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Transcriptome signatures of *p,p′*-DDE-induced liver damage in *Mus spretus* mice☆

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ABSTRACT

The use of DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane) in some countries, although regulated, is contributing to an increased worldwide risk of exposure to this organochlorine pesticide or its derivative *p,p'*-DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene]. Many studies have associated *p,p'*-DDE exposure to type 2 diabetes, obesity and alterations of the reproductive system, but their molecular mechanisms of toxicity remain poorly understood. We have addressed this issue by using commercial microarrays based on probes for the entire *Mus musculus* genome to determine the hepatic transcriptional signatures of *p,p'*-DDE in the phylogenetically close mouse species *Mus spretus*. High-stringency hybridization conditions and analysis assured reliable results, which were also verified, in part, by qRT-PCR, immunoblotting and/or enzymatic activity. Our data linked 198 deregulated genes to mitochondrial dysfunction and perturbations of central signaling pathways (kinases, lipids, and retinoic acid) leading to enhanced lipogenesis and aerobic glycolysis, inflammation, cell proliferation and testosterone catabolism and excretion. Alterations of transcript levels of genes encoding enzymes involved in testosterone catabolism and excretion would explain the relationships established between *p,p′*-DDE exposure and reproductive disorders, obesity and diabetes. Further studies will help to fully understand the molecular basis of *p,p′*-DDE molecular toxicity in liver and reproductive organs, to identify effective exposure biomarkers and perhaps to design efficient *p,p'*-DDE exposure counteractive strategies.

1. Introduction

Organochlorine pesticides (OPs) have been widely used to control pests and to prevent the loss of crops and human diseases. These chlorinated hydrocarbonated backbones are considered persistent organic pollutants (POPs) due to their high lipophilicity, bioaccumulation capability, long half-life and potential for long range transport. Despite their efficacy as biocides, the deleterious effects of OPs in both human and environmental health have led to a partial prohibition of most of them. However, their use in tropical and equatorial countries to fight vector-borne diseases, and even for agricultural purposes, make them a worldwide risk (Harada et al., 2016; Jayaraj et al., 2016).

DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane) has been the most commonly used OP in agricultural practice in the past century. The observed relationship between DDT exposition and eggshell thinning and other effects in non-target animals led to its ban in the early 1970s (Vos et al., 2000; Mora et al., 2016). The rising incidence of malaria, yellow fever, dengue and the Zika virus outbreak motivated the World Health Organization (WHO) to allow its use in 2006 as an indoor-spray in some tropical countries where is necessary to fight the disease vector, *Anopheles* sp. (Sadasivaiah et al., 2007; WHO, 2007). The analogous compound *p,p'*-DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene] is an impurity in commercial DDT pesticide formulations and has never been produced commercially. It is also produced as a breakdown product of DDT by abiotic degradation or metabolism (EPA, 2008). Even more persistent than DDT, *p,p'*-DDE can be found in soils decades after the last DDT treatment because its recalcitrance makes it difficult to remediate (Thomas et al., 2008). As a consequence, *p,p'*-DDE currently poses a risk for environmental and human health since

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not only is the population from tropical countries exposed but so are people living far away who consume fruit and fish of tropical origin (Harada et al., 2016; Jurgens et al., 2016).

Despite the documented relationship of *p,p'*-DDE with impaired glucose and lipid metabolism (Cetkovic-Cvrlje et al., 2016; Kim et al., 2016, 2017; Ward et al., 2016; Morales-Prieto and Abril, 2017; Pestana et al., 2017), oxidative stress (Song et al., 2014; Morales-Prieto and Abril, 2017), neurotoxicity (Wnuk et al., 2016), endocrine disruption (Monteiro et al., 2015; Hoffmann and Kloas, 2016; Morales-Prieto and Abril, 2017) and reproductive system alterations (Song et al., 2011), the mechanisms underlying the toxicity of this organochlorine are still poorly understood. Due to the huge variety of different effects, *omics* approaches seem to be a powerful tool for elucidating the mechanisms of *p,p'*-DDE toxicity, as previously shown (Morales-Prieto and Abril, 2017).

Microarray-based transcriptomics have been widely used to demonstrate the possible effects of pesticides (*i.e.*,Dondero et al., 2011; Sharma et al., 2011; Mesnage et al., 2015; Kimura-Kuroda et al., 2016). By using this technology, is possible to investigate the molecular mechanisms of chemical toxicity and to obtain valuable information about adverse health effects following environmental exposure.

Usually, suitable species for the study of pesticides and other environmental pollutants are non-model organisms. The interest in using these environmentally relevant, non-model animals, has forced the development of in-house (custom) microarrays (*i.e.*, Watanabe et al., 2008; Dondero et al., 2011; De Smet et al., 2017), which usually contain fewer probes than commercial microarrays designed for classical model animals and require the design of cDNA libraries.

Our group and others have demonstrated the suitability of commercial microarrays when working with environmentally relevant non-target organisms as long as both species (the sequence of which is contained in the commercial platform and the RNA donor) are phylogenetically close (Osuna-Jimenez et al., 2009; Abril et al., 2014; van Ommen Kloeke et al., 2014; Ruiz-Laguna et al., 2016). *Mus spretus* is a non-protected rodent species that is of great environmental interest because it has been widely used in biomonitoring studies, i.e., (Tanzarella et al., 2001; Festa et al., 2003; Mateos et al., 2008; Abril et al., 2014, 2015; Drouhot et al., 2014; Garcia-Sevillano et al., 2014; Ruiz-Laguna et al., 2016). *Mus spretus* SPRET/EiJ mice, like other strains derived from recently trapped progenitors, retain features of their wild relatives (Dejager et al., 2009), complicating the administration of laboratory treatments because they have remained aggressive and sensitive to stress. In turn, *M. spretus* present a high level of phenotypic variation, which makes them a good model for studying diseases such as inflammation and cancer (Mahler et al., 2008; Fleming et al., 2013; Dejager et al., 2009), and infections (Perez del Villar et al., 2013). The genome of *M. spretus* is not fully sequenced. However, the phylogenetic proximity of *M. spretus* with the model organism *M. musculus*, the classical laboratory mouse species, has allowed the use of commercial microarrays as well as *M. musculus* genomic and proteomic databases (Abril et al., 2015; Ruiz-Laguna et al., 2016; Morales-Prieto and Abril, 2017; Morales-Prieto et al., 2017) in this *M. spretus* study. Here, we evaluated the consequences of chronic *p,p'*-DDE exposure on the hepatic transcriptional profile of *M. spretus* mice consuming aprox. 115 μg/g bw day, a dose 3-fold higher than that used in previous works (Morales-Prieto and Abril, 2017; Morales-Prieto et al., 2017), with the aim of obtaining more defined effects to help unravel the molecular basis of *p,p'*-DDE toxicity. The microarray data have been partially verified by qRT-PCR, immunoblotting and enzymatic activity measurements.

2. Materials and methods

2.1. Animals and experimental design

This study was authorized by the Bioethical Committee of the University of Cordoba (UCO, Spain). Eight seven-week-old *Mus spretus* SPRET/EiJ male mice were housed in individual cages under a controlled environment in the UCO Experimental Animals Service facility (temperature 25 ± 2 °C, humidity 55 ± 5%, and 12-h light/12-h dark cycle) with access to food and water *ad libitum*. After acclimation (3 days), the mice were separated into two groups. The "Control" group was fed Teklad Global 14% Protein Rodent Maintenance diet-Envigo 2004 containing 3% refined corn oil. For the "Exposed" group, *p,p'*-DDE (SUPELCO® Analytical, SIGMA-ALDRICH) was diluted in corn oil to a final concentration of 0.45 mg of *p,p'*-DDE per gram of feed. Both water and food were renewed every second day. The removed chow was weighted to calculate the real ingested *p,p'*-DDE dose, which was found to be 112.5 μg/g bw day. The treatment lasted for 30 days. Mice were ethically sacrificed by cervical dislocation and the liver removed, washed (0.9% NaCl), flash-frozen in liquid nitrogen and stored at −80 °C. The frozen livers were homogenized with a Freezer/Mill® (SPEX Sample Prep).

2.2. Histological analysis

Before freezing liver, a fragment was removed from the same hepatic lobule in each animal and fixed in 10% formalin. Standard histological techniques proportionated 4 mm sections that were stained with hematoxylin-eosin and analyzed under an Olympus BX51 photomicroscope (Morales-Prieto and Abril, 2017).

2.3. RNA isolation

Total RNA from each liver was individually isolated using the RNeasy® MiniKit (QIAGEN®) according to the manufacturer's guide. This protocol included an on-column DNase clean-up to ensure the complete removal of gDNA from RNA samples, which was subsequently confirmed by qRT-PCR using the RNA without the previous step of retrotranscription as a template. RNA purity and concentration were determined by measuring the absorbance at 260/280 nm in a DU800 Spectrophotometer BECKMAN COULTER using a Hellma cuvette. The RNA integrity was monitored with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

2.4. One-color RNA labeling and microarray hybridization

Gene expression microarrays analyses were performed according to the Agilent protocol for One-Color Microarray-Based Gene Expression Analysis, with the help of the staff of the Genomics Section at the Central Service for Research Support (SCAI) of the University of Cordoba. RNA samples (50 ng RNA) and RNA positive controls (Agilent One Color RNA Spike-It Kit) were labeled (cyanine 3-CTP, Cy3) and amplified for hybridization to the gene expression microarrays with the Agilent Low-Input QuickAmp Labeling Kit according to the manufacturer's protocol. The yield and specific activity of each reaction met the requirements recommended for our microarray format. Hybridizations were performed in the HD Mouse GE 4 × 44K v2 Microarray (60-mer, *Mus musculus* probes) (G2519F-026655) at 65 °C for 17 h in an Agilent G2545A Hybridization Oven, using the Agilent Gene Expression Hybridization Kit. Four biological replicates per experimental group were performed. The slides were washed with Agilent Gene Expression Wash Buffer Kit with Triton X-102 detergent to reduce background artifacts and allowed to dry at room temperature in an ozone-free atmosphere.