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Transgenerational inheritance of heart disorders caused by paternal bisphenol A exposure

Marta Lombó [a], Cristina Fernández-Díez [a], Silvia González-Rojo [a], Claudia Navarro [a], Vanesa Robles [b], María Paz Herráez [a, *]

[a] Department of Molecular Biology, Faculty of Biology, University of León, Campus Vegazana s/n, León, 24071, Spain
[b] Spanish Institute of Oceanography (IEO), Promontorio de San Martín s/n, 39004, Santander, Spain

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ABSTRACT

Bisphenol A (BPA) is an endocrine disruptor used in manufacturing of plastic devices, resulting in an ubiquitous presence in the environment linked to human infertility, obesity or cardiovascular diseases. Both transcriptome and epigenome modifications lie behind these disorders that might be inherited transgenerationally when affecting germline. To assess potential effects of paternal exposure on offspring development, adult zebrafish males were exposed to BPA during spermatogenesis and mated with non-treated females. Results showed an increase in the rate of heart failures of progeny up to the F2, as well as downregulation of 5 genes involved in cardiac development in F1 embryos. Moreover, BPA causes a decrease in F0 and F1 sperm remnant mRNAs related to early development. Results reveal a paternal inheritance of changes in the insulin signaling pathway due to downregulation of insulin receptor β mRNAs, suggesting a link between BPA male exposure and disruption of cardiogenesis in forthcoming generations.

1. Introduction

Several studies support that emerging contaminants, and especially endocrine disruptors (EDCs), may have a central role in the increasing loss of male fertility because of their ability to interfere with the synthesis, metabolism and action of endogenous hormones related to spermatogenesis (Tse et al., 2013). Nowadays, bisphenol A (BPA: 2,2-bis(4-hydroxyphenyl)propane), an intermediate in the production of epoxy resins and polycarbonates, very common in domestic and industrial use (Furhacker et al., 2000), is one of the most studied compounds among these chemicals due to its ubiquitous presence. Ecotoxicological concerns of BPA rely on the increasing exposure through both environment and food chain (Lam et al., 2011). Human exposure to BPA occurs through diet (as it can be leached into food and drinks) and through dermal contact (with thermal paper used in tickets) (Kundakovic and Champagne, 2011). There are several mechanisms of bisphenol A action, being the most studied those interfering with the activity of endogenous estrogens (Wetherill et al., 2007). It has been proven that BPA can also act via membrane receptor GPER (Pupo et al., 2012), androgen receptors (Xu et al., 2005), thyroid receptors (Moriyama et al., 2002) and insulin receptors (Fang et al., 2015). Moreover, its range of toxicity is relatively high as a result of their multiples effects on reproduction, metabolism or obesity development (Kang et al., 2002) being those involving the cardiovascular system one of the main health concerns.

Direct exposure to BPA (or gestational exposure in mammals) has been reported to cause epigenetic effects (Miao et al., 2014; Singh and Li, 2012; Corrales et al., 2014) and DNA fragmentation (Iso et al., 2006). Therefore, its effects on reproductive health could go beyond the decrease in the production of spermatozoa since changes affecting the germline could be transmitted transgenerationally to subsequent generations (Manikkam et al., 2013; Lam et al., 2011). Some reports state that the inheritance of the effects promoted by the toxic when applied to embryos or pregnant females is sperm-mediated, showing that primordial germ cells exposed to the toxic during the period of germ cell migration (Huang et al., 2011) or gonadal determination (Hiyama et al., 2011), suffer from permanent alterations of the information contained in the sperm that are transmitted to the next generation.

* Corresponding author. Tel.: +34 0034987291912.
E-mail address: paz.herraez@unileon.es (M.P. Herráez).

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The paternal information provided by the spermatozoa is contained in the sperm nuclei and in the cytoplasmic RNAs. Albeit transcriptionally inactive, the spermatozoa have remnant mRNAs from spermatogenesis whose function is being elucidated (Ostermeier et al., 2004; Fang et al., 2014). Some of them, which are delivered into the oocyte at fertilization (Lalancette et al., 2008; Fang et al., 2014), are supposed to have crucial functions in the control of early embryogenesis. In fact, whether these sperm-specific transcripts (which are absent in the oocyte) are somehow altered, this might lead to embryo problems at early stages of development (Boerke et al., 2007). BPA is known to modify the transcriptome of different organs (Corrales et al., 2014; Fang et al., 2015; Chapalamadugu et al., 2014) so it is likely that alterations of the testicular transcription during early spermatogenesis could lead to a differential content of transcripts in the mature spermatozoa, affecting their contribution to the proper progeny development.

In addition, epigenetic modifications are essential for sperm cells to their own development as well as to the developmental program of future embryo (Yamauchi et al., 2011; Kumar et al., 2013; Carrell, 2011). Epigenetic mechanisms are thought to represent the most plausible target through which BPA could have long-lasting effects, being reported for the first time in yellow agouti mouse model (A^{vy}), when maternal exposure led to a change in coat coloration pattern of the next generation due to a sharp decrease in certain CpG sites methylation (Dolinoy, 2008). Moreover, as a result of its lipophilic nature, BPA can accumulate in adipose tissue of testicles allowing modifications in spermatozoal DNA methylation pattern (Soubry et al., 2014).

Consequently, the transcriptional activity and the epigenetic modifications that take place during spermatogenesis in the adult testes could be sensitive to BPA exposure. Then, changes in the sperm profile with further long-term effects on the progeny could be promoted. Nevertheless, all the studies are focused on direct exposure of embryos or pregnant females, neglecting the risk of adult male exposure to the toxic during fertile life.

Since George Streisinger firstly proposed zebrafish (*Danio rerio*) as model species (Streisinger et al., 1986) it has become more and more used in developmental biology and human disease (including cardiovascular one (Bakkers, 2011)), due to its high fecundity, rapid life cycle, transparence during development and high gene homology with humans (71%) (Howe et al., 2013; Asnani and Peterson, 2014). Moreover, it has become widely used in ecotoxicology as reviewed by Dai and colleagues (Dai et al., 2014) and more specifically for the study of the effects of BPA (Little and Seebacher, 2015; Cypher et al., 2015; Lam et al., 2011; Tse et al., 2013).

In this scenario, the aim of our work was to examine whether paternal BPA exposure (F0) at adult stages altered global DNA methylation pattern of spermatozoa or the expression of certain transcripts related to embryo development (likely to be transmitted throughout paternal via) and whether this exposure had adverse effects on the offspring phenotype (F1 and F2), with special focus on heart disease.

2. Materials and methods

2.1. Ethics statement

This work is included in a project from the Spanish Ministry of Economy and Competitiveness (AGL2011-27787) specifically approved by the University of León Bioethical Committee. All the animals were manipulated in accordance with the Guidelines of the European Union Council (86/609/EU, modified by 2010/62/EU), following Spanish regulations (RD 1201/2005, abrogated by RD 53/2013) for the use of laboratory animals.

2.2. Zebrafish maintenance

5-month-old zebrafish (*D. rerio*), AB strain (wildtype), were maintained in 2.5 L aquaria (ZebTEC, Tecniplast System) with a recirculating water system (pH 7.0–7.5, 30 mg/L Instant Ocean, at 27–29 °C, 14:10 light–dark cycle according to the instructions of The Zebrafish Book (Sprague et al., 2003). Animals were fed once a day with dry food (Special Diets Services®) and live artemia.

2.3. Exposure to BPA

Adult zebrafish males (3 replicates of 6 males each per treatment) were exposed to concentrations of 100 and 2000 μg/L BPA (0.44 μM and 8.8 μM) with final concentration 0.014% (v/v) of ethanol (vehicle) in 1.5 L of zebrafish water. Experimental fishes were exposed to the toxic during 14 days and control fish (3 replicates of 6 males each) were maintained in water with the same concentration of vehicle (Fig. 1). From day 14 to day 21 the animals were kept in water under the standard conditions described before. The tested BPA concentrations were the lowest concentration causing an increase of mortality (100 μg/L) and that which leads to a 100% of mortality by direct exposure in zebrafish embryos (2000 μg/L) (Lam et al., 2011). The lowest dose of BPA was also the total allowed concentration by Environmental Protection Agency (EPA) in drinking water (Willhite et al., 2008) whereas the highest dose was inside the levels of BPA in leachates from a hazardous waste landfill (Kang et al., 2007). To improve the assessment of cardiac malformations, 3 adult males of the transgenic line *Tg*(fli1:EGFP; mlc2a:mCherry) were used as control and 3 were exposed to 2000 μg/L BPA and mated with females from the same strain under the standard conditions.

2.4. Mating and progeny evaluation

As indicated in Fig. 1 one week after the treatment had finished 3 males per treatment were mated with non-treated females (*sex ratio* 1:2, male:female). Obtained embryos were rinsed 2 min with 0.5% (v/v) bleach and 10 s with 70% (v/v) ethanol. Then, they were transferred to embryo medium (7.5 mM NaCl, 0.25 mM KCl, 0.5 mM $MgSO_4$, 0.7 mM, KH_2PO_4, 0.02 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 0.35 mM $NaHCO_3$, 0.05% (v/v) methylene blue and 0.1% ampicillin/streptomycin; pH 7.2). Embryos were kept at 28 °C in darkness until further analysis: RNA extraction at 48 hpf and assessment of phenotypes at 7 dpf under a fluorescence stereomicroscope (Leica MZ16F) or a confocal microscope (Nikon Eclipse TE-2000). Malformations were evaluated and the percentage of malformed embryos per batch was established. Males from F1 were maintained in the standard conditions for 4 months until sexual maturity and then squeezed to obtain sperm or mated with non-exposed females to obtain the next generation. F2 embryos were processed as previously stated and phenotypes were analyzed at 7-dpf.

For routine histology 7 control and 7 malformed larvae at 7-dpf were fixed overnight in 4% paraformaldehyde and paraffin embedded. Sections of 4 μm thick were stained with hematoxylin and eosin and observed under light microscope (Nikon Eclipse E400).

Testes of 3 males per treatment were carefully removed after sacrificing the fishes with 300 mg/L of tricaine methanesulfonate (MS222, Sigma–Aldrich, USA) under a dissecting microscope (Sakai, 2002). After washing, they were incubated in 2% collagenase (Sigma–Aldrich, USA) in PBS 1X at 37 °C for 30 min and washed twice in PBS 1X until DNA extraction.

Sperm from 3 males per treatment was mixed, obtaining 3 sperm pools per treatment. Once the fishes were anesthetized with 168 mg/L of MS222, sperm collection was performed by ventral