may indicate directions of future proteomic and functional studies.

2. Material and methods

2.1. Culture of porcine granulosa cells

Porcine granulosa cell line AVG-16 was obtained from The European Collection of Authenticated Cell Cultures (ECACC; 06062701). AVG-16 cells were cultured and passaged as previously described (Sadowska et al., 2015). Briefly, to perform target experiment, granulosa cells were cultured in six-well plates, seeding density: 1×10^6 cells/3 ml culture medium: Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acid (NEAA), 2.5 ng/ml fibroblast growth factor-basic human (bFGF) and antibiotics mixture (100 U penicillin, 100 μg streptomycin and 0.25 μg amphotericin B/ml) (Sigma Aldrich, St. Louis, MO, USA). After preculture, *i.e.*, after reaching 60–65% confluency, AVG-16 cells were treated with TCDD (100 nM; Sigma Aldrich) for 3, 12 or 24 h ($n = 2$). The TCDD concentration was selected based on data from earlier studies (Gregoraszczuk et al., 2000; Gregoraszczuk, 2002; Jablonska et al., 2014). The 100 nM of TCDD was found to affect granulosa cell steroidogenesis of pigs and did not affect cell viability (Jablonska et al., 2014). At the end of culture, medium was removed, cells were washed twice with a phosphate-buffered saline (PBS) and designed for total RNA isolation.

2.2. Total RNA isolation and evaluation of RNA integrity

Total RNA was isolated from cells using peqGold TriFast (Peqlab Biotechnologie GmbH, Erlangen, Germany). RNA concentration and quality were determined spectrophotometrically (NanoVue Plus, GE Healthcare, Little Chalfont, UK) and RNA integrity was evaluated by microfluidic electrophoresis using a 2100 Bioanalyzer with RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA integrity number (RIN; 28 S/18 S ratio) above 8.0 were used for NGS.

2.3. Construction and sequencing of Illumina cDNA libraries

Depleted RNA obtained from 400 ng of total RNA was used to construct cDNA libraries (TruSeq RNA Sample Preparation Kit; Illumina, San Diego, CA,USA). Following RNA purification and fragmentation, first and second cDNA strands were synthesized. Next steps included 3′ ends adenylation, adapter ligation and library amplification (PCR). Quantification of the cDNA library templates was performed using KAPA Library Quantification Kit (KapaBiosystem, Wilmington, MA, USA). Library profiles were estimated using the DNA High Sensitivity LabChip kit on the 2100 Bioanalyzer (Agilent Technologies). Afterwards, libraries were sequenced on HiSeq2500 high throughput sequencing instrument (Illumina) with 100 paired-end sequencing.

2.4. Bioinformatic analysis of gene expression

The quality of cDNA fragments obtained after sequencing (raw reads) was first evaluated using FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Next, the reads were trimmed using Trimmomatic tool (Bolger et al., 2014) to remove from the dataset any remaining Illumina adapter sequences as well as reads shorter than 50 bp. In addition, PHRED33 score was used to evaluate the identification quality of the nucleobases generated by NGS. Then, the trimmed fragments were mapped to the whole porcine genome (Sus_scrofa.Sscrofa10.2; Ensembl database) using TopHat splice junction mapper (Trapnell et al., 2009).