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Integrative transcriptome and microRNome analysis identifies dysregulated pathways in human Sertoli cells exposed to TCDD

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ABSTRACT

Male fertility and spermatogenesis are directly linked to the Sertoli cell's ability to produce factors associated with germ cell development. Sertoli cells express receptors for FSH and testosterone, and are the major regulators of spermatogenesis. Recent studies report that regulatory RNA molecules, such as microRNAs (miRNAs), are able to modulate testicular function during spermatogenesis and that their altered expression may be involved in male infertility. miRNAs may play a role in the response to xenobiotics that have an adverse consequences to health. An important group of xenobiotic organic compounds with toxic potential are dioxins, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Experimental models of TCDD exposure in mice demonstrated that TCDD exposure causes low sperm count and delayed puberty. This study below examines the mechanism of TCDD's action in human Sertoli cells, through interrogating the expression profile of miRNAs and mRNAs, that enabled us to identify dysregulated molecular pathawys in Sertoli cell. 78 miRNAs presented altered expression, with positive regulation of 73 and negative regulation of 5 miRNAs when compared to the control group. Regarding gene expression profile, 51 genes were deregulated, of which 46 had positive regulation and 5 genes with negative regulation. Important pathways have been altered by the action of TCDD as AhR pathway, GPR68, FGF2 and LIF. This study has opened the door to new perspectives on the TCDD toxicity pathway as it affects Sertoli cells physiology that can ultimately lead to male infertility.

1. Introduction

The disruption of male reproductive health is increasing in frequency in industrialized countries. Environmental pollution has been suggested as the main source (Sharpe et al., 2003).

Endocrine disruptors are estrogen-like and/or anti- androgenic chemicals in the environment that have potentially hazardous effects on male reproductive axis capable of triggering morphological and functional changes (Sikka and Wang, 2008). Studies in animals showed that the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is toxic and detrimental to human health (Denison and Nagy, 2003), its effect was clearly evident after the Seveso accident (Bertazzi et al., 2001; Warner et al., 2013). TCDD is introduced to our environment largely as an unwanted by-product of manufacturing, such as incineration and burning of fossil fuels, although volcanic eruptions and forest fires also contribute to the overall environmental burden of dioxins. Among human and animal populations, ingestion of contaminated food is the primary source of dioxin exposure (Schecter et al., 2002; Harrad et al., 2003; Pompa et al., 2003; Malisch and Kotz, 2014). Wolf et al. (1999) reported that the administration of low doses of TCDD during gestation, in mice, alters the reproductive development of the fetus. In females this leads to a reduction of the uterine weight and in male can decrease fertility.

The male gonad, i.e., testis, consists of seminiferous tubules surrounded by a fibrous coat, tunica albuginea. Inside the seminiferous tubules are large Sertoli cells, supported by the basement membrane of the germinal epithelium, which act as the nurse cells for germ cell development (Croxford et al., 2011). Sertoli cells are pyramidal somatic cells with a large surface area, supporting the development of a large number of germ cells in the estimated ratio of 1:50 in the adult rat testis

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(Weber et al., 1983). Sertoli cell number can determine testicular size, the number of spermatogonial stem cells in the testis and spermatid production. They are responsible for providing an immunologically privileged and highly specialized environment inside the seminiferous tubules. Sertoli cells are connected by gap junctions, enabling chemical and ion exchange, helping to coordinate the seminiferous epithelium cycle (Mruk and Cheng, 2004; Gao et al., 2015). Adjacent Sertoli cells are joined by tight junctions in the lateral membrane, which creates the Blood-Testis Barrier (BTB) (de Freitas et al., 2016). At the onset of meiosis, germ cells beyond the BTB move into the adluminal compartment of the seminiferous tubule, and become dependent on Sertoli cells to provide nutrients and growth factors for their development (Cheng and Mruk, 2004). Therefore, any compound that disturbs Sertoli cell function could disrupt spermatogenesis, ultimately leading to male infertility.

The canonical TCDD pathway is though aril hydrocarbon receptor (AhR) that is an important regulator of the male reproductive tract (Hansen et al., 2014). When AhR is inactive, it binds to a chaperone in the cytoplasm. Upon interaction with the ligand, i.e., TCDD, AhR is activated and dissociates from the chaperone, translocating to the nucleus to interact with ARNT (aryl hydrocarbon receptor nuclear translocator). The AhR/ARNT complex becomes active and binds to Xenobiotics Responsive Elements (XRE) throughout the genome, thereby modifying the expression of various genes, mainly *AHRR, CYP1A1* and *CYP1B1*, triggering a cellular response (Jackson and Mitchell, 2011). Among the genes that are triggered by AhR/TCDD is *AHRR* that competes with ARNT to bind to AhR, thus repressing the cellular TCDD pathway. *TIPARP* is also an AhR target gene that is expressed in many different tissues, including liver, heart, spleen, brain, and reproductive organs. MacPherson et al. (MacPherson et al., 2014, 2013a) recently showed that TIPARP is a transcriptional repressor of AhR. This, revealed a novel negative feedback loop in AhR signaling.

TCDD could affect the expression of a variety of genes dependent on the organism and cell type including those that participate in the inflamatory response pathways like *Il1B* and *LIF*. Similarly, small nocoding RNAs (sncRNAs) might play an important role when responding to xenobiotics and impact health. Recent studies suggest that small noncoding regulatory RNAs (sncRNA), such as microRNAs, can modulate testicular function during spermatogenesis and that their altered expression may be factors involved in male infertility (Abu-Halima et al., 2013; Lian et al., 2009).

There is a lack of data regarding the role of miRNAs and mRNAs in human Sertoli cells as regulatory factors of human spermatogenesis. There are no previous studies about potential effects of TCDD on the response of microRNAs from male reproductive cells. Thus, the identification of miRNAs as targets for TCDD, inducing altered gene expression, offers a new path to understand the toxicological mechanisms and the role of environmental pollutants on male reproductive tract.

2. Material and methods

2.1. Culture, chemicals and cellular stress

Human primary Sertoli cells (HSeC) were purchased from Lonza (USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM/Ham F12) (Sigma Aldrich, St. Louis, MO, USA)([1/1] v/v, supplemented with 15% fetal bovine serum, 100 U/ml penicillin, 100 µ/ml streptomycin and 0.25 µg/ml fungizone (Gibco, Invitrogen, USA). Cells were incubated with TCDD (Sigma Aldrich, St. Louis, MO, USA), diluted in culture medium and DMSO as a carrier to 0.05%, achieving a 10 nM concentration of the chemical; concentration was determined after MTT assay (Mosmann, 1983). Exposure was performed in triplicate for 72 hs, including a control group also in triplicate, exposed to 0.05% DMSO for the same time period. Sertoli cells were divided into aliquots containing approximately 1×10^6 cells and subsequently underwent total RNA and protein extraction.

2.2. Total RNA extraction

RNA extraction was performed with Trizol (Ambion, USA) with 1% of β-Mercaptoethanol, following manufacturer's instructions. The RNA was quantified by spectrophotometry using a NanoDrop (Thermo Scientific, USA). RNA quality, as measured with ribosomal RNAs by the RNA Integrity Number (RIN), was obtained using the 2100 Bioanalyzer system (Agilent, USA).

2.2.1. mRNA purification, library construction, and sequencing

RNAs were sequenced using the HiSeq2500 platform (Illumina) within the provision of Animal Biotechnology Laboratory Services, School of Agriculture Luiz de Queiroz (ESALQ). A single 500 ng aliquot of total unfractionated RNA was submitted for library construction, and sequencing. During library preparation Ribo-Zero rRNA Removal Kit (Illumina,USA) was used for rRNA depletion. Messenger RNA purification and library construction was carried out with total RNA using the mRNA TruSeq Stranded LT Sample Preparation Kit (Illumina), following the manufacturer's specifications. The sequencing was performed with the HiSeq Sequencing System using the sequencing by synthesis methodology with modified chemical and labeled with fluorescent nucleotides.

2.2.2. High performance sequencing – sncRNAs

A single 500 ng aliquot of total unfractionated RNA was also submitted to the Animal Biotechnology Laboratory sequencing facility for library construction, and sequencing of sncRNAS. The construction of the sncRNAs libraries was performed using the TruSeq Small RNA Sample Preparation Kit following the manufacture's protocol. The sequencing was also performed with the HiSeq Sequencing System using the sequencing by synthesis methodology with modified chemical and labeled with fluorescent nucleotides.

2.2.3. Sequencing analysis

To determine the levels of each RNA, RNA-seq reads were mapped to the Human reference genome hg38 ("http://www.gencodegenes.org/," n.d.) using HiSat (Kim et al., 2015). Raw data were calculated as the TPM (Transcripts per Million mapped) of each gene in each sample using RSEM (Li and Dewey, 2011). Genes with a TPM of zero in one or more samples were excluded from analysis. Filtered data were logarithmically transformed and normalized using a normalization method by DESeq2 and EBSeq (26, 27). A Log 2 Fold Change (Log2 FC) between TCDD and control groups was calculated of each gene pair. Differentially expressed genes were determined by adjusting Log2 FC < -0.41 or > 0.26 [False Discovery rate (FDR)- corrected p-value ≤ 0.05] when comparing the experimental groups.

For miRNA analysis, all the reads were demultiplexed according to their index sequences using CASAVA version 1.8 (Illumina) and reads that did not pass the Illumina chastity filter were removed from the dataset. Small RNA sequencing reads of good quality were subjected to analysis by sRNAbench toolbox (Rueda et al., 2015). Data analysis included read quality assessment using FastQC (Andrews, 2010) and read cleaning assessment by CutAdapt (Chen et al., 2014). Read alignment was performed using Bowtie1 (Langmead et al., 2009) followed by HT-Seq (Anders et al., 2014) for annotation and quantification of aligned sequences. Lastly sncRNA tags were annotated also using the RepeatMasker library (Kent et al., 2002). Raw data were calculated as normalized mean count in each sample. Data normalization (Robinson and Oshlack, 2010) and differential expression analysis was performed using edgeR (Bioconductor/R) v.3.0 (Gentleman et al., 2004; Robinson et al., 2010), which implements a negative binomial distribution, to identify differential miRNAs (Anders and Huber, 2010). Read counts were transformed logarithmically and normalized using a quantile normalization method. For each miRNA, fold change between case and control was calculated. Differentially expressed miRNAs were determined by adjusting Log2 FC < -0.60 or > 0.58 with FDR- corrected