Differential Effects of Exposure to Single Versus a Mixture of Endocrine-Disrupting Chemicals on Steroidogenesis Pathway in Mouse Testes

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ABSTRACT

Endocrine-disrupting chemicals (EDCs) generate reproductive dysfunctions affecting the biosynthesis of steroid hormones and genes of the steroidogenic pathway. EDCs effects are mainly reported as a result of exposure to single compounds. However, humans are environmentally exposed to a mixture of EDCs. Herein, we assess chronic exposure to single alkylphenols and phthalates versus a mixture in mouse testes histology and steroidogenesis. Pregnant mice were exposed through drinking water to: 0.3 mg/kg-body weight (BW)/d of each phthalate (bis (2-ethylhexyl) phthalate, dibutyl phthalate, benzyl butyl phthalate), 0.05 mg/kg-BW/d of each alkylphenol (4-nonylphenol, 4-tert-octylphenol), or their mixture, covering from 0.5 postcoital day to weaning, continuing in the male offspring each exposure until adulthood (60-days old). Body and relative testis weight were increased in mixture-exposed mice along with histological alterations. Intratesticular testosterone (T) changed only in mice exposed to DBP, whereas estradiol (E2) levels were altered in all groups (except benzyl butyl phthalate), 0.05 mg/kg-BW/d of each alkylphenol (4-nonylphenol, 4-tert-octylphenol), or their mixture, covering from 0.5 postcoital day to weaning, continuing in the male offspring each exposure until adulthood (60-days old). Body and relative testis weight were increased in mixture-exposed mice along with histological alterations. Intratesticular testosterone (T) changed only in mice exposed to DBP, whereas estradiol (E2) levels were altered in all groups (except benzyl butyl phthalate). mRNA levels of genes encoding hormones of the steroid pathway (Cyp11a1, Hsd3b1, Cyp17a1, and Cyp19a1), cholesterol transporters (Star), and transcriptional factors (Sp1) showed that mice exposed to single or mixed compounds had alterations in at least 2 transcripts. However, none of the different types of exposure induced changes in all transcripts. In addition, changes at the mRNA or protein levels with single compounds were not always the same as those with a mixture. In conclusion, the effects of a chronic exposure to a mixture of EDCs on the expression of genes and proteins of the steroidogenic pathway and hormonal status were different from those exposed to single EDC.

Key words: phthalates; alkylphenols; aromatase; estradiol; testosterone; testis.

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Endocrine-disrupting chemicals (EDCs) are widely present in the environment, contributing to its contamination. These compounds are generally man-made molecules used as by-products in the manufacture of a wide range of daily-use utensils such as bottled waters, food packaging, personal care products, cleaners, etc (Dodson et al., 2012). EDCs are considered "an exogenous chemical or mixture of chemicals, which interferes with any aspect of hormone action" (Gore et al., 2015). Although human data are limited, it has been possible to measure the exposure levels of some of these compounds in certain populations showing high variability between individuals, age, or sex (Meeker et al., 2009; Ye et al., 2011).

Early reports have shown that phthalates and alkylphenols exposure is associated with damage observed in male reproductive health mainly since both are known to have estrogenic and antiandrogenic potential (De Jager et al., 1999; Gray et al., 2000; Puy-Aurumendi et al., 2014; Satoh et al., 2005). Within the group of phthalates, the most well-studied are: bis (2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and benzyl butyl phthalate (BBP); while within the alkylphenols are: 4-nonylphenol (NP) and 4-tert-octylphenol (OP) (Chen et al., 2014; Fang et al., 2003).

Most of the studies concerning the effects of these compounds have been performed during embryonic and/or pubertal development and using single EDCs exposure, often at doses non-related to the exposure levels shown in humans. However, widespread exposure in populations occurs throughout life, and at different low doses of EDCs (Diamanti-Kandarakis et al., 2009). More recently, it has been suggested that in the case of mixed exposure with one or more families of different EDCs, several mechanisms are involved, further each compound could interact with each other resulting in additive, synergistic or antagonistic effects which leads to complex dose-response relationship (Kortenkamp, 2007; Sanderson, 2006). Examples of some of these effects are the malformations of external sex organs in male rats induced by a combined exposure to different EDCs that were reported as synergistic effects (Christiansen et al., 2009).

Mathematical models combining the concepts of concentration, addition, independent action, and toxicokinetic chemical interaction have been used to predict the cumulative effects in a mixture of EDCs (Howard and Webster, 2009; Rider and LeBlanc, 2005). Yet, new evidences are showing that the predictive models of steroid hormones synthesis based on single chemicals effects fail when the doses of the compounds used in the mixtures do not present a defined potency or when the chemicals have opposite effects (Conley et al., 2016; Hadrup et al., 2013). These facts usually result in under- or overestimated predictive approaches of the in vivo potential effects. In addition, preliminary reports have suggested that mixtures of EDCs at small doses may induce different effects from those observed in single EDCs exposure (Kortenkamp, 2014). Therefore, it is important to evaluate the in vivo effect of single exposure to phthalates and alkylphenols in comparison with the exposure to mixed EDCs, which could mimic the risks in human health.

Pituitary gonadotropins induce the production of testosterone in Leydig cells, while estradiol synthesis takes place in Leydig, Sertoli, and germ cells. Thus, a normal spermatogenesis in adulthood depends on the correct secretion of both hormones (Ramaswamy and Weinbauer, 2014; Schulster et al., 2016). In this context, in males, single exposure to phthalates and alkylphenols showed interference in testosterone and estradiol biosynthesis. This is because these compounds could act as inhibitors or activators of key proteins implicated in steroidogenic synthesis such as: transcriptional factors, cholesterol transporters, enzymes of cytochrome P450 system, steroid dehydrogenases, and reductases (Chen et al., 2013; Hampel et al., 2016).

Another important effect of EDCs exposure can be detected as alterations in the enzyme that converts androgen to estrogen: aromatase (Cyp19a1). Although, the in vitro outcomes of single and mixed exposure to different EDCs have been contradictory (Benachour et al., 2007; Castro et al., 2012), it has been suggested that exposure to a mixture of EDCs in vivo may not only seriously affect aromatase but also other enzymes implicated in the steroidogenesis pathway (Hadrup et al., 2013). However, this has not been confirmed yet.

The aim of this work was comparing the effects of chronic exposure to DEHP, DBP, BBP, NP, and OP on mouse testes, individually or in a mixture, in testosterone and/or estradiol biosynthesis and the expression of mRNAs and proteins implicated in the steroidogenic pathway.

MATERIALS AND METHODS

Ethical statement and animals. All procedures related to care and handling of animals were performed in accordance with the regulations of the Centro de Investigaciones Biológicas (CSIC) and Pontificia Universidad Católica (PUC), following the European Commission (EC) guidelines (directive 86/609/EEC), and the guides for the Care and Use of Agricultural Animals in Agricultural Research and Teaching by the National Research Council of Chile, respectively. The General Direction of Environment of CAM in Spain (Ref. PROEX 054/15) and the National Fund of Science and Technology (FONDECYT) (No. 1150532) in Chile reviewed and approved all the experimental protocols in this work. C57BL/6j mice were bred at the CSIC and PUC animal facilities under specific pathogen-free, temperature-controlled and humidity-controlled conditions in 12-hour light/dark cycles with ad libitum access to food and water.

Chemicals. DEHP, DBP, BBP, NP, OP, and DMSO (dimethyl sulfoxide) were purchased from Sigma-Aldrich Co, (St Louis, Missouri, USA). Ethanol was acquired from Winkler (Chile).

Exposure to endocrine disruptors chemical. For comparative in vivo study of single versus mixed EDCs exposure, firstly we established a single exposure to DEHP, DBP or BBP at doses of 0.3 mg/kg-body weight (BW)/d and NP or OP at doses of 0.05 mg/kg-BW/d. Then, we studied these compounds in the form of a mixture at the same individual concentrations given previously, which resulted in a final dose of 1 mg/kg-BW/d (Table 1). The dose of each EDC was chosen based on the reported for non-occupational and occupational exposure and the exposure during the lactation period reported for these compounds (Table 1). As an example, for DEHP a range of doses No-observed Adverse Effect Level (NOAEL) between 60 and 3 mg/kg/d (European Commission, 2016) was defined. The level of nonoccupational human exposure to DEHP by indirect sources (from the environment) has been estimated to be close to 0.017 mg/kg/d. Based on this data and exposure levels by occupational and medical sources, it has been possible to define the intake tolerable daily for DEHP at 0.6 mg/kg-BW/d (U.S. Food and Drug Administration, 2003; European Commission, 2016). The concentration
of DEHP in our mixture is 0.3 mg/kg-BW/d. This indicates that we used equivalent doses of DEHP at exposure levels in humans. Furthermore, the dosages used for each EDC in the present work were at least approximately 1000-fold lower than the LOAEL (Lowest Observed Adverse Effect Level) values for reproductive effects in experimental male animals, but being environmentally relevant low-doses (Chapin et al., 1999; Nagao et al., 2001; Rider et al., 2010). We reported in a previous work, a pilot studies to evaluate the effect of 2 high dose of the mixture of EDCs, relative to LOAEL and NOAEL. But, these doses were no considerer, due to the embryonic toxicity and the no-effect in hormonal status, respectively (Burjay et al., 2017).

Phthalates (DEHP, DBP, and BBP) were diluted in DMSO and alkylphenols (NP, OP) were diluted in ethanol at a dose of 0.25 and 0.06 g/kg-BW/d, respectively. The NOAEL doses for DMSO an ethanol have been established in 2.5 and 2.4 g/kg-BW/d, respectively. For the control group, we used a mixture of DMSO and ethanol (vehicles) diluted in the drinking water in a concentration equivalent to that used in the experimental EDCs mixture exposures (Table 1). We defined some bulk stocks for control and single or mixture of EDCs that were individually dissolved and administrated in the drinking water of C57BL/6 J mice in independent bottles covered with foil and with ad libitum access to food and water. Single and mixture diluted doses were administrated continuously and the ingest of drinking water was controlled each day by the animal facility personal that were unfamiliarized with the experiment. This chronic exposure was a modification of a previous work (López-Casas et al., 2012). The final doses were calculated according to the BWS and to the volume of ingested water, recorded in a previous pilot study and in agreement with the data in the literature referring to these parameters (Bachmanov et al., 2002).

To emulate human exposure of EDCs throughout life, single or mixed compounds were administrated in the drinking water to random pregnant female mice (evidenced by the presence of vaginal plug) from postcoital day 0.5, during all pregnancy and lactation. At weaning, male offspring were selected and maintained within a maximum of 4 individuals per cage. The exposure of EDCs was maintained until adulthood (endpoint: postnatal day 60).

For the initial toxicological analyses of the exposure model, we evaluated the number of newborn pups (male and female) in pregnant mice exposed to single and mixed EDCs and compared it to the control (vehicles) and unexposed mothers. Data were considered biological replicates.

Evaluation of testicular damage and germ cell apoptosis. Body and testes relative weights were quantified for each group of mice exposed to single or mixed EDCs and controls. Then, one testis was fixed in Bouin solution, embedded in paraffin, and histological sections of 7 μm were mounted on slides and analyze by PAS staining (periodic acid-Schiff, counterstained with hematoxylin) and recorded using an Olympus CX31 microscope (Olympus, Japan). Pictures were taken using a 5XC-3 digital camera (Olympus) and morphometric analyses were performed using ImageJ software (NIH, Bethesda, Maryland, USA) at 1 mg/ml, followed by incubated with a biotiny- lated secondary antibody, streptavidin–biotinylated–peroxidase complex, amplification reagent (biotinyltyramide), and peroxidase-conjugated streptavidin for 30 min each. Between each step, slides were washed 3 times for 5 min with tris–HCl buffer, pH 7.6 with 0.3 M NaCl, 0.1% Tween-20. Finally, we applied a substrate–chromogen solution (Tris–HCl and 3,3-diaminobenzidine tetrahydrochloride solutions [chromogen]) to the slides for 30s and washed them in distilled water. The sections were counter-stained with hematoxylin and then evaluated using an Olympus CX31 microscope (Olympus).

Intratesticular testosterone and estradiol analyses. We isolated seminiferous tubular fluid (STF) from whole testes according to...

**Table 1. List of EDCs Doses and Time of Exposure**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Symbol</th>
<th>Doses</th>
<th>References</th>
<th>Start</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEHP</td>
<td>DEHP</td>
<td>0.3 mg/kg-BW/d</td>
<td>Hines et al., (2011), National Toxicology Program (2006), and U.S. Food and Drug Administration (2003)</td>
<td>Postcoital day 0.5</td>
<td>Postnatal day 60</td>
</tr>
<tr>
<td>DBP</td>
<td>DBP</td>
<td>0.3 mg/kg-BW/d</td>
<td>Hines et al. (2011) and National Toxicology Program (2003b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBP</td>
<td>BBP</td>
<td>0.3 mg/kg-BW/d</td>
<td>Hines et al. (2011) and National Toxicology Program (2003a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>NP</td>
<td>0.05 mg/kg-BW/d</td>
<td>Ademollo et al. (2008) and WHO (2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP</td>
<td>OP</td>
<td>0.05 mg/kg-BW/d</td>
<td>Ademollo et al. (2008) and Bian et al. (2006)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixture of EDCs</td>
<td>(NP + OP)</td>
<td>1 mg/kg-BW/d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO/ethanol</td>
<td>Control</td>
<td>1 mg/kg-BW/d</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Intratesticular testosterone and estradiol analyses.** We isolated seminiferous tubular fluid (STF) from whole testes according to...
Expression of mRNA by RT-qPCR. We isolated RNA from decapsulated testes using TRIzol Reagent, measured its concentration on a ND-1000 spectrophotometer (NanoDrop, USA) and determined the integrity using a 2100 Bioanalyzer (Agilent, USA). Then, we performed retrotranscription to cDNA using 500 ng of total RNA with Oligo dT17, 1X first-strand buffer (Invitrogen), 0.01 M dithiothreitol (DTT), 0.1 mM of each dNTP, and 200 U of superscript II (Invitrogen) in final reaction volume of 20 μl diluted in RNase-free water. Quantitative PCR reactions were performed using 10 μl of 2 × SYBR Green PCR supermix (Bio-Rad, USA), 2 μl of cDNA template and 0.0625 μM of each specific primer (Supplementary Table 1) in 20 μl reaction volume. We obtained PCR profiles using the IQ5 Detection System (Bio-Rad). Data were normalized using the 2−ΔΔCt method (Livak and Schmittgen, 2001) using Gapdh, H2afz, and Ppia as endogenous reference genes, and following the MIQE guidelines (Bustin et al., 2009).

RESULTS

Single or Mixed EDCs Exposures Induced Different Testicular Damage

First, we evaluated the obesogenic and testicular effect of single or mixed exposure to DEHP, DBP, BBP, NP, and OP, and compared it to the control. Data showed that, except for NP, all mice exposed to EDCs, single or mixed, presented higher BW than control mice. However, mice exposed to OP or the mixture presented lower relative testes weight (gonadosomatic index) (Table 2). In addition, the seminiferous tubules of mice exposed to DBP, BBP, OP, and the mixture of EDCs displayed smaller diameter (Table 2).

Histological evaluation of testis showed seminiferous tubules with different types of alterations such as loss of lumens, germ cells exfoliated towards tubular lumen, partial or complete loss of germ cells and areas of hypertrophic/hyperplasia of Leydig cells (Figure 1). The quantification of those alterations showed that exposure to DEHP, DBP, OP, and the mixture of EDCs increased the percentage of seminiferous tubules with germ cells exfoliated toward the lumen, and exposure to DEHP, DBP and to the mixture of EDCs increased the percentage of seminiferous tubules without lumen (Table 2).

Next, we evaluated spermatogenesis progression based on the frequency of seminiferous tubules stages, which was relatively similar in all exposed mice when compared with the controls (see Supplementary Figure 2). Nonetheless, decrease in the frequency of the stages IV–V could be observed in mice exposed to DEHP and DBP and stages VI–VII in those exposed to NP, OP, and the mixture of EDCs. On the other hand, only OP and the mixture induced increase in the frequency of seminiferous tubules that were not possible to classify in any specific stage due to the high level of degeneration/atrophy (Table 2 and Supplementary Figure 2).

Germ cell death has been described in single exposure to phthalates and alkylphenols (Lagos-Cabre and Moreno, 2012) and recently we have also described that exposure to a mixture of EDCs induces germ cell apoptosis (Bunay et al., 2017). Here, we detected an increase in germ cell death (evaluated through the presence of pyknotic cells) in mice exposed to all 3 phthalates and the mixture of EDCs but not to the alkylphenols (Table 2). However, positive caspase-3 cells, increased in all exposed mice as compared with controls (Table 2). Furthermore, based on its location in the seminiferous tube, most of death germ cells were associated to spermatocytes. Thus, both methods indicate that phthalates induce germ cell death, whilst alkylphenols induced increase in caspase-3 positive cells, but not in pyknotic cells. Since caspase-3 activation is not always related to apoptosis progression, the result observed with NP and OP could not be attributed only to germ cell death. These data suggest that within the mixture, phthalates rather than alkylphenols promote germ cells death in testes.

The results showed that the effects of the mixture could be due to a specific group of compounds or even to a single compound, some phthalates or alkylphenols show effects that are not observed when they are in a mixture.

Changes in Testosterone and Estradiol in Testis of Mice Exposed to EDCs

To assess local hormonal changes directly traceable in the testis, we measure testosterone and estradiol from intratesticular fluid. The data showed that only chronic exposure to DBP induced decrease in intratesticular testosterone levels (Figure 2A).
Table 2. Testis Damage Induced by Exposure to Single or Mixed EDCs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>DEHP</th>
<th>DBP</th>
<th>BBP</th>
<th>NP</th>
<th>OP</th>
<th>Mixture</th>
</tr>
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<tr>
<td>BW (g)</td>
<td>n = 12</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 11</td>
<td>n = 7</td>
<td>n = 6</td>
</tr>
<tr>
<td></td>
<td>22.41</td>
<td>24.93</td>
<td>25.19</td>
<td>25.36</td>
<td>21.90</td>
<td>26.18</td>
<td>25.27</td>
</tr>
<tr>
<td></td>
<td>± 1.66</td>
<td>± 1.14</td>
<td>± 0.45</td>
<td>± 1.08</td>
<td>± 3.55</td>
<td>± 1.84</td>
<td>± 2.57</td>
</tr>
<tr>
<td>Testis relative weight (UA)</td>
<td>n = 12</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 7</td>
<td>n = 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.37</td>
<td>0.36</td>
<td>0.37</td>
<td>0.35</td>
<td>0.38</td>
<td>0.30*</td>
<td>0.30*</td>
</tr>
<tr>
<td></td>
<td>± 0.03</td>
<td>± 0.02</td>
<td>± 0.02</td>
<td>± 0.03</td>
<td>± 0.08</td>
<td>± 0.11</td>
<td>± 0.04</td>
</tr>
<tr>
<td>Diameter of seminiferous tubules (μm)</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 5</td>
<td>n = 6</td>
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<tr>
<td></td>
<td>221.8</td>
<td>210.5</td>
<td>139.8</td>
<td>135.5*</td>
<td>136.4*</td>
<td>105.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>± 64.23</td>
<td>± 60.63</td>
<td>± 2.95</td>
<td>± 4.27</td>
<td>± 4.27</td>
<td>3.30</td>
<td>± 9.40</td>
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<tr>
<td>Seminiferous tubules with germ cells exfoliated (%)</td>
<td>n = 8</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.77</td>
<td>9.67***</td>
<td>11.60**</td>
<td>5.00</td>
<td>3.25</td>
<td>9.00**</td>
<td>10.75**</td>
</tr>
<tr>
<td></td>
<td>± 1.36</td>
<td>± 5.24</td>
<td>± 1.52</td>
<td>± 4.98</td>
<td>± 1.17</td>
<td>± 4.47</td>
<td>± 1.98</td>
</tr>
<tr>
<td>Seminiferous tubules without lumen (%)</td>
<td>n = 8</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.75</td>
<td>12.33**</td>
<td>9.67*</td>
<td>7</td>
<td>6.50</td>
<td>7.33</td>
<td>9.40*</td>
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<td></td>
<td>± 1.39</td>
<td>± 5.78</td>
<td>± 1.53</td>
<td>± 3.61</td>
<td>± 3.51</td>
<td>± 4.51</td>
<td>± 4.04</td>
</tr>
<tr>
<td>Spermatogenesis stages IV and V (relative frequency)</td>
<td>n = 8</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 5</td>
<td></td>
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<tr>
<td></td>
<td>0.17</td>
<td>0.09*</td>
<td>0.10*</td>
<td>0.14</td>
<td>0.15</td>
<td>0.16</td>
<td>0.15</td>
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<tr>
<td></td>
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<td>± 0.04</td>
<td>± 0.01</td>
<td>± 0.02</td>
<td>± 0.06</td>
<td>± 0.02</td>
<td>± 0.03</td>
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<tr>
<td>Spermatogenesis stages VI and VII (relative frequency)</td>
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<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 5</td>
<td></td>
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<tr>
<td></td>
<td>0.16</td>
<td>0.13</td>
<td>0.15</td>
<td>0.14</td>
<td>0.09**</td>
<td>0.08**</td>
<td>0.11*</td>
</tr>
<tr>
<td></td>
<td>± 0.03</td>
<td>± 0.01</td>
<td>± 0.02</td>
<td>± 0.03</td>
<td>± 0.01</td>
<td>± 0.06</td>
<td>± 0.02</td>
</tr>
<tr>
<td>Seminiferous tubules with undetermined stage (relative frequency)</td>
<td>n = 8</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.08</td>
<td>0.13*</td>
<td>0.08</td>
<td>0.06</td>
<td>0.15**</td>
<td>0.13**</td>
</tr>
<tr>
<td></td>
<td>± 0.01</td>
<td>± 0.03</td>
<td>± 0.03</td>
<td>± 0.04</td>
<td>± 0.02</td>
<td>± 0.09</td>
<td>± 0.08</td>
</tr>
<tr>
<td>Apoptotic index (Active caspase-3 cells/Seminiferous tubules)</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.30*</td>
<td>0.60**</td>
<td>0.43**</td>
<td>0.45**</td>
<td>0.40**</td>
<td>0.47**</td>
</tr>
<tr>
<td></td>
<td>± 0.27</td>
<td>± 0.99</td>
<td>± 1.19</td>
<td>± 1.03</td>
<td>± 1.15</td>
<td>± 1.00</td>
<td>± 1.35</td>
</tr>
<tr>
<td>Apoptotic index (Pyknotic cells/Seminiferous tubules)</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td></td>
</tr>
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Evaluation of mouse testes exposed to DEHP, DBP or BBP at doses of 0.3 mg/kg·BW/d, NP or OP at doses of 0.05 mg/kg·BW/d and the mixture of EDCs in fixed doses, compared with control mice. All data represent the mean ± SE. One-way ANOVA followed by Dunnett’s post hoc test.

*p < .05; **p < .01; ***p < .001. Bold Text, differences statistically significant.

Figure 1. Mice chronically exposed to single phthalates, alkylphenols or the mixture presented alterations in testis histology. Histological evaluation of mouse testes by PAS/hematoxylin staining. Animals exposed to DEHP, DBP, or BBP at doses of 0.3 mg/kg·BW/d, NP or OP at doses of 0.05 mg/kg·BW/d, the mixture of all EDCs in fixed doses and control. Arrows at the top show degeneration/atrophy of seminiferous tubule, and arrows at the bottom show specific type of testicular damage included caspase 3 positive cells by immunohistochemistry. Representative pictures.

However, we found that intratesticular estradiol levels decreased in all EDCs-exposed animals except for BBP (Figure 2B). In addition, we only observed a decrease in E2/T ratio on mice exposed to the mixture of EDCs (Figure 2C) and a tendency to decrease by DEHP exposure. But not observed any changes in the E2/T ratio with the single exposure to DBP, BBP, NP, and OP. This suggested that the expression and/or activity of aromatase in testes of mice exposed to the mixture of EDCs might be altered.

Differential Deregulation of Genes Involved in the Steroidogenic Pathway After EDCs Exposure

The biosynthesis of steroid hormones in testes is regulated by the expression of genes encoding proteins involved in the
steroidogenic pathway such as: Cyp11a1, Hsd3b1, Cyp17a1, and Cyp19a1, cholesterol transporters such as Star, and transcriptional factors upstream of the steroidogenic pathway gene such as Sp1 (Supplementary Table 2). We analyzed the mRNAs levels of these genes in testes of adult mice exposed to single phthalates, alkylphenols, or mixed EDCs. We detected changes in the mRNAs levels in all exposed mice (Figure 3). However, neither individually nor in the mixture, the EDCs changed the mRNAs levels of all transcripts (Figure 3). All transcripts, except Hsd3b1, were deregulated in mice exposed to mixture of EDCs (Figure 3). In single exposure to EDCs, the level of Sp1 decreased in all mice exposed to phthalates and mixture of EDCs but not after exposure to NP or OP (Figure 3A). Star levels only decreased in testis of animals exposed to DBP and OP. Surprisingly, exposure to the mixture of EDCs increased Star levels when compared with the controls. A decreased expression of Cyp11a1 was detected in mouse testes exposed to DBP and OP, whilst also Cyp17a1 mRNA decreased after NP exposure (Figs. 3B and 3C). In addition, DBP, NP, and OP compounds significantly decreased the mRNA levels of Hsd3b, but this effect was not observed in mice exposed to the mixture of EDCs. Interestingly, among all the studied genes, only the mRNA levels of Cyp19a1 (aromatase) were found reduced in mice exposed to all compounds alone or in the mixture (Figure 3F). In short, differential expression of proteins such as Star and Cyp19a1 between exposure to single and mixed EDCs was observed (Figure 4).

**Differential Deregulation of Protein Levels of Star and Cyp19a1 Between Exposure to Single and Mixed EDCs**

To investigate whether the changes observed in mRNAs levels were reflected in the protein level, we evaluated if STAR and CYP19A1 proteins levels were affected by any exposure to single or mixed EDCs compared with control. Our data showed that STAR increased only in testes of adult mice that were exposed to DEHP despite the level of the corresponding transcript not being significantly affected, as previously mentioned. However, the analyses of protein levels of STAR showed a tendency to increase in mice exposed to the mixture in agreement with mRNA levels (Figure 5).

On the other hand, CYP19A1 levels decreased in mice testes exposed to BBP, NP and the mixture of EDCs (Figure 5). This suggested that different EDCs affect protein stability in different ways and their effect, as individual compounds, are not always reflected in a mixture.

**DISCUSSION**

Previous research on EDCs has been based using individual compounds with exposure doses close to or even higher than those defined as LOAEL, which cannot predict the effect of low-doses exposure in humans (Vandenberg et al., 2012). Moreover, many of them have been done in a specific developmental window, rather than long time exposure from prenatal period onwards, which does not simulate the chronic exposure to mixed EDCs throughout life occurring in wildlife species and humans beginning at the prenatal life.

In this work, we designed an experimental procedure to emulate chronic exposure to environmental EDCs from fertilization up to adulthood. Our model allowed us to study the impact of exposure to a specific mixture of EDCs versus the effect of the single compounds on male reproductive health, which is a necessary step to understand the influence of these compounds on humans (Kortenkamp, 2014).

Epidemiological approaches suggested that weight at birth is inversely associated with exposure to different EDCs (Vilahur et al., 2013). At this point, our study determined that chronic exposure to single or mixed EDCs (except NP) induced obesogenic effects in adult male mice that seem to be independent of the type of EDCs exposure (single or mixed). EDCs can alter lipid homeostasis by increasing in the number and size of adipocytes. The molecular targets of single exposure to phthalates and...
alkylphenols are the peroxisome proliferator-activated receptors (PPARs, PPAR-α, and PPAR-γ) (Heindel et al., 2015) and steroid receptors mainly ERα, ERβ, and GPER (Klöting et al., 2015; Nadal et al., 2016). However, according to Biemann et al. (2014) “the adipogenic effect of EDCs mixtures [in vitro] is not deducible from single compound experiments”. Therefore, further in vivo studies are required to elucidate this effect and the molecular pathways implicated.

Our data showed a reduction of the seminiferous tubules diameter in mice exposed to DBP, BBP, OP, or the mixture. However, solely the exposure to OP or the mixture of EDCs decreased the testis relative weight. This is in concordance with the report of Bian et al. (2006) for chronic exposure of OP in rats but in opposition with the report of (Fisher et al., 1999; Gregory et al., 2009; Nagao et al., 2001). The difference between these reports and our work is basically the longer period of exposure.

Figure 3. Effects of exposure to single and mixed phthalates and alkylphenols in mRNA levels involved in the steroidogenic pathway. mRNAs levels by qPCR of: (A) Sp1; (B) Star; (C) Cyp11a1; (D) Cyp17a1; (E) Hsd3b1; (F) Cyp19a1; in testes of mice exposed to DEHP, DBP, BBP, NP, OP, the mixture of EDCs and control. Quantifications of mRNAs levels are relative to the expression of Gapdh, H2afz and Ppis as endogenous reference genes, using the 2^(-ΔΔCt) method. Each bar is the mean ± SE, n = 3, technical replicates = 4, 1-way ANOVA followed by Dunnett’s post hoc test, *p < .05; **p < .01; ***p < .001.

Figure 4. Genes associated with steroid hormones biosynthesis pathway and expression profiles induced by single and mixed exposure of EDCs. Adaptation of steroid hormones biosynthesis KEGG pathway (mmu 04913). Genes represented: Star, Cyp11a1, Cyp17a1, Hsd3b1. Cyp19a1. Sp1 was present in other related pathways but not in this one (see Supplementary Table 1). Heatmap represent the mRNA fold changes for each gene compared with control.
and doses approximately 1000 lower. This was an indicator that chronic exposure to low doses of OP was enough to make an important reduction in the gonadosomatic index.

Among the alterations detected in testes of mixed-exposed mice, several of them were also found in single-exposed mice to DEHP and DBP, such as sloughed germ cells, seminiferous tubules without lumen or abnormal frequency of stages IV and V of spermatogenesis. On the other hand, changes in the stages VI and VII were only observed with alkylphenols or the mixture of EDCs. These suggested 2 aspects: (i) there are alterations in the testes more sensitive to a family of compounds than other and (2) there are compounds with greater potential for a specific damage. Apoptosis is a hallmark of different toxicants that target germ cells (Creasy et al., 2012; Lagos-Cabre and Moreno, 2012). Here we show that all chemicals and the mixture used in this study induced increased germ cells death in some cases clearly defined as apoptosis, suggesting that all of them share some mechanistic pathway at this level. The differences observed between both apoptotic assays might be associated with the apoptosis rate but also to necrosis progression of germ cells apoptosis and not with low androgens. Our data indicate that decrease in intratesticular estradiol as a common mechanism after exposure to a mixture of EDCs, which could have a direct effect on the testicular physiology and be one of the causes of the reported alterations. Previous reports in humans have shown that estradiol is a germ cell survival factor (Suomalainen et al., 2000). However, in humans and murine models germ cells apoptosis and seminiferous tubule damage is associated with increased estradiol levels (Chaki et al., 2006; Handelsman et al., 2000). In this sense, our data suggest that most probably the damage observed in mice testicle is due to a direct action of the EDCs or their metabolites on estradiol biosynthesis. However, we cannot rule out that the decrease in estradiol or other changes in the level of hormones, genes, and/or proteins specific of germ cells and somatic cells not tested in this study may account for these effects.

Sp1 is required for transcriptional activity of steroid receptors specially Erα (Porter et al., 1997). Interestingly, mRNA level of this transcription factor was only affected by phthalates and the mixture of EDCs, not by alkylphenols. In this case, the decrease in estradiol and Sp1 mRNA levels, could suggest that phthalates and its mixtures induce changes in the expression levels of steroidogenic enzymes.

The expression of Star and Hsd3b1 on mice exposed to the mixture EDCs was the opposite of the expression detected in mice exposed to single compounds. These data support the fact that the effects on mRNAs expression due to exposure to mixed EDCs cannot be directly correlated with the exposure to single EDCs. It is important to note that in our study we observed, for the Star gene, an antagonist effect of the exposure to mixed EDCs when compared with single exposure (Figure 3). This result goes in concordance with Fiandanese and collaborators (Fiandanese et al., 2016) who reported that exposure to a binary mixture of DEHP/PCB antagonized (when compared with single exposure) the expression levels of genes involved in pituitary-

**Figure 5.** Mice exposed to phthalates, alkylphenols and their mixture present changes in STAR and CYP19A1 protein levels. STAR and CYP19A1 protein levels relative with the expression of β-ACTIN in exposed mice compared with control. Each bar is the mean ± SE, n = 3, 1-way ANOVA followed by Dunnett’s post hoc test, *p < .05; **p < .01. Abbreviation: AU, arbitrary units.
gonadal cross-talk. Furthermore, exposure to the mixture of EDCs induced an increase in Star mRNA and caused a tendency to increase protein levels (Figs. 3 and 4), in a similar way to the one observed in combined exposure to Genistein/DEHP in Leydig cell lines (Jones et al., 2015). In this case, the increase of Star due to mixed EDCs could be related to a complex mechanism that causes changes in the cAMP/PKA pathway and its transcriptional regulators including Sp1-5F1, p-Creb, Gata4, Dax-1, C/EBP, and p-Fos/Jun-DNA interactions (Manna et al., 2009). Also, in steroidogenic cells, the existence of 2 Star isoforms has been reported: a predominant isoform with an extended 3′ untranslated region (UTR) and an alternate with short 3′-UTR (Ariyoshi et al., 1998). This short 3′-UTR isoform presented differences in its stability which could indicate it is being submitted to regulatory processes (Zhao et al., 2005) such as polyadenylation (poly[A]) by mRNA-binding proteins or is being targeted by microRNAs (miRNAs). Our previous studies indicated the involvement of miRNAs in alteration in testsis induced by EDCs mixture exposure, in particular the decrease of miR-18a-5p, miR-20 b-5p, miR-15 b-5p, miR-1981-5p, and miR-382-5p induced by exposure to mixtures of EDCs, which might be directly targeting Star (Buyun et al., 2017). Interestingly, DEHP increased STAR protein levels, but not Star mRNA, which proposes that DEHP exposure could induce post-transcriptional modifications in Star that are not induced by exposure to mixed EDCs, suggesting that single exposure to DEHP induces different effects not yet reported inside steroid biosynthesis that are not reflected into the mixture exposure.

Among all genes, aromatase (Cyp19a2) was the only one that showed a reduction of mRNAs levels with exposure to all single compounds and the mixture, along with significant decrease at the protein level after exposure to BBP, NP and the mixture (Figs. 3 and 4). Early approaches have suggested that aromatase expression is modulated by environmental chemicals (Chen, 2002) and many reports have shown that down-regulation of Cyp19a1 might be a common mechanism of action caused by exposure to single or mixed EDCs (Hany et al., 1999; Laville et al., 2006; Lephart, 2015; Sanderson, 2006). In concordance with the literature, we suggested that the expression of aromatase could be a good biomarker for exposure to single or mixed EDCs. Comparable to Star, it seems that posttranscriptional mechanisms are important to regulate aromatase protein levels in mice exposed to EDCs.

In addition, we submitted our data to several mathematical models; namely, dose addition, response addition and integrated addition (Rider et al., 2008), and compared the predicted responses for the mixture with the observed responses (Supplementary Material and Methods). We observed that parameters such as apoptotic index in mixture models were able to capture the experimental results when assuming that the maximum effect was achieved with the mixture (see Supplementary Figure 3C). But, for other endpoints such as the intratesticular testosterone, none of the models were able to capture the experimental data for the mixture despite the maximum effect or slope considered (see Supplementary Figure 3D). These results were expected, in concordance with the data showed, since the response for DBP was stronger than the response for the mixture and the models considered did not take into account opposite or antagonistic effects. Therefore, the response for the mixture is expected to be at least as strong as for any individual compound.

The lack of agreement between model predictions and experimental data puts into question the monotonicity assumption. Other study in our laboratory with a female mice model also points in this direction showing higher effects at lower doses. Further experiments performed at different doses are needed to properly characterize the individual dose response curves. Moreover, nonmonotonic models of the dose response curve (Zhang et al., 2013) and more complex models of the mixture response might be needed.

In conclusion, our study shows that the effects on mouse testes of chronic exposure to a mixture of EDCs are more complex when compared with the corresponding single exposure. Therefore, it is necessary to conduct more specific human epidemiological studies to evaluate the direct impact of EDCs in human male reproductive health (Bliatka et al., 2017) considering mix of compounds in studies of the effect of EDCs or assessing the mix context in studies of individual compound exposure. For this, in future studies, we suggest taking a special interest in the detection of different kinds of EDCs in several populations and study them over a long period of time, to be able to compare to the time of exposure used in this work.

SUPPLEMENTARY DATA
Supplementary data are available at Toxcolical Sciences online.

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