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Alterations in oxidative responses and post-translational modification caused by *p,p´*-DDE in *Mus spretus* testes reveal Cys oxidation status in proteins related to cell-redox homeostasis and male fertility

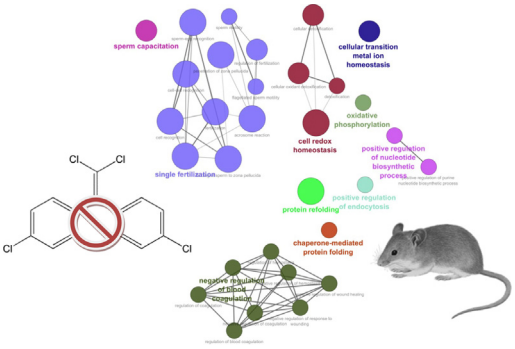
José Alhama [a], Carlos A. Fuentes-Almagro [b], Nieves Abril [a], Carmen Michán [a,*]

[a] Departamento de Bioquímica y Biología Molecular, Campus de Excelencia Internacional Agroalimentario CeiA3, Universidad de Córdoba, Campus de Rabanales, Edificio Severo Ochoa, E-14071 Córdoba, Spain
[b] Servicio Central de Apoyo a la Investigación (SCAI), Unidad de Proteómica, Universidad de Córdoba, Campus de Rabanales, Edificio Ramón y Cajal, E-14071 Córdoba, Spain

HIGHLIGHTS

- *p,p´*-DDE alters antioxidant enzyme levels in *Mus spretus* testes.
- *p,p´*-DDE decreases global carbonylation and phosphorylation levels in mice testis.
- *p,p´*-DDE changes the oxidation/reduction state of cysteine-containing proteins in testes.
- *p,p´*-DDE modulates oxidation in redox homeostasis, fertilization and sperm capacitation proteins.

GRAPHICAL ABSTRACT



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ABSTRACT

The major derivate of DDT, 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (*p,p´*-DDE), is a persistent pollutant previously associated with oxidative stress. Additionally, *p,p´*-DDE has been linked to several metabolic alterations related to sexual function in rodents. In this study, we analysed the effects of a non-lethal *p,p´*-DDE dose to *Mus spretus* mice in testes, focusing on oxidative damage to biomolecules, defence mechanisms against oxidative stress and post-translational protein modifications. No increase in lipid or DNA oxidation was observed, although antioxidative enzymatic defences and redox status of glutathione were altered in several ways. Global protein carbonylation and phosphorylation were significantly reduced in testes from *p,p´*-DDE-exposed mice; however, the total redox state of Cys thiols did not exhibit a defined pattern. We analysed the reversible redox state of specific Cys residues in detail with differential isotopic labelling and a shotgun labelling-based MS/MS proteomic approach for identification and quantification of altered peptides. Our results show that Cys residues are significantly affected by *p,p´*-DDE in several proteins related to oxidative stress and/or male fertility, particularly those participating in fertilization, sperm capacitation and blood coagulation. These molecular changes could explain the sexual abnormalities previously described in *p,p´*-DDE exposed organisms.

1. Introduction

A large variety of synthetic organic chemicals, such as organochlorine pesticides (OCPs), have been released into the environment over the last few decades (Valeron et al., 2009). As widespread

* Corresponding author at: Department of Biochemistry and Molecular Biology, Building Severo Ochoa, 2nd floor, Campus de Rabanales, University of Córdoba, 14071, Córdoba, Spain.
E-mail address: bb2midoc@uco.es (C. Michán).

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environmental pollutants, OCPs are highly lipophilic and chemically stable compounds that persist in the environment and accumulate in both the food chain and human tissues (Alvarez-Pedrerol et al., 2008); 2,2-bis (4-chlorophenyl)-1,1,1-trichloroethane (*p,p´*-DDT), the first widely used synthetic organochlorine pesticide, was given credit for having helped one billion people live malaria-free. Although banned for agricultural use in the 1970s–1980s, several kilotons had already been released into the environment (Stemmler and Lammel, 2009), and *p,p´*-DDT's bioaccumulation, long-range transport, persistence in the environment, and anti-androgenic properties have caused concern about its ecological effects. Furthermore, although having being banned or restricted for three decades, *p,p´*-DDT is still being used for the control of vectors in public health in some developing countries (Aulakh et al., 2007; Lopez-Carrillo et al., 1996; Rivero-Rodriguez et al., 1997; Zhang et al., 2013).

1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (*p,p´*-DDE) is DDT's major metabolite and, as such, is the form most commonly found in human tissues at the highest concentration (Rogan and Chen, 2005). In 2002, it was reported that this pollutant was present in the sera of >90% of the population of North America (Daxenberger, 2002). Recently, high levels of DDT metabolites, including *p,p´*-DDE, have been detected in human milk samples, particularly from less industrialized countries (van den Berg et al., 2017). Moreover, new determinations of these pollutants in China show that their levels have not significantly declined over time, and are reaching very toxic concentrations in harbour environments, both in biotic and abiotic samples, where dietary seafood intake can cause deleterious effects in human health (Zhang et al., 2013). Additionally, *p,p´*-DDE has been reported to be a widespread environmental endocrine disrupting chemical, associated with abnormalities in sexual development in rats and wildlife (Gray Jr and Kelce, 1996; Kelce et al., 1995). Moreover, *p,p´*-DDE is anti-androgenic and can inhibit androgen binding to the androgen receptor (Kelce et al., 1995; Xu et al., 2006), and high *p,p´*-DDE-DDT levels significantly increase the risk for low sperm count (Messaros et al., 2009).

Despite the molecular mechanisms for *p,p´*-DDE adverse androgenic effect being incompletely understood, ROS generation has been suggested to play a critical role in disrupting testis function (Shi et al., 2009; Song et al., 2008). Certain pesticides induce oxidative stress through generation of ROS, highly toxic and mutagenic species, mediating damage to biomolecules and altering the content and redox status of glutathione (Sies, 1986). To protect from oxidative stress, aerobic organisms use several lines of defence. Primary antioxidant enzymes, such as catalase (CAT), detoxify ROS, while others act as ancillary antioxidant enzymes, such as glutathione reductase (GSR), which uses NADPH to turn oxidized glutathione (GSSG) into its reduced form (GSH). Glutathione is an abundant thiol that keeps the cytosol reduced and is related to a third line of defence that cooperates with enzymatic defences (Sies, 1986). Both damage to biomolecules and antioxidative defence mechanisms are highly sensitive to pollutants that generate oxidative stress (López-Barea, 1995).

ROS are considered important second messengers since they mediate redox-signalling cascades that are critical to numerous physiological and pathological processes. As a consequence of ROS exposure, a range of reversible and irreversible post-translational modifications (PTMs) have been described that play essential roles in cellular localization, protein-protein interactions, protein structure and biological activity (Cabiscol and Ros, 2006; Davies, 2005; Eaton, 2006; Levine, 2002; Sheehan et al., 2010). Redox proteomics aim to detect and analyse redox-based changes within the proteome both in redox signalling scenarios and during oxidative stress (Sheehan et al., 2010). Formation of non-reversible carbonyl groups (Chaudhuri et al., 2006; Yan and Forster, 2011) and reversible modification of redox-sensitive thiol groups on cysteine (Cys) residues (Fernandez-Cisnal et al., 2014; Sheehan et al., 2010; Ying et al., 2007) are major forms of protein oxidation that are widely used indicators of oxidative stress. Unlike irreversible oxidative damage, redox signalling operates as a reversible "redox switch" that allows rapid responses to physiological and environmental cues (Morales-Prieto and Abril, 2017; Sheehan et al., 2010; Ying et al., 2007). Phosphorylation/dephosphorylation of proteins is another common PTM that is an important modulator of protein function, thus playing a major role in the regulation of various bio-signalling pathways (Graves and Krebs, 1999; White, 2008). Gel electrophoresis-based redox proteomics approaches are commonly used to evaluate protein oxidation/modification levels. Several chemical probes have been used for specific detection and quantification of different PTMs. Among these, fluorescence labelling offers advantages when used in gel-based analysis for the following reasons: i) it provides high sensitivity with short analysis times; ii) unbound probes are separated from proteins during gel electrophoresis; and iii) the same gel can be used for both specific PTM staining and total protein imaging (Yan and Forster, 2011).

Redox signalling has been proposed as the central mechanism underlying toxicological effects of many environmental toxicants, including pesticides such as *p,p´*-DDE (Morales-Prieto and Abril, 2017). Although cysteine residues are scarce in proteins, representing only 1–3% of total protein residues, they are one of the most reactive residues. The electronegativity of sulphur atoms in the thiolate group of cysteine side chain renders Cys vulnerable to many electrophiles, such as ROS, leading to redox modification (Eaton, 2006; Sheehan et al., 2010; Winterbourn and Hampton, 2008; Ying et al., 2007). Redox proteomics refers to different methods used for the detection, quantification and identification of oxidant-sensitive thiol proteins (Eaton, 2006; Sheehan et al., 2010). However, the current trend is to use gel-free proteomics techniques, which are less time-consuming and labour-intensive than 2DE and allow for higher sample throughput (Sheehan et al., 2010). A novel redox proteomic approach uses liquid chromatography-tandem mass spectrometry (LC-MS/MS analysis) to identify and quantify the reversible redox state of specific Cys residues after differential labelling of reversibly oxidized and reduced Cys residues in peptides with light (d0) and heavy (d5) forms of the thiol alkylating reagent *N*-ethylmaleimide (NEM) (McDonagh et al., 2014).

Mus musculus mouse strains commonly used for laboratory analysis are artificial hybrids that present few natural genetic polymorphisms (Dejager et al., 2009). In contrast, *Mus spretus* strains are only moderately inbred and therefore have a larger reservoir for phenotypic variation. Additionally, these mice belong to an unprotected species broadly found in Southern Spain and Northern Africa. These qualities make *M. spretus* rodents valuable bioindicators for studying complex effects (Dejager et al., 2009; Dejager et al., 2010); as such, they have been utilized in several laboratories, including our own, for environmental monitoring and in-lab exposure experiments (Abril et al., 2015; Garcia-Sevillano et al., 2014; Morales-Prieto and Abril, 2017).

As described above, *p,p´*-DDE is a hormone disruptor that alters male fertility in humans and other animals, affecting semen quality (Quan et al., 2016; L. Song et al., 2014a). Molecularly, DDT/DDE-induced toxicity has been associated with induction of oxidative stress and mitochondrial dysfunction by several groups (Harada et al., 2016; Li et al., 2017; Morales-Prieto and Abril, 2017; Morales-Prieto et al., 2017). Thus, this study was designed to explore the biological effects of *p,p´*-DDE in mice testes, particularly those related to oxidative stress. Specifically, we aimed to determine the molecular effects of a sub-lethal dose of *p,p´*-DDE on mice testes by studying several biochemical parameters related to stress and oxidative response. We investigated antioxidative enzymes, lipid peroxidation, DNA damage, glutathione levels and protein posttranslational modifications (carbonylation, thiol oxidation and phosphorylation). Furthermore, we assessed the redox state of the testis proteome by massive mass spectrometry after differential isotopic labelling of oxidized and reduced Cys-containing peptides.

2. Materials and methods

2.1. Animals and experimental design

This study was performed with all ethical requirements demanded by the Bioethical Committee of the University of Córdoba (Spain).