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"Phthalates effects on male reproductive tract"

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INDEX

Riassunto

Gli ftalati rappresentano un interessante gruppo di distruttori endocrini ampiamente utilizzati per la plastificazione del PVC. Il dibutilftalato (DBP) ed il di-2-etilexilftalato (DEHP) sono gli ftalati più commercialmente utilizzati e studi tossicologici presenti in letteratura hanno dimostrato un effetto tossico di tali sostanze per la riproduzione.

Per tale motivo, in questo progetto di ricerca sono stati valutati gli effetti del DBP e del DEHP sull'apparato riproduttivo maschile mediante studi *in vitro* ed *in vivo*.

Mediante studi *in vitro* sono stati valutati gli effetti del DBP sulla linea cellulare umana di adenocarcinoma prostatico LNCaP e, parallelamente, si è indagato sulle azioni determinate dall'estrogeno endogeno 17β-estradiolo (E2), al fine di valutare possibili effetti sovrapponibili tra le due sostanze su questo modello sperimentale.

In primo luogo, è stato valutato il loro effetto sulla vitalità cellulare delle LNCaP dopo 24 h di esposizione. Il DBP induce una riduzione della vitalità cellulare alla concentrazione di 10^{-8} M, al contrario E2 induce un aumento della vitalità alla concentrazione di 10^{-9} M.

Mediante esperimenti di RT-qPCR e western blot è stata valutata l'espressione di geni e proteine chiave coinvolte nella regolazione del ciclo cellulare come *MCT4, Ciclina D1, Ki-67*. Per comprendere attraverso quale pathway il DBP induce una riduzione della vitalità cellulare, mediante western blot è stata valutata l'espressione di Bax e Bak, due proteine pro-apoptotiche coinvolte nella via intrinseca dell'apoptosi. Il trattamento con DBP determina un significativo aumento di espressione sia di Bax che di Bak suggerendo un coinvolgimento del DBP nel processo di morte cellulare programmata. Inoltre, per studiare il possibile coinvolgimento dei recettori degli estrogeni (ERs) e degli androgeni (AR) è stata valutata la loro espressione proteica mediante western blot dopo 24h di trattamento e la loro localizzazione mediante immunofluorescenza indiretta dopo diversi tempi di esposizione (30', 2h, 4h). Il DBP è in grado di indurre una minore espressione di ERα e la sua traslocazione dal citoplasma al nucleo solo dopo 4h di trattamento e non determina alcuna variazione né di espressione né di localizzazione di ERβ ed AR.

I risultati indicano che il DBP riduce la proliferazione cellulare delle LNCaP probabilmente andando ad alterare i meccanismi coinvolti nella regolazione del ciclo cellulare attraverso l'interazione con il pathway recettoriale degli estrogeni.

Mediante studi *in vivo* sono stati studiati gli effetti di differenti dosi di DEHP sull'istopatologia del testicolo di ratto durante il periodo neonatale, dopo esposizione *in utero* e durante la lattazione. Ratte gravide Wistar sono state trattate oralmente dal giorno di gestazione (GD) 7 al GD 21 e dal primo giorno dopo la nascita (PND) 1 al PND 6 con 0, 30, 300 e 900 mg/kg bw/day DEHP.

La presenza di gonociti grandi e multinucleati è stata osservata solo dopo esposizione ad alte dosi di DEHP ed il trattamento a nessuna delle dosi considerate ha determinato effetto sulle cellule neonatali del Sertoli. Mediante immunofluorescenza con 3βHSD, è stato possibile evidenziare che le cellule del Leydig in seguito al trattamento tendono ad aggregarsi in clusters già dopo esposizione alla bassa dose di 30mg/kg-d di DEHP. Nei ratti trattati con DEHP 900 mg/kg/bw-d, si è notato anche la presenza di cellule del Leydig positive alla 3βHSD all'interno di tubuli malformati. Si è inoltre osservata una riduzione del diametro dei tubuli dopo esposizione a tutte le dosi considerate di DEHP. Il trattamento con DEHP non induce iperproliferazione dei gonociti o iperplasia delle cellule Leydig e non determina l'innescarsi del fenomeno apoptotico. Per comprendere il meccanismo attraverso il quale il DEHP determina i suoi effetti antiandrogenici è stata eseguita una IHC per AR e PPARγ. Il trattamento non determina alcuna variazione di espressione di AR. Nelle cellule del Leydig del gruppo di ratti trattati con DEHP 900mg/kg/bw-d si è osservato una riduzione di espressione di PPARγ.

I risultati ottenuti ci permettono di concludere che il DEHP influenza lo sviluppo del testicolo. Durante il periodo neonatale, gli effetti sulle cellule del Leydig sono marcati ed evidenti già dopo esposizione a basse dosi della sostanza e le azioni del DEHP sono probabilmente modulate da PPARγ.

Abstract

Phthalates are an interesting group of endocrine disruptors widely used in the manufacture of PVC. Dibutylphthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) are the two most commonly used phthalates and toxicological studies showed their toxic effect for the reproduction.

Thus, in the present research project, the effects of DBP and DEHP on male reproductive system have been evaluated using *in vitro* and *in vivo* studies.

In vitro studies were used to evaluate DBP and 17-β-estradiol (E2) effects on human prostate adenocarcinoma epithelial cells (LNCaP). First we assessed the effects of DBP and E2 on the cell viability after 24h of exposure. DBP induced a cell proliferation decrease at 10^{-8}M, instead E2 at 10^{-9}M stimulated cell viability. RT-qPCR and western blot analysis were used to evaluate the expression of genes and proteins involved in the regulation of cell cycle such as *MCT4*, *Cyclin D1*, *Ki-67*. Then, to evaluate through which pathway DBP induced a decreased cell viability, we performed western blot for Bax an Bak, two pro-apoptotic proteins involved in intrinsic apoptosis pathway. DBP treatment strongly enhanced both Bax and Bak expression, suggesting its involvement in programmed cell death processes. Moreover, in order to study estrogen (ER) and androgen (AR) receptors involvement, we evaluated their expression with western blot after 24 h of exposure and their cellular localization with immunofluorescence after three different times of exposure (30', 2h, 4h). DBP induced a minor expression of ERα and its cytoplasm-nucleus translocation after 4h of treatment; whereas DBP had no effects on ERβ and AR expression and cell localization. Results confirm that DBP may be involved in the deregulation of prostate cell cycle and it may interfere with estrogen hormonal receptor pathway.

In vivo studies were used to evaluate the effects of different doses of DEHP on testis histopathology in neonatal rats after *in utero* and lactation exposure. Pregnant Wistar rats were gavaged from gestation day (GD) 7 to GD 21 and from postnatal day (PND) 1 to 6 with vehicle, 30, 300, 900 mg/kg bw/day DEHP. Gonocytes appeared to be enlarged and multinucleated only after treatment with high DEHP doses and the treatment did not affect neonatal Sertoli cells. Immunofluorescence for 3β hydroxysteroid dehydrogenase (3β-HSD) revealed that Leydig cells tended to group together in clusters dose dependently from DEHP 30 mg/kg/bw-d. Moreover, in rats treated with DEHP 900 mg/kg/bw-d, it was possible to note malformed cords with positive 3βHSD Leydig cells inside the tubules. Furthermore, DEHP treatment reduced cord diameters after exposure to all DEHP doses. DEHP did not induce gonocytes proliferation or Leydig cells hyperplasia and did not cause apoptosis. To highlight a mechanism for DEHP antiandrogenic effects, immunohistochemistry for AR and PPARγ has been performed. Treatment did not interfere with AR expression, instead it induced a reduced expression of PPARγ in Leydig cells of rats treated with DEHP 900 mg/kg/bw-d.

In conclusion, DEHP impairs testis development during neonatal period; in particular, the most evident effects are registered on Leydig cells through PPARγ involvement.

1. Background

1.1 Endocrine Disruptor Chemicals (EDCs)

In recent years, a growing number of studies associate the worrisome trends in the incidence of reduced fertility rate observed in industrialized countries, to human exposure to specific chemicals identified as endocrine disruptors (EDCs).

EDCs are described as "exogenous chemicals or mixtures of chemicals that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations" (World Health Organization, 2012).

EDCs are an heterogeneous group of compounds and they may be divided into anthropogenic and natural chemicals or classified based on your chemical properties, origin and applications. According to document from WHO and UNEP 2012, in **Table 1** EDCs classification is reported: the chemicals are grouped in 4 groups and divided into eleven broad classes based on their physical-chemical characteristics or origin/application areas.

Table 1: Endocrine Disruptor Classification

Persistent and bioaccumulative halogenated chemicals
Persistent Organic Pollutants (POPs) (Stockholm Convention) [PCDDs/PCDFs, PCBs, HCB, PFOS, PBDEs, PBBs, Chlordane, Mirex, Toxaphene, DDT/DDE, Lindane, Endosulfan]
Other Persistent and Bioaccumulative Chemicals [HBCDD, SCCP, PFCAs (e.g. PFOA), Octachlorostyrene, PCB methyl sulfones]
Less persistent and less bioaccumulative chemicals
Plasticizers and Other Additives in Materials and Goods [Phthalate esters (DEHP, BBP, DBP, DiNP), Triphenyl phosphate, Bis(2-ethylhexyl) adipate, n-Butylbenzene, Triclocarban, Butylated hydroxyanisole]
Polycyclic Aromatic Chemicals (PACs) including PAHs [Benzo(a)pyrene, AnthraceneBenzo(a)anthracene, Pyrene]
HalogenatedPhenolicChemicals (HPCs) [2,4-Dichlorophenol ,Pentachlorophenol, Hydroxy-PCBs, Hydroxy-PBDEs, Tetrabromobisphenol A, 2,4,6-Tribromophenol, Triclosan]
Non-halogenatedPhenolicChemicals (Non-HPCs) [Bisphenol A, Bisphenol F, Bisphenol S, Nonylphenol, Octylphenol, Resorcinol]

Pesticides, pharmaceuticals and personal care product ingredients

Current-use Pesticides
[2,4-D, Atrazine, Carbaryl, Malathion, Mancozeb, Vinclozolin, Procloraz, Procymidone, Chlorpyrifos, Fenitrothion, Linuron]

Pharmaceuticals, Growth Promoters, and Personal Care Product Ingredients
[Endocrine active (Diethylstilbestrol, Ethinylestradiol, Tamoxifen, Levonorgestrel), Selective serotonin reuptake inhibitors (SSRIs; e.g. Fluoxetine), Flutamide, Octylmethoxycinnamate, Parabens, Cyclic methyl siloxanes (D4, D5,D6), Galaxolide, 3-Benzylidene camphor]

Other chemicals

Metals and Organometallic Chemicals
[Arsenic, Cadmium, Lead, Mercury, Methylmercury Tributyltin, Triphenyltin]

Natural Hormones
[17β-Estradiol, Estrone, Testosterone]

Phytoestrogens
[Isoflavones (e.g. Genistein, Daidzein), Coumestans (e.g. Coumestrol), Mycotoxins (e.g. Zearalenone), Prenylflavonoids (e.g. 8-prenylnaringenin)]

1.2 EDC molecular mechanisms

EDCs can interfere with the endocrine system at multiple levels, by agonizing or antagonizing the target receptors or by disrupting the synthesis of the hormones or hormonal release, transport, metabolism and excretion (Gore et al., 2015; Giulivo et al., 2016). The more frequent EDC targets are nuclear receptors such as thyroid receptors (TR), progesterone receptors (PR), estrogen receptors (ER), androgen receptors (AR) but they can also interact with membrane receptors, non steroidal receptors and orphan receptors (Diamanti-Kandarakis et al., 2009; Wuttke et al., 2010; Yang et al., 2015). Moreover, several studies demonstrate the ability of these substances to act on hormone metabolizing enzymes including aromatase, 5-reductase, 3-β-hydroxysteroid dehydrogenase (3βHSD), 11-β-hydroxysteroid dehydrogenase (11βHSD) (Kalfa et al., 2009; Ye et al., 2011; Guo et al., 2012). Furthermore, EDCs can also act through epigenetic mechanisms which are particularly useful to understand how EDC exposure during the development can cause adverse effects in adulthood (Prusinski et al., 2016).

It is important to note that EDC responses, do not follow the classical monotonic dose responses typically used in toxicological risk assessments. Experimental studies investigating EDC effects

frequently detect non monotonic dose-response (NMDR) relationships (Lagarde et al., 2015), indicating that Paracelsus principle of *"the dose makes the poison"* is not valid to assess their toxicity. NMDR relationship can result from multiple mechanisms and presents a bell-shaped profile. U-shaped profiles are characterized by the highest response at low and high exposure levels; instead, inverted U-shape is characterized by response at intermediate dose(s) and a decrease response or no response at low and high exposure levels (Vandenberg et al., 2013; Vandenberg, 2014).

Risk assessment of toxicological effects is currently focused on thresholds for effects of single compounds such as the lowest observed adverse effect levels (LOAEL) and the no observed adverse effect levels (NOAEL) which are not easy to establish, according to their dose-response relationships. However, mixture effects have been investigated in several studies showing that exposure to several endocrine disrupting chemicals, with similar or different modes of action, leads to "cocktail" effects and combined exposure may lead to additive, synergistic or antagonistic effects (Kortenkamp et al., 2007).

1.3 Human and wildlife exposures to EDCs

EDCs are lipophilic compounds and thus they are persistent in the environment. EDCs may biomagnificate and bioaccumulate during their production, their use and disposal. The main source of human exposure to EDCs is estimated to be the diet, particularly ingestion of contaminated food and water (Rudel et al., 2011). Dietary habits are influenced by different factors such as socioeconomic status, culture and religion as well as individual choices and these factors have a major impact on human daily consumption of nutrients, bioactive constituents, residues and contaminants.

Considering human exposure patterns through oral intake, these xenobiotics can be divided in four major classes:

- EDCs with bioaccumulation ability (e.g., polychlorinated biphenyls -PCBs-, polybrominated flame retardants, perfluorinated chemicals);
- Compounds utilized in food production (e.g., pesticides);
- Chemicals present in food due to contact materials, processing aids, etc. (e.g., alkylphenols, phthalates);
- Endocrine-active substances naturally present in food (e.g., phytoestrogens).

Besides ingestion, another important source of human exposure to EDCs is the indoor environment. Indoor air contamination in buildings and houses may induce airborne exposure noticeably above

background levels. One group of the relevant compounds associated with indoor exposures are PCBs (Bräuner et al., 2016).

It has also been shown that exposure may occur also through skin contact (Jeng, 2014) or medical devices such as blood bags, catheters, breathing and respiratory equipments containing different mixtures of EDCs (Ponzo, Carbone, 2013).

Humans are daily exposed to EDCs during all stages of life, from conception and fetal development through adulthood and senescence. EDC exposures health risks are closely associated to particular stages of life with concomitant critical windows of exposure (Mantovani, 2016). Considering EDC mechanisms of action, embryonic development and early life stages are certainly critical windows of human exposure to these compounds. In fact, during early development is required accurate timing of hormone action to endorse organogenesis and tissue differentiation. EDCs can affect the endogenous functioning of these hormones and also enzymes implicated in xenobiotic biotransformation. Additionally, it must be consider that, at these stages, elimination processes are not fully developed (Choudhary, D. et al., 2003). Thus, exposure during this "window of susceptibility" through maternal blood and/or milk may reprogram physiologic processes prompting health dysfunctions later in life (Prusinski et al., 2016).

1.4 EDC effects on male reproductive tract

The epidemic increase of male reproductive disorders, which cannot be explained by genetic changes, has occurred contemporaneously with cumulative exposures to various environmental factors through modern lifestyle. Increasing evidences demonstrate that males appear to be particularly vulnerable to exposure of certain compounds (Hauser et al., 2015). Androgens are the most important hormones involved in the normal development and homeostasis of the male reproductive tract (Knez, 2013) but recently it has been proposed a role for estrogens involvement in testicular function (Zhang et al., 2014). Thus, the balance between androgens and estrogens is really important in maintaining normal spermatogenesis. Therefore, exposure to xenoestrogens and anti-androgens during early life development, has been linked to male reproductive disorders (Zhang et al., 2014; De Falco et al., 2015). Many epidemiological data support the hypothesis that *in utero* exposure to dioxins, phthalates and PCBs may induce hypospadia and crtptorchidism (Jeng, 2014; Svechnikov et al., 2014).

Bisphenol A (BPA) has been positively correlated with male infertility, in fact BPA-exposed male workers had consistently higher risk of sexual dysfunctions than unexposed workers (Li et al., 2010). It has been demonstrated that BPA mainly induces germ cell apoptosis and reduces total sperm count, vitality and motility (Li et al., 2011). Moreover, it can also affects prolactin, estradiol

and sex hormone-binding globulin (SHBG) serum levels (Liu et al., 2015). PCB exposure has been associated with increased anogenital distance and prostate size and reduced sperm count (Faroon et al., 2015). Several studies have demonstrated that alkylphenols (AP) are able to influence the correct development and physiology of male reproductive system specifically target organs important for the quality of spermatozoa and reproductive fitness. NP and octylphenol (OP) have been shown to induce testicular damage with decreased testis weight and size in rodents (Knez et al., 2013; Ponzo and Carbone, 2013). Furthermore, neonatal exposure to NP, during early stage of sexual maturation and in adulthood, led to histological disorganization of testis, reduction in testis, epididymis and seminal vesicle size and it also may induce and increased incidence of cryptorchidism (Fan et al., 2010; Ponzo and Carbone, 2013). BPA and NP are also involved in increased prostatic cells proliferation (Forte et al., 2016; Prins et al., 2014). Organohalogen pollutants and phthalates may affect testicular descent in humans and spermatogenesis by affecting spermatogonia or damaging Sertoli or Leydig cells number and function or changing the morphology and motility of spermatozoa (Svechnikov et al., 2014).

1.5 Phthalates

Phthalate esters (PAEs), derived from phthalic acid are ubiquitous industrial chemicals in our surrounding environment. PAEs are widely used as plasticizers to add softness, flexibility, transparency and longevity to polyvinyl chloride-based (PVC) products (Giulivo et al., 2016).

They are divided into two groups based on their molecular weight: high molecular weight compounds (with alkyl chain length from 8 to 13 carbons), such as di-2-ethilhexyl-phthalate (DEHP) and diisononyl phthalate (DiNP), and low molecular weight compounds (with alkyl chain length from 1 to 4 carbons) such as diisobutyl phthalate (DiBP) and dibutyl phthalate (DBP). High molecular weight phthalates are primarily used as plasticizers in the manufacture of flexible vinyl plastic present in consumer products, flooring and wall covering and medical devices. PAEs with low molecular weight are mainly used in personal care products as solvents, perfumes, nail varnish, hair sprays and plasticizers for cellulose acetate (ATSDR, 2002; Chen et al., 2011, Jeng, 2014). PAEs group also includes three DEHP metabolites: mono-(2-ethyexyl) phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) (Jeng, 2014).

Phthalates do not accumulate appreciably in the body and show a relatively short half life, in fact they are rapidly metabolized to their respective monoesters which in turn undergo to oxidation reactions and form conjugates with glucuronic acid (Latini et al., 2003; Calafat et al., 2011).

Moreover, they are volatile compounds and do not covalently bound to the polymer so with age, use and ultraviolet exposure, they can easily migrate from plastics to food, beverages and body fluids (Cheng et al., 2013; Ponzo and Carbone, 2013). Different studies confirm PAEs metabolite presence in human urine, breast milk and serum (Wittasek et al., 2011; Moody et al., 2013).

The major source of exposure for humans is diet. Nevertheless, different studies have suggested that depending on their properties, the routes of human exposure can differ. For example, diet is thought to be the main source of exposure to DEHP (Clark et al., 2011; Guo et al., 2012). DEHP exposures were reduced when individuals' diets were restricted to those that have limited contact with packaging materials. Further, foodstuffs can be contaminated also during production or food chain transfer of these compounds (Rudel et al., 2011). Inhalation is the predominant source of human exposure to dimethyl phthalate (DMP) and inhalation and dermal contact are important sources of exposure to DBP (Guo and Kannan, 2011; Wormuth et al., 2006). Maternal exposure is the first source of fetal exposure to phthalates through amniotic fluid and umbilical cord blood (Latini et al., 2003, 2006).

Every year it has been calculated that around 6 million tons of phthalates are produced worldwide (Knez et al., 2013) and individuals, especially children exposure is 2 to 4-fold higher than adults (Moody et al., 2013).

1.6 Phthalate effects on male reproductive system

PAEs are considered to be one of the major groups of antiandrogenic substances and among these, DEHP and DBP are the most commonly found in the environment (Chen et al., 2011; Knez, 2013). There is a large body of evidence about association between phthalates exposure and congenital male reproductive disorders such as reduced anogenital distance (AGD) and hypospadia (Jeng, 2014), smaller testis and penis size, adult pathologies such as Leydig cell aggregation (Hu et al., 2009), tubules only containing Sertoli cells, poor spermatogenesis (Nistal et al., 2006), reduced semen quality and testicular germ cell tumors (Yao et al., 2012).

It has been shown that DEHP impaired steroidogenesis and can cause birth defects (Jeng, 2014; Ponzo and Carbone 2013) producing the "phthalate syndrome" which comprises non descent testis, poor semen quality, malformation of external genitalia (Johnson et al., 2012). DEHP has been shown to disrupt the androgen-regulated development of the male reproductive system reducing prostate weight and altering spermatogenesis processes; moreover, it strongly affects Leydig cells hormonal influence by decreasing testosterone biosynthesis and increasing LH and estradiol (Ponzo and Carbone, 2013). DBP exposure produces marked changes in the growth and development of male reproductive tract and decreased sperm count by altering testosterone levels (Chen et al.,

2011). Both DEHP and DBP, such as other PAEs, may interfere with normal steroidogenesis, suppressing the expression of steroidogenic enzymes (Gao et al., 2017) or disrupting the regulation of cholesterol and lipid homeostasis (Knez, 2013). A relation between urinary and environmental levels of phthalates (DEHP, DBP, DEP) has been related with reduced sperm motility and concentration and DNA damage (Pant et al., 2014). However, compared to other anti-androgen products, both DEHP and DBP affect male reproductive tract by disrupting testosterone biosynthesis instead of directly contacting with androgen receptors (Kabir et al., 2015).

2. Aim of the studies

The purpose of these studies was to improve the knowledge about phthalates impact on male fertility by examining the following topics:

- Interference of dibutylphthalate (DBP) on human prostate cell viability.
- Diethylhexyl phthalate (DEHP) affects testicular histopathology in neonatal rats.

- Interference of dibutylphthalate on human prostate cell viability.

Prostate gland plays a key role in determining a good quality of seminal fluid and therefore of male fertility, thus it may represent one of the targets through which EDCs cause infertility. In this view we examined the effects of DBP and 17-β-estradiol (E2) on human prostate adenocarcinoma cells (LNCaP) in order to investigate a possible mimetical behaviour.

- Diethylhexyl phthalate affects testicular histopathology in neonatal rats.

Phthalate effects on testis are thought to be dependent on age and dose of exposure. Thus, the goal of this study is to evaluate the effect of different doses of DEHP on testicular histopathology in neonatal rats after prenatal and lactation exposure. We wished to examine how exposure during window of susceptibility would affect male rats at a different stage of life.
This project was carried out at the National Food Institute of the Technical University of Denmark (DTU Food) in Copenhagen, under the supervision of Senior Scientist Julie Boberg.

3. Experimental setup

3.1 *In vitro* study

3.1.1 Published paper:

Interference of dibutylphthalate on human prostate cell viability.

LNCaP cells were treated for 24h with DBP and E2 from 10^{-5} M to 10^{-13} M and effects on cellular viability were evaluated using MTT assay.
RT-qPCR and western blot analysis were used to evaluate the expression of genes and proteins involved in the regulation of cell cycle after 24h of exposure.
Androgen and estrogen receptors involvement has been studied through western blot and immunofluorescence analysis.

3.2 *In vivo* study

3.2.1 Manuscript in preparation:

Diethylhexyl phthalate affects testicular histopathology in neonatal rats.

Pregnant Wistar rats were orally gavaged from gestation day (GD) 7 to GD 21 and from postnatal day (PND) 1 to 6 with vehicle, 30, 300, 900 mg/kg bw/day DEHP. At PND 6 rats were anesthetized, decapitated and testes were removed and used for histological analysis.
Testis morphology was studied using hematoxylin and eosin staining and Leydig cells distribution was evaluated using immunohistochemistry.
Proliferation and apoptosis were studied using immunohistochemistry and TUNEL assay.
Moreover, immunohistochemistry was also used to assess androgen receptor and peroxisome proliferator-activated receptors involvement.

Detailed descriptions of *in vitro* and *in vivo* studies can be found in the subsequent manuscripts.

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Interference of dibutylphthalate on human prostate cell viability

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ABSTRACT

Dibutylphthalate (DBP) is an environmental pollutant widely used as plasticizer in a variety of industrial applications worldwide. This agent can be found in personal-care products, children's toy, pharmaceuticals, food products. Exposure to DBP can occur via ingestion and inhalation as well as intravenous or skin contact. DBP belongs to the family of endocrine disrupting chemicals (EDCs) and its effects on reproductive system were demonstrated both *in vivo* and *in vitro*. In the present study we evaluated the effects of DBP on human prostate adenocarcinoma epithelial cells (LNCaP) in order to highlight xenoestrogens influence on human prostate. Moreover, we have compared DBP effects with 17β-estradiol action in order to investigate possible mimetical behaviour. We have assessed the effects of both compounds on the cell viability. After then, we have evaluated the expression of genes and proteins involved in cell cycle regulation. Furthermore, we have observed the expression and the cell localization of estrogen (ERs) and androgen (AR) receptors. In conclusion, we have demonstrated that DBP interacts with estrogen hormonal receptor pathway but differently from E2. DBP alters the normal gland physiology and it is involved in the deregulation of prostate cell cycle.

1. Introduction

Phthalates are heterogeneous group of xenobiotics widely used to enhance products flexibility, durability and transparency (Alam et al., 2010; Howdeshell et al., 2007). Phthalate plasticizers are esters of phthalic acid and based on their alcohol chain length, they may be divided into two groups: low and high molecular-weight (Barlow et al., 2004; Barlow, Foster, 2003; Blount et al., 2000). Both of them are not chemically bound to products and with age, use and ultraviolet light they can easily end up into the environment (Johnson et al., 2012; Thomas, Thomas, 1984), therefore, human exposure can occur through diet, inhalation and dermal absorption (Schettler, 2006; Wormuth et al., 2006). Many studies corroborate phthalate metabolite presence in human serum, urine and breast milk (Frederiksen et al., 2011; Göen et al., 2011; Moody et al., 2013; Wittasek et al., 2011). These compounds are endocrine disruptors: they can affect thyroid signaling and metabolic homeostasis (Borch et al., 2006; Gray et al., 2000; Lyche et al., 2009; Zhai et al., 2014) and they are also reprotoxic; their negative effects for reproductive system depend on their alkyl chain (Fujii et al., 2005).

Phthalates exposure during sensitive window of perinatal development may result in developmental effects in human babies (Christiansen et al., 2010). The presence of different phthalate monoesters in breast milk seems to be correlated with increased levels of luteinizing hormone (LH), sex hormone-binding globulin (SHBG) and with an increased ratio of LH/free testosterone in 3 months age boys (Main et al., 2005). Furthermore, infant boys, whose mothers presented elevated levels of phthalate metabolites in urine, have reported shortened anogenital distance (AGD) (Swan et al., 2005).

Men's phthalate exposure has been associated with hypospadias, gynecomastia, cryptorchidism, abnormal spermiogram and sperm DNA damage and with abnormal sexual hormones levels; instead, women's exposure can cause infertility, endometriosis, breast cancer, early menarche and breast development and pregnancy complications (De Falco et al., 2015; Hannon, Flaws, 2015; Heudorf et al., 2007; Kay et al., 2013; Zhang et al., 2015). Experimental studies on early gestation exposure to phthalates in rats, show that they may display phthalate syndrome. This syndrome symptoms look like the effects of phthalate exposure in human male and it is characterized by the presence of seminiferous tubules with reduced diameter, hypospadias, cryptorchidism, reduced anogenital distance and malformation of vas deferens, epididymis, seminal vesicles and prostate gland (Christiansen et al., 2010; Kay et al., 2013; Lioy et al., 2015).

Dibutylphthalate (DBP) is short-chain phthalate prepared from

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butanol (Shirai et al., 2013; Wakui et al., 2014, 2013). It is commonly used in paints, inks, adhesive, insecticides, solvents, cosmetics, perfumes and medications (Guo and Kannan, 2013; Hubinger, 2010; Schettler, 2006; Xu et al., 2014); so human population appears to be predominantly exposed to it (Barlow, Foster, 2003; Blount et al., 2000).

DBP belongs to the subclass of endocrine disrupting chemicals (EDCs) that mimic the endogenous estrogens. DBP mainly damages male reproductive system inducing negative effects on testicular function and steroidogenesis (Dobrzynska et al., 2011; Kay et al., 2013; Li et al., 2016). It rapidly crosses the placenta barrier and embryos from rats *in utero* exposed show several reproductive abnormalities: hypospadias, nipple retention, reduced AGD and retarded testis descent and spermatogenesis dysfunction (Liu et al., 2012; Silva et al., 2007). DBP *in utero* exposure may also causes age-related morphological changes of Leydig cells smooth endoplasmic reticulum (LCs-ER) corresponding to reduced testicular testosterone biosynthesis (Motohashi et al., 2016).

The mechanism by which phthalates, including DBP, exert their actions on reproductive functions are not yet fully cleared. Phthalates and their metabolites, have been suggested to interfere with normal steroidogenesis, dropping the expression of steroidogenic enzymes and disrupting the regulation of cholesterol and lipid homeostasis or insulin signaling (Barlow et al., 2003; Knez, 2013; Liu et al., 2005; Moody et al., 2013).

Prostate is an accessory gland of the male reproductive tract. Both androgens and estrogens hormones play a pivotal role in its differentiation, development and maintenance of adult homeostasis. *In vivo* and epidemiological studies suggest a positive relationship between EDC men exposure and prostate diseases (Alavanja et al., 2003).

In this study, we evaluated the effects of DBP on human adenocarcinoma prostate cells (LNCaP). LNCaP cells are a useful prostate model *in vitro* because they are hormone responsive and express all prostate specific markers (Horoszewicz et al., 1983). We analyzed the effects of DBP on the expression of genes and proteins that can be altered after exposure to endocrine disruptor chemicals (EDCs). Particularly, we have observed the DBP action on cell viability, the expression of key genes (MCT4, Ki-67 and cyclin D1) involved in the regulation of cell proliferation and proteins (mct4, cyclin D1, Bax, Bak) involved in cell cycle and apoptosis, and the expression and cellular localization of estrogen ERs (ERα and ERβ) and androgen AR receptors. Cells were also treated with the endogenous hormone 17 β-estradiol to better understand exogenous and endogenous compounds involvement in prostate gland and to investigate possible mimetical behaviour by DBP.

2. Materials and methods

2.1. Cell culture

LNCaP cells (CRL-1740™ American Type Culture Collection, Manassan, VA) were grown in RPMI 1640 (Sigma-Aldrich), supplemented with 10% FBS, 2 mM glutamine, 1X non essential amminoacid, 1X penicillin/streptomycin, 10 μg/mL gentamycin (Euroclone) at 37 °C, 5% CO_2 in an humidified incubator. When 70% confluent, cells were enzymatically detached with trypsin-edta (Sigma-Aldrich) and seeded in a new cell culture flasks. The medium was changed every 2 days. Cells were used from passage 9–20.

Table 1
a) Details of primers used for RT-qPCR, b) Details of primary antibodies used for western blot and immunofluorescence assays.

a)

Gene	*Forward*	*Reverse*
MCT4	5′-	
ACCCACAAGTTCTCCAGTGC-3′		
5′-AGCAAAATCAGGGAGGAGGT-3′		
Cyclin D1		
5′-CGTGGCCTCTAAGATGAAGGA-3′		
5′-CGGTGTAGATGCACAAGCTTCTC-3′		
Ki-67		
5′-CCCGTGGGAGACGTGGTA-3′		
5′-TTCCCGTGACGCTTCCA-3′		
HPTR1		
5′-GACTTTGCTTTCCTTGGTCAGGCA-3′		
5′- ACAATCCGCCCAAAGGGAACTGA-3′		

b)

Antibody	*Source*	*Species*	*Dilution*
MCT4	sc-50329, Santa Cruz, CA, USA	Rabbit	1:200
Cyclin D1	ab-74646, Abcam, Cambridge	Rabbit	1:200
Bak	sc-832, Santa Cruz, CA, USA	Rabbit	1:200
Bax	sc52b, Santa Cruz, CA, USA	Rabbit	1:200
ERα	sc544, Santa Cruz, CA, USA	Rabbit	1:200
ERβ	sc-8974, Santa Cruz, CA, USA	Rabbit	1:200
AR	ab-74272, Abcam, Cambridge	Rabbit	1:300
β-actin	sc-7210, Santa Cruz, CA, USA	Rabbit	1:200

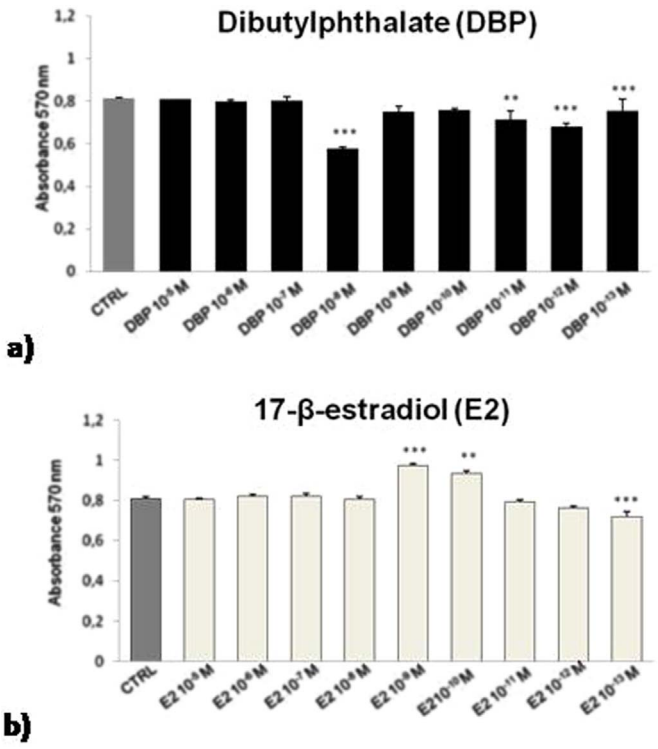

Fig. 1. MTT assay after 24 h of exposure to dibutylphthalate (DBP) and 17β-estradiol (E2) from 10^{-5} M to 10^{-13} M. In graphs are reported the absorbencies measured at 570 nm which correlates with number of living cells. (**a**) DBP induce a decrease of cell viability at 10^{-8} M; (**b**) E2 stimulates prostate cell viability, reaching the most notable effect at 10^{-9} M. (**$p < 0,01$; ***$p < 0,001$).

2.2. Chemicals

Dibutylphthalate (DBP) and 17 β-estradiol (E2) were purchased from Sigma-Aldrich and dissolved in DMSO. Then, DBP and E2 were diluted in RPMI 1640 red-phenol free at the concentrations used for the experiments. Control cells were treated with vehicle (DMSO 0,01%).

2.3. Treatment

LNCaP cells were treated with DBP and E2 from 10^{-5} M to 10^{-13} M for 24 h in order to perform the MTT assay. MTT assay allowed us to establish DBP and E2 concentration to use for the further experiments. RT-pPCR and western blot analysis were performed after 24 h of exposure with DBP 10^{-8} M and E2 10^{-9} M. Immunofluorescence was carried out after three different times (30 min, 2 h, 4 h) of exposure to 10^{-8} M DBP and 10^{-9} M E2.

2.4. MTT assay

MTT assay was performed to evaluate the effects of DBP and E2 on cell viability. LNCaP cells were cultured at a density of $1,5 \times 10^4$/ well in 96 multiwell, starved (FBS 1%) for 24 h and treated with DBP and E2 from 10^{-5} M to 10^{-13} M for 24 h. Then, 10 µL of MTT were added to each well for 4 h at 37 °C, 5% CO_2. In order to dissolve the formazan crystals produced in each well, the medium was aspirated and was added a solution of isopropanol and DMSO (1:1). Then, the assorbance of the solution was read at 570 nm using a microplate reader. Each MTT assay was performed in triplicate.

2.5. RNA extraction and RT-qPCR

mRNA expression levels of genes were analyzed using real-time PCR. Total RNA was extracted from control and treated cells for 24 h with DBP 10^{-8} M and E2 10^{-9} M using PureLink™ RNA Mini Kit (Life Technologies). TURBO DNA-free™ Kit (Life Technologies) was used for purification from genomic DNA. After purification total RNA was quantified with a NanoDrop spectrophotometer. cDNAs were synthesized from 1 µg RNA using the High Capacity cDNA Reverse Transcriptase (Life Technologies) and quantitative PCR (RT-PCR) was performed using the 7500 Real-Time PCR System and SYBR®Select Master Mix 2X assay (Applied Biosystem). All primers used, are listed in the Table 1 and were designed according to the sequences published on GenBank using Primer Express software version 3.0. The amount of target cDNA was calculated by comparative threshold (Ct) method and expressed by means of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). Three different experiments were performed for RT-qPCR and each sample was tested in triplicate.

2.6. Protein extraction

Proteins were extracted from LNCaP cells after 24 h of treatment with DBP 10^{-8} M and E2 10^{-9} M. Control cells were treated only with vehicle (DMSO 0.01%). 10 cm cell dishes with confluent LNCaP cells were placed on ice for 10 min and washed twice with ice cold PBS. Then PBS-EDTA was added and cells were scraped and transferred to microcentrifuge tubes. The collected cells were centrifuged at 3000 rpm for 5 min at 4 °C and pellets were resuspended wit RIPA lysis buffer containing protease and phosphatase inhibitors cocktail (Santa Cruz) for 30 min. Homogenates were centrifuged at 12,000*g* for 20 min at 4 °C. Total protein amounts of samples were determined by the BCA protein assay reagent kit (PIERCE).

2.7. Western blot

50 µg of proteins for each samples were boiled for 5 min in SDS buffer [50 mM Tris-HCl (pH 6.8), 2 g 100 mL^{-1}SDS, 10% (v/v) glycerol, 0.1 g 100 mL^{-1}Bromophenolblue], separated on 10% SDS-PAGE and transferred to a PVDF membrane for blotting (Trans-Blot® Semi-Dry Transfer Cell, Biorad). Membranes were incubated for 1 h at room temperature with blocking buffer (TBS, 0.05% Tween-20% and 5% milk). After blocking, membranes were incubated overnight at 4 °C with primary antibodies diluted in TBS-T containing 2% milk. Primary antibodies used were listed in the Table 1. The membranes were washed four times for 10 min in TBS, 0.05% Tween-20 before a 1 h incubation with secondary antibody diluited in TBS-T containing 2% milk. Secondary antibody used was goat anti-rabbit IgG (HRP) (1:3000; Abcam ab-6721). Then, the membranes were washed four times for 10 min and specific protein bands were detected with chemiluminescence using the C-DiGit Chemiluminescent Western Blot Scanner (LI-COR). Western blot were analyzed using Image Studio Software to determine optical density (OD) of the bands. The OD reading was normalized to β-actin to account for variations in loading. All experiments were performed in triplicates. Western blots were performed as reported in Zizza et al. (2017).

2.8. Immunofluorescence

LNCaP cells were seeded overnight at a density of 5×10^4/well in 4-well chamber slides (Sarstedt, Nürnbrecht, Germany) and after 24 h 1% FBS, cells were treated with DBP 10^{-8} M and E2 10^{-9} M for three different times: 30 min, 2 h and 4 h, instead control cells were treated only with vehicle (DMSO 0.01%). Then, control and treated groups were fixed with ice cold methanol for 10 min at RT, permeabilized with 0.4% Triton X-100 in PBS for 10 min at RT, washed in PBS and blocked in 5% Normal Goat Serum (NGS) for 30 min. Subsequently, cells were incubated overnight at 4 °C with the primary antibodies: rabbit polyclonal anti-human ERα, rabbit polyclonal anti-human ERβ, and rabbit polyclonal anti-human androgen receptor, diluited in 1% NGS. The day

after cells were incubated with goat anti-rabbit Alexa Fluor 488 (ab 150077), diluited 1:300 in 1% NGS. Cell nuclei were stained with 0,1 μg/mL Höechst and negative controls were performed by avoiding incubation with primary antibodies. Fluorescent images were captured with Axioshop (Carl Zeiss, Milano, Italy) epifluorescence microscope using a 40x objective. Axiocam MRc5 and the acquisition software Axiovision 4.7 (Carl Zeiss) were used to take the images in different channels (Alexa Fluor 488, Höechst 33258). Each immunofluorescence was performed in triplicate and for data analysis different fields were randomly chosen.

The immunofluorescence was performed as reported in Forte et al. (2016).

2.9. Statistical analysis

Data showed in graphs are expressed as means ± SEM for the indicated number of independent determinations. The statistical significance was calculated by the one way ANOVA with Bonferroni's multiple comparison test and differences were considered statistically significant when the P values was at least $p < 0.05$. All experiments were repeated at least three times and performed in triplicate.

3. Results

3.1. MTT Assay

MTT assay was performed to evaluate the effects of DBP and E2 on cellular viability. We exposed LNCaP cells to DBP and E2 range from 10^{-5} M to 10^{-13} M. After 24 h of exposure, DBP induced a decreased cells viability with the greatest effect at 10^{-8} M (Fig. 1a). Instead, E2 increased LNCaP viability with the higher effect showed at 10^{-9} M (Fig. 1b).

3.2. RT-qPCR analysis

After 24 h of exposure with DBP 10^{-8} M and E2 10^{-9}M, RT-qPCR was performed to evaluate expression of genes involved in cell cycle regulation such as *MCT4*, *Ki67* and *Cyclin D1*. DBP didn't interfere on mRNA levels of *MCT4* (Fig. 2a); on the contrary DBP strongly decreased expression of *Ki67* (Fig. 2b) and *Cyclin D1* (Fig. 2c) of 50% and 40% respectively. Conversely, E2 enhanced *MCT4* expression of 30% (Fig. 2a), and it didn't significantly interfere on *Ki67* (Fig. 2b) and *Cyclin D1* expression (Fig. 2c).

3.3. Western blot analysis

Western blot analysis was performed after 24 h of exposure with DBP 10^{-8} M and E2 10^{-9} M in order to evaluate the expression of MCT4 and Cyclin D1 involved in cell cycle regulation, the expression of pro-apoptotic proteins such as Bax and Bak and protein expression of estrogen and androgen receptors. Densitometric analysis were normalized with β-actin (42 kDa).

Western blot results showed MCT4 (43 kDa), Cyclin D1 (33 kDa), Bax (23 kDa), Bak (30 kDa), ERα (66 kDa), ERβ (56 kDa) and AR (99 kDa) both in control and treated cells (Fig. 3a).

The densitometric analysis revealed higher levels of MCT4 and Cyclin D1 proteins in E2 treated cells (Figs. 3b, c) compared to DBP treated and control cells. Treatment with DBP significantly enhanced both Bax (Fig. 3d) and Bak (Fig. 3e) protein expressions, instead treatment with E2 significantly decreased Bax expression (Fig. 3d) and didn't interfere with Bak expression (Fig. 3e).

ERα protein expression was drastically reduced by DBP treatment (Fig. 3f), in contrast E2 strongly increased its expression (Fig. 3f). Regarding to ERβ and AR only the treatment with E2 induced a significant increase of their expressions (Figs. 3g, h).

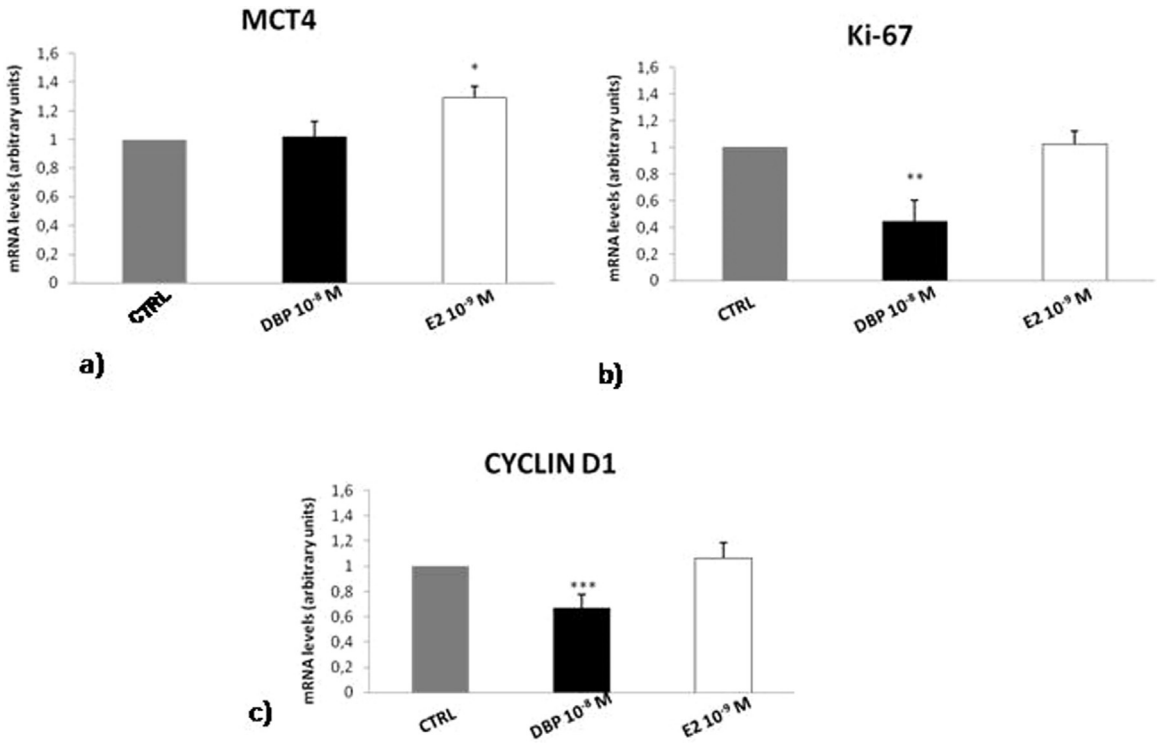

Fig. 2. qPCR analysis after 24 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β- estradiol (E2) 10^{-9} M. RT-qPCR was performed to evaluate expression of genes involved in cell cycle regulation such as MCT4, Ki67 and Cyclin D1. To note the different actions on gene expression of DBP and E2 (a, b,c). (*$p < 0,05$; ** $p < 0,01$; ***$p < 0,001$).

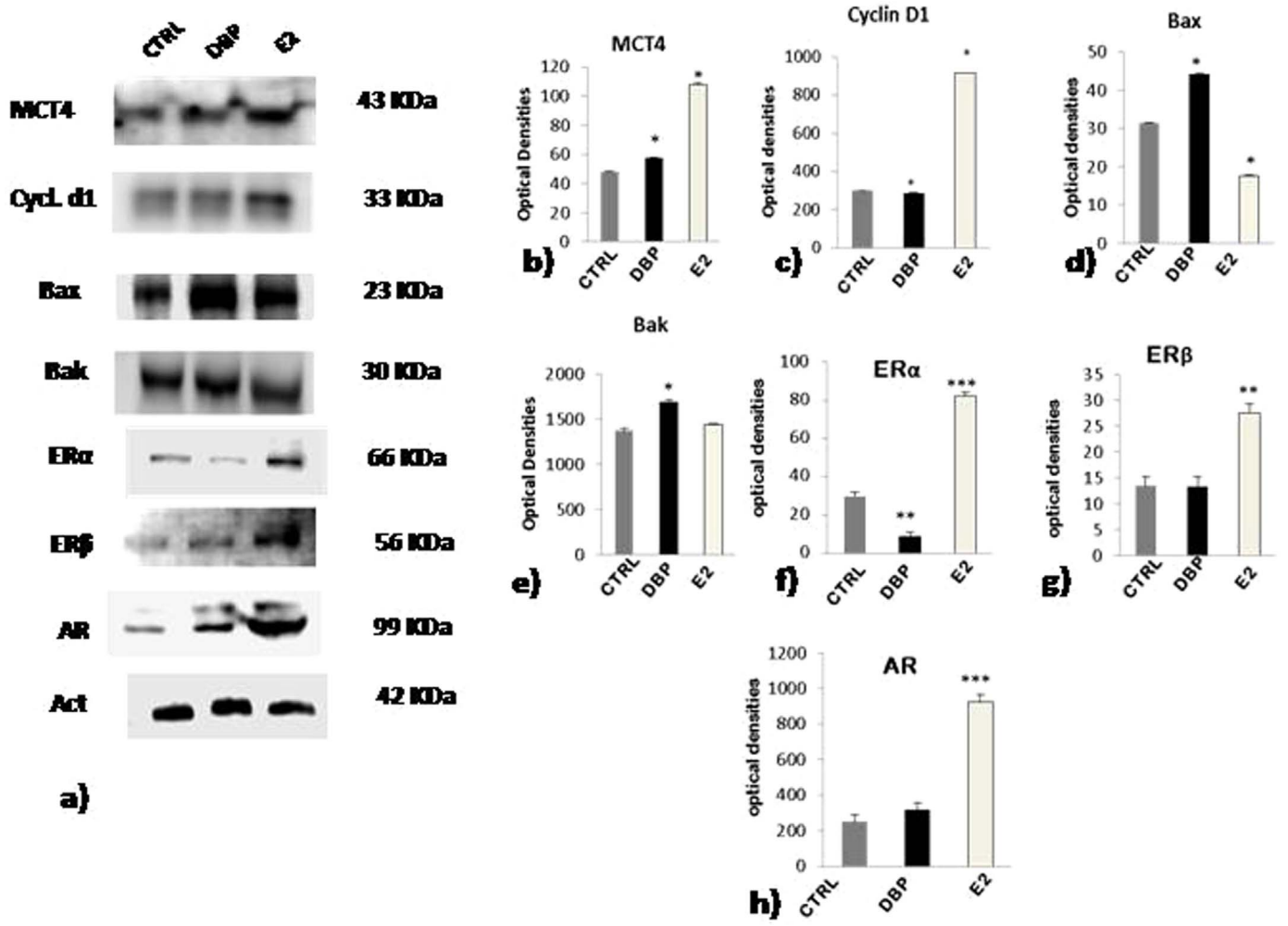

Fig. 3. Western blot analysis after 24 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β- estradiol (E2) 10^{-9} M. The graphs represented the optical density (O.D.) ratio of MCT4 (b), cyclin D1 (c), Bax (d), Bak (e), ERα (f), ERβ (g), AR (h) normalized on β actin. Look at the text for more details. (*$p < 0,05$; ** $p < 0,01$; ***$p < 0,001$).

3.4. Immunofluorescence

In order to investigate a possible interaction among DBP with estrogen and androgen receptors in the cells, we performed immunofluorescence after 30 min, 2 h and 4 h of exposure to DBP 10^{-8} M and E2 10^{-9}M.

3.4.1. Localization of ERα

After 30 min of treatment, control cells and treated with DBP and E2 showed ERα in the cytoplasm. After 2 h DBP did not affect ERα localization, that was localized in the cytoplasm as in control cells. ERα shifted from cytoplasm to nucleus after 4 h of treatment with DBP. On the contrary, both after 2 h and 4 h, E2 induced ERα translocation from the cytoplasm to the nucleus that appeared to be strongly positive (Fig. 4).

3.4.2. Localization of ERβ

DBP did not affect ERβ localization at any time of exposure: ERβ was localized in the cytoplasm of LNCaP cells with no fluorescent signal in cell nuclei as in control cells. Instead E2 translocated ERβ from the cytoplasm to the nucleus after 4 h (Fig. 5).

3.4.3. Localization of AR

AR localization was also investigated and data showed that DBP did not interfere with AR localization which was perinuclear in both control and treated cells after 30′, 2 h and 4 h of exposure. Only after 4 h of treatment E2, AR translocated from the cytoplasm to the nucleus (Fig. 6).

4. Discussion

Prostate gland plays a key role in male fertility. Its main function is to produce secretion (20–30% of the total ejaculation) that provides essential components for sperm quality and survival. Androgens have a significant function in prostate development and differentiation. Also estrogens have been demonstrated to have direct effects on prostate gland development and adult homeostasis but small changes in their levels might play a role in the etiology of prostatic diseases (McPherson et al., 2008; Prins, Korach, 2008). Many epidemiological studies reveal that chronic or intermittent exposure to different classes of EDCs may affect the development and progression of prostate disorders (Van Maele-Fabry et al., 2006).

Hence, the aim of this study is to investigate the effects of dibutylphthalate (DBP) on adenocarcinoma prostate cells (LNCaP); to highlight a possible xenoestrogenic effects on this cell line. The same experiments have been performed also with the estrogen endogenous 17 β-estradiol (E2) in order to compare the effects of these compounds. First of all, we studied the effects of DBP and E2 on cellular viability and data obtained showed a reduced cellular viability with DBP 10^{-8} M; on the contrary E2 at 10^{-9} M stimulated cellular viability of prostate cell line. Particularly interesting is the reduction of cellular viability obtained at a low concentration of DBP, according to Hrubá

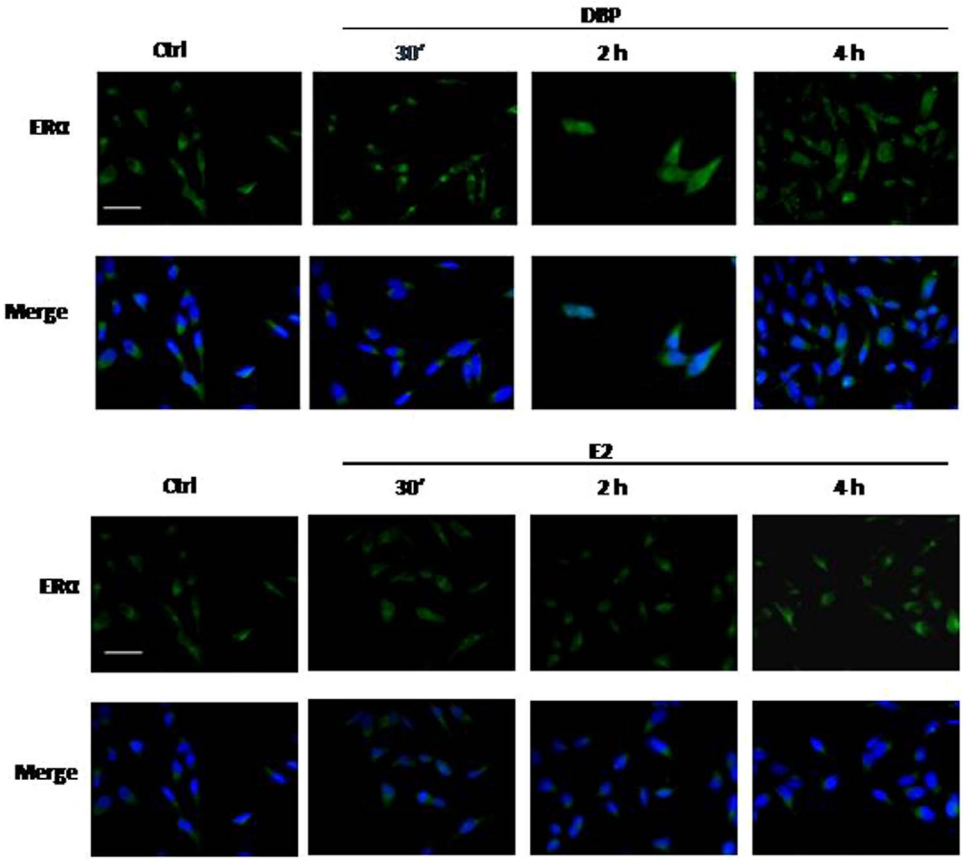

Fig. 4. ERα localization after 30′, 2 h and 4 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β-estradiol (E2) 10^{-9} M. ERα appears to be localized in the cytoplasm in control cells. DBP induced a cytoplasm-nuclear translocation after 4 h of exposure. E2 switch cytoplasm-nucleus after 2 h and 4 h. (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10 μm.

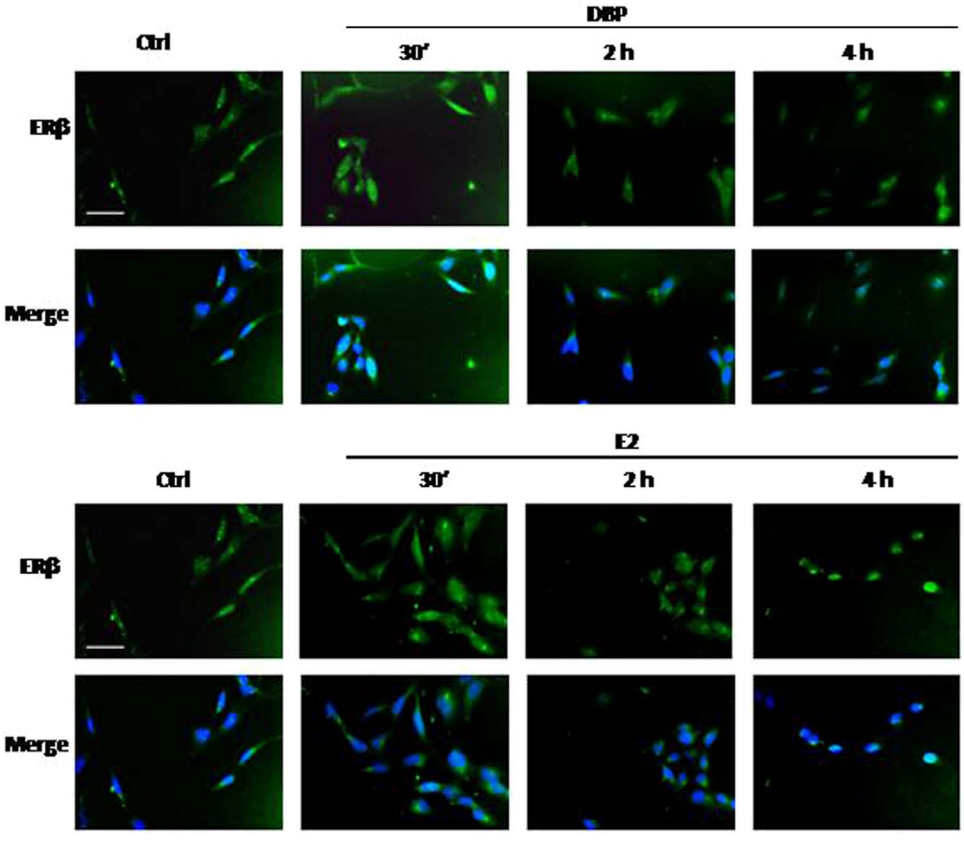

Fig. 5. ERβ localization after 30′, 2 h and 4 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β-estradiol (E2) 10^{-9} M. ERβ appears to be localized in the cytoplasm in control cells. DBP did not affects ERβ localization. E2 induced a cytoplasm-nucleus translocation only after 4 h. (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10 μm.

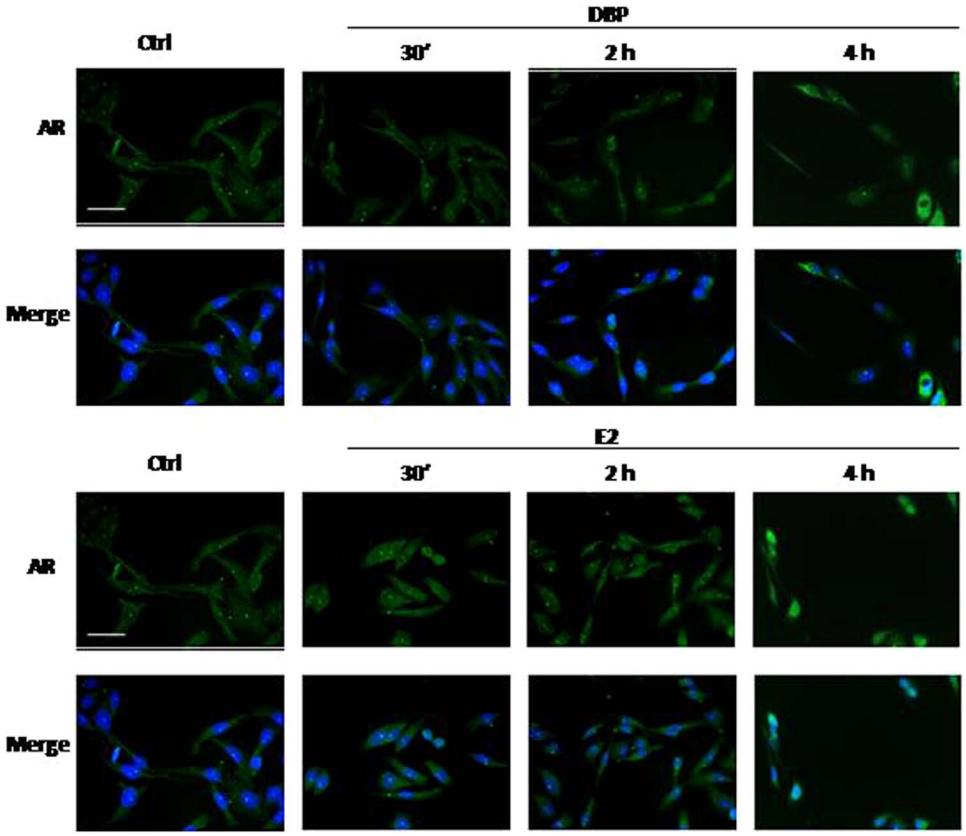

Fig. 6. AR localization after 30′, 2 h and 4 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β-estradiol (E2) 10^{-9} M. DBP did not affects AR localization. E2 induced AR cytoplasm-nucleus translocation only after 4 h. (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10 μm.

et al. (2014) that showed DBP inhibition of LNCaP cell proliferation (Hrubá et al., 2014). These data are in agreement with two common EDC features: the biological effect at low doses and the non monotonic trend of the dose-response curve (Vandenberg et al., 2012). In this view, Lee et al. (2014) have reported a positive influence of DBP on LNCaP proliferation, but the authors have tested high concentrations (10^{-5} and 10^{-6} M) and more prolonged exposure times (Lee et al., 2014). This apparent discrepancy is not surprising since EDCs such DBP may exert opposite effects at different concentrations and exposure times.

Then, in order to evaluate expression of genes and proteins involved in cellular proliferation and in cell cycle regulation, after 24 h of treatment with DBP 10^{-8} M and E2 10^{-9} M, we performed two different approaches: qPCR and western blot analysis. DBP treatment did not interfere with MCT4 gene and protein expression, instead E2 enhanced both of them. MCT4 belongs to the family of the monocarboxylate transporter and it is thought to be involved in the cellular efflux of lactic acid/H^+ (Dimmer et al., 2000); highly MCT4 expression has been associated in cancer progression by promoting several oncogenic processes (Sanità et al., 2014). Gene expression of Ki67, a well known marker of cell proliferation, is decreased after treatment with DBP but not after E2 treatment. Some studies have shown that estrogens might play a decisive role in some processes such as the development of prostate cancer (Bosland, 2000; Griffiths, 2000; Henderson, Feigelson, 2000; Lee et al., 2014; Susa et al., 2015). DBP was able to decrease gene and protein expression of cyclin D1, on the contrary E2 induced a strongly increase of cyclin D1 protein expression. Cyclin D1 is an estrogen response target and it promotes G1/S phase transition of cell cycle (Kastan, Bartek, 2004). These results are all in agreement with the showed reduced proliferation caused by DBP and promoted by E2.

To better understand trough which pathway DBP induced a decreased cell viability, we evaluated protein expression of two different pro-apoptotic proteins involved in intrinsic apoptosis pathway: Bax and Bak. DBP, contrarily to E2, strongly enhanced their expression, suggesting a DBP involvement in programmed cell death processes.

Moreover, to assess estrogen (ER) and androgen (AR) receptors participation, we evaluated the expression of ERs and AR with western blot technique. We showed a reduced expression of ERα after treatment with DBP and a significant increase of its expression after E2 treatment. It has been demonstrated that ERα appears to be involved in cellular proliferation and carcinogenesis of prostate (Prins, Korach, 2008), hence our results suggest that the anti-proliferative effects of DBP. Furthermore, DBP did not interfere with ERβ and AR expression, instead E2 increased the expression of both of them. The E2 action on both ERs and AR expression is in agreement with Susa et al. (2015) that showed E2 involvement in the activation of AR pathway (Susa et al., 2015).

Finally, we studied ERα, ERβ and AR localization after, 30 min, 2 h and 4 h of treatment. DBP induced ERα cytoplasm-nucleus translocation only after 4 h of treatment; conversely E2 affected ERα localization after 2 h and 4 h. ERα nuclear translocation is linked to its activation and it was not surprising that E2 had highest effects than DBP because of its best binding affinity with ERs (Laws, 2000).

DBP did not interfere with ERβ and AR localization indicating that its effects on LNCaP cells are not linked with AR interaction as also reported by Hrubá et al. (2014). On the contrary E2 was able to induce ERβ and surprisingly also AR cytoplasm-nucleus translocation after 4 h. It has been demonstrated that AR might be activated by other steroid hormones and E2 shows affinity for its LBD domain so it can be able to activate transcription of AR target genes (Taplin et al., 1995; Yeah et. al., 1998; Susa et al., 2015).

5. Conclusions

In conclusion, we demonstrated that DBP acts on LNCaP cells through the activation of ERα pathway. Moreover, DBP exerts different effects than E2. We showed that DBP reduced cell viability probably

activating molecular pathway involved in the programmed cell death processes as suggested by the obtained strong increase of both Bax and Bak protein expression. Phthalates are only one of the component of the mixture of EDCs which human population is non-stop exposed. Thus, it is very important to compare the effects of environmental compounds with anti androgenic and anti estrogenic properties in order to explore the crosstalk between different hormonal signaling pathways.

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DIETHYLHEXYL PHTHALATE AFFECTS TESTICULAR HISTOPATHOLOGY IN NEONATAL RATS.

Abstract

Phthalates are synthetic chemicals widely used as plasticizers since they provide flexibility in plastics. Diethylhexyl phthalate (DEHP) is commonly used in a wide range of consumer products.
Several studies have shown that DEHP exposure can lead to serious anomalies for male reproductive tract like cryptorchidism, hypospadias, impaired spermatogenesis and reduced fertility.
In this study we examined the effects of DEHP on neonatal testicular histopathology at PND 6. Pregnant Wistar rats were gavaged from gestation day (GD) 7 to GD 21 and from postnatal day (PND) 1 to 6 with vehicle, 30, 300, or 900 mg/kg bw/day DEHP. Histopathological investigations revealed alterations in testis morphology at all doses tested. At the highest DEHP doses, gonocytes appeared to be enlarged and multinucleated, diameter of cords were reduced and it was possible to find malformed cords. Interestingly, effects on Leydig cells were seen at lower doses than effects on gonocytes, and Sertoli cells were not affected by the treatment at this stage. DEHP treatment did not interfere with proliferation/apoptosis, but changes in PPARγ expression indicated possible involvement in the observed effects.

Key words: phthalate, rat, testis, histopathology

1. Introduction

Human population is steadily exposed through general environmental contamination or direct contact to several different compounds with endocrine disrupting properties.
An endocrine disrupting chemical (EDC) has been described as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"[1,2]. More than 200 compounds are considered to be EDCs, such as pesticides, herbicides, metals, lubricants and solvents, natural plant metabolites, and plasticizers [3-9] and exposure to most of them during development is suggested to contribute to different reproductive tract disorders [10].
Among EDCs, phthalates are an important group of multifunctional and environmental chemicals widely used as plasticizers and solvents in many different applications [11,12]. Phthalate esters are ubiquitous in our surrounding environment and because they are not covalently bound to the polymer, they can easily be available for biological exposure [13-15]. Human exposure can occur by absorption, inhalation, dermal exposure and ingestion which is the major source of exposure [16-18]; moreover, maternal exposure represents the first source of fetal exposure to phthalates [19,20].
Di-(2-ethylhexyl)phthalate (DEHP) is one of the most found phthalate in the environment [21] and it is commonly used to convey flexibility and transparency to numerous consumer products [22].
DEHP is well known for being toxicant for the male reproductive system [23,24] and it exerts antiandrogenic effects by suppressing fetal testosterone biosynthesis which in turn led to male reproductive tract anomalies [25-27]. The wide range of DEHP effects is characterized by disrupted

androgen dependent development and increased incidence of reduced anogenital distance (AGD) cryptorchidism, hypospadias, impaired spermatogenesis and testicular cancer, which jointly comprise the testicular dysgenesis syndrome (TDS) [22,28]. The TDS hypothesis proposes that an *in utero* insult to testicular development may result in failure to develop normal Leydig and Sertoli cell function during male sexual differentiation with adverse consequences for male reproductive health [29].

DEHP effects depend on developmental stage of organism at the time of exposure [30]. Recent published studies reveal that at low dose of 10mg/kg-d DEHP caused adverse anti-androgenic effects on male rat development such as reduced anogenital distance, increased nipple retention, reduced weight of levator ani and bulbocavernosus muscles (LABC) and mild dysgenesis of external genitalia; instead with higher doses it was possible to note histopathological effects on juvenile testis, reduced testicular and prostate weight with associated reduction in the expression of androgen-regulated genes [31]. Hence in this study we evaluated the effects of different doses of DEHP on testicular histopathology during neonatal period after gestation and lactation exposure. These periods of exposure are more sensitive than adulthood and young animals have been found to be more sensitive to DEHP than adult rats [32].

2. Materials and methods

2.1 Chemicals

DEHP (di(2-ethylhexyl)phthalate), CAS No. 117-81-7, purity 99% was obtained from Sigma-Aldrich 20, 115-4. DEHP was dissolved in corn oil, used as vehicle and obtained from the Royal Veterinary Agriculture Pharmacy, Copenhagen, Denmark.

2.2 Animals and treatment

Sixty-four time-mated, young adult Wistar rats, with a body weight approximately around 200g (Han-Tac: WH, Taconic M&B, Denmark), were supplied at day 3 of pregnancy. The day following mating was designated gestational day (GD) 1, and postnatal (PND) 1 was the day of birth. Upon arrival, the dams were randomly distributed in pairs and housed under standard conditions: semitransparent plastic cages (15 cm x 27 cmx 43 cm) with Aspen bedding (Tapvei), situated in an animal room with controlled environmental conditions (12 h light- dark cycles with light starting at 9 p.m., light intensity 500 lux, temperature 21±2°C, humidity 50±5 %, ventilation 8 air changes/h). All dams were provided with a complete rodent diet for growing animals (Altromin Standard Diet 1314) and acidified tap water *ad libitum*. Before starting the treatment, an acclimatization period of 4 days was allowed and the animals were weighted every day to calculate the dosing volume of 2ml/kg bw; then from GD 7 to GD 21 (day before expected birth) and from PND 1 until PND 6 they were gavaged with vehicle (corn oil) or 30, 300, 900 mg DEHP/ kg bw/day. Animals were inspected for general toxicity twice daily. On PND 6, pups were anesthetized in CO_2/O_2 and decapitated and testes were removed for histopathological investigation.

2.3 Haematoxylin and eosin staining

One or two testes per litter were placed in Bouin's fixative, and one testis per litter was placed in neutral buffered formaldehyde for histopathology and immunohistochemistry. Sections from testes fixed in Bouin's fixative were paraffin embedded and stained with haematoxylin and eosin (alternately right and left testis). In all dose groups, Bouin's fixed testes were evaluated for presence or absence of multinucleated gonocytes, small or large Leydig cell clusters and malformation of cords. In testes from all dose groups, cord diameters were investigated by measuring the diameter of tubular cross sections perpendicular to the tubular length direction. In each tissue section of testes of optimal quality, all cords were measured as previously described [33] using a computer assisted microscope (Leica DMR).

2.4 Immunohistochemistry

In all dose groups immunofluorescence for 3-β-hydroxysteroid dehydrogenase (3-β-HSD) was performed. Before starting, slides with paraffin sections were incubated for 30 min at 60°C and then deparaffinized. After microwave treatment in appropriate buffer, slides were washed in PBS 3x5 min and blocked with 5% BSA in PBS for 30 min at room temperature. Then slides were incubated with primary antibody overnight at 4° C. Primary antibody used was 3-β-HSD (sc-30820, Santa Cruz Biotechnology), diluted 1:200 in 1% BSA in PBS. After washing in PBS, slides were incubated with donkey anti-goat GFP 488 (A11055, Molecular Probes) diluted 1:500 in 1% BSA in PBS for 1h at room temperature. This was followed by 30 min of incubation with Sudan black 0,1% and after washing in PBS, slides were counterstained with DAPI diluted 1:1000 in PBS for 3 min. Slides were then washed in PBS and mounted with ProLong Gold AntifadeMountant (P10144, ThermoFisher Scientific).

In all dose groups double immunostaining for α-smooth muscle actin (SMA) and 3-β-HSD was performed as previously described [34]. Immunostaining for ki-67, androgen receptor (AR) and PPARγ were performed only in 0 and 900 mg DEHP/kg/bw/day groups. Immunohistochemistry was performed on one section per testis and except for microwave pre-treatment and secondary antibodies, the staining was performed using comparable protocols for all antibodies. Following pre-treatment, sections were blocked for endogenous peroxidase activity in 3% H_2O_2 in PBS for 10 min and blocked for 30 min in 1% BSA in PBS. Then sections were incubated overnight at 4°C with the following antibodies: α-smooth muscle actin (SMA) 1:100 (clone 1A4, DAKO) ki-67 1:100 (NCL MM1, Leica), androgen receptor (AR) 1:200 (N20SC816P, Santa Cruz Biotechnology), and PPARγ 1:100 (Cell Signaling, Beverly, MA). Sections were then incubated for 30 min with secondary antibody anti-mouse Envision+ (DAKO) for SMA and ki-67 and anti-rabbit Envision+ (DAKO) for 3-β-HSD, AR and PPARγ. Finally, sections were stained in diaminobenzidine (DAB+Substrate Chromogen System, DAKO) and counterstained in Meyer's haematoxylin.

2.6 TUNEL

In order to study the apoptosis, TUNEL was carried out in 0 and 900 mg DEHP/kg/bw/day groups using ApoTag-peroxidase kit (Cat.No. S7100, Intergen Co, NY) following the supplier's guidelines. Deparaffinized sections were microwaved in Citrate Buffer (pH 6) 2x5min at 99°C. Sections were

rinsed in PBS and then blocked for endogenous peroxidase activity in 3% H_2O_2 in PBS for 10 min at room temperature. After a second rinse, equilibration buffer was added for 30 min at room temperature, removed and TdT enzyme diluted in reaction buffer was added and incubated for 1h at 37°C. Sections were then washed in Stop Wash Buffer for 10 min at room temperature, rinsed, dried and incubated with Anti Digoxigenin Peroxidase for 30 min at room temperature. Sections were stained in diaminobenzidine (DAB+Substrate Chromogen System, DAKO) and counterstained in Meyer's haematoxylin.

2.7 Image capture

Nonfluorescent images were examined and photographed using a LEICA DMR microscope fitted with Leica DFC295 Digital Camera.
Fluorescent images were captured using Axioshop (Carl Zeiss) epifluorescente microscope fitted with Axiocam MRc5.
Images were compiled using Photoshop 7.0 (Adobe System Inc., Mountain View, CA, USA).

2.8 Statistical Analysis

Data showed in graphs are expressed as means ± SD for the indicated number of independent determinations. The statistical significance was calculated by the one way ANOVA with Dunnett's multiple comparison test and differences were considered statistically significant when the P values was at least $p< 0.05$.

3. Results

3.1 Testicular histopathology

Testicular histopathology was examined in one or two testis per litter from PND 6 rats exposed to DEHP 0, 30, 300, 900mg/kg bw/d from GD 7 to GD 21 and from PND 1 to PND 6. After DEHP treatment, especially with the higher dose, gonocytes appear to be enlarged and multinucleated (**Table 1**). Changes in the distribution of Leydig cells were clearly visible in all treated groups when compared to control group. After exposure to ≥ 30 mg/kg of DEHP Leydig cells tended to group together abnormally. Sertoli cells are not significant affected by the treatment (**Fig.1,Table 1**).Cord diameters were also investigated and results showed that cords had a reduced diameter in all the exposed rats at all doses tested (**Fig.2**).

3.2 Immunohistochemistry

Immunofluorescence for 3β-HSD revealed that Leydig cells tended to group together in clusters dose dependently from DEHP 30 mg/kg and with DEHP 900 mg/kg Leydig cell clusters were particularly large and tendend to be centrally located in the testes with corresponding decrease in the number of small Leydig clusters (**Fig.1**).

Immunohistological double staining for 3β-HSD and SMA revealed that SMA staining was stronger in peritubular cells of control group. Treated groups showed a lower SMA staining especially in the area neighboring Leydig cell clusters. After high dose DEHP treatment it was also possible to see

malformed cords with 3β HSD positive Leydig cells inside the tubules (**Fig.1** and **Table 1**). Only few positive ki-67 gonocytes were found both in control and treated groups (**Fig.3**). AR staining was similar among groups and was strongly detected in peritubular myiod cells and Leydig cells and also a weak presence was detected in Sertoli cells nuclei (**Fig.3**). PPARγ immunostaining was seen in Leydig cells cytoplasm and nuclei of control cells. Testes from the high dose DEHP exposed group showed much weaker PPARγ staining and it was mainly located in Leydig cells cytoplasm and almost absent in the nuclei especially in Leydig cell clusters (**Fig.3**).

3.4 Tunel

No positive TUNEL cells were seen in control or DEHP exposed groups (**Fig.3**).

4.Discussion

In recent decades, higher incidences of male reproductive disorders such as cryptorchidism, hypospadias, poor semen quality and impaired fertility have been associated with exposure to EDCs during perinatal life [22,35].

Our first interesting finding regard Leydig cells which are not uniformly distributed in the interstitial space but are found in clusters. Leydig cell clusters become notable after low dose DEHP (30mg/kg-d) and become enlarged after high dose treatment. It has already been demonstrated that DEHP 300mg/kg/bw/d promotes fetal Leydig cells aggregation [36]. Moreover, this phenomenon has not been observed in adult Leydig cells after postnatal exposure [26, 37-39]. Phthalates mode of action on Leydig cells is thought to be dose and age dependent and results from affected testosterone production and signals from other testicular cells, including myoid cells and Sertoli cells [40,41]. In high doses exposed rats, Leydig cells positively stained for 3βHSD were also found inside the cords and the presence of dysgenetic areas characterized as malformed tubules was also detected in DEHP exposed rats at PND 22 and 190 [42] but not at GD21 [34]. The dysgenetic cords develop in late gestation and may indicated malfunction of androgen dependent peritubular cells and in the current study testis treated with high DEHP showed weaker SMA staining than normal.

Gonocytes were enlarged and multinucleated only after high dose DEHP treatment and these results are in agreement with data about DEHP effects in fetal rat testis [36]. Moreover, neonatal Sertoli cells were not affected from DEHP treatment, and it is proposed that Sertoli cells changes are mainly seen after exposure during adulthood [43].The diameter of the cords was decreased dose-dependently from 30mg/kg/d and this reduction in testis size was also seen in previous studies at PND 16 [31] and PND 22 [25]. Testis size is associated with Sertoli cells number and function so in the reported studies serum inhibin B levels were investigated. Inhibin B is a well-known marker of Sertoli cells function and number and results showed that levels were reduced after higher DEHP doses at PND 22 [25] but not at PND 16 [31] indicating that effects on Sertoli cells are seen at later time of development.

To better understand if DEHP induces gonocytes proliferation and if Leydig cell clusters are due to hyperplasia we performed immunohistochemistry analysis for ki-67, a specific marker of proliferation and very low incidences of positive cells were seen. DEHP treatment did not induce proliferation indicating that at this stage proliferation is not a significant consequence of DEHP treatment. Leydig cells aggregation in clusters but not their increase in number was also found in

fetal testis of DBP exposed rats [44]. Apoptosis was not induced by treatment either at this stage, as only few TUNEL positive cells were seen. However, DEHP may induce apoptosis at later stage of testis development as supported by several studies [40,45,46] and may be related with observed poor Sertoli cells number and function [47].

Some of the antiandrogenic effects seen after pre or perinatal exposure of rats to phthalates are likely caused by a reduction in testosterone production seen in late gestation [25]. Normally testosterone levels in male rats increase from GD 17 to GD 20 [26] and DEHP and many other phthalates have been found to reduces testosterone levels between GD 17 and PND 2 [25-27]. The mechanisms by which phthalates exert their effects are not yet fully elucidated and to understand a potential mechanism for their impairing testis development, we performed AR and PPARγ immunostaining. Treatment with DEHP did not cause AR mislocalization indicating that DEHP is not AR antagonist [26]. One possible mechanism of phthalate-mediated toxicity is binding to peroxisome proliferator-activated receptors (PPARs). The PPAR family contains three subtypes PPARα, PPARβ, PPARγ encoded by different genes [38, 48]. PPARγ regulates genes involved in cholesterol uptake and transport which is essential for testosterone production. In our study, DEHP at the high dose reduces PPARγ staining in Leydig cells and it is predominatly located only in Leydig cells cytoplasm. Similar results were observed in fetal rat testis after *in utero* exposure to DEHP [36] and may support the idea of PPARs-mediated effects of phthalates impairing testis development [49-51]. Interference with the aryl hydrocarbon receptor is another signalling pathway that might be affected by phthalates [52], and these findings warrant further studies.

5. Conclusions

In summary, DEHP impairs testis development in a way dependent on age and dosing. During neonatal period profound effects on Leydig cells were seen at lower doses of DEHP than effects seen on gonocytes. DEHP effects may be modulated by PPARγ. Phthalate effects on developing testes causes concern that human testis may also be affected, thus leading to TDS postnatally.

Fig. 1

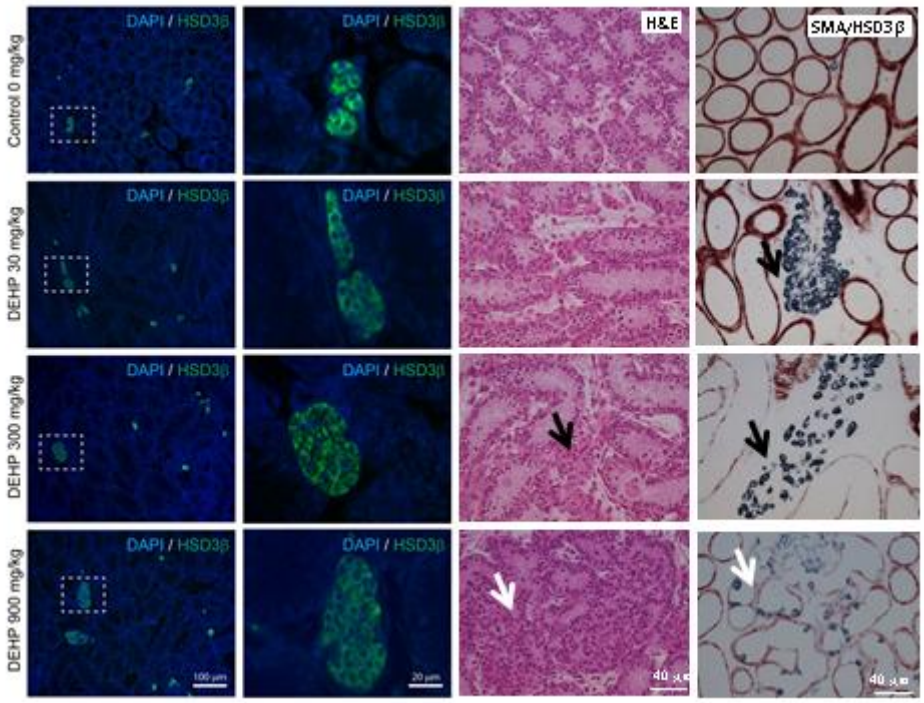

Table 1

	CTRL	Dehp 30mg/kg	Dehp 300mg/kg	Dehp 900mg/kg
Multinucleated gonocytes	12,5%	83,3%	83,3%	100%
Small Leydig cell clusters	12,5%	83,3%	25%	25%
Large Leydig cell clusters	0%	16,6%	75%	75%
Malformed chords	0%	0%	0%	50%

Fig. 2

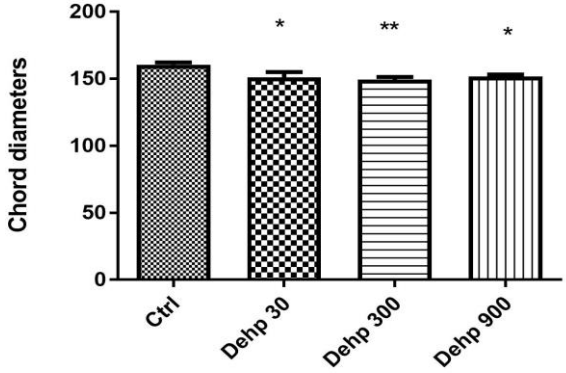
Chord diameters
200
150
100
50
0
*
**
*
Ctrl
Dehp 30
Dehp 300
Dehp 900

Fig. 3

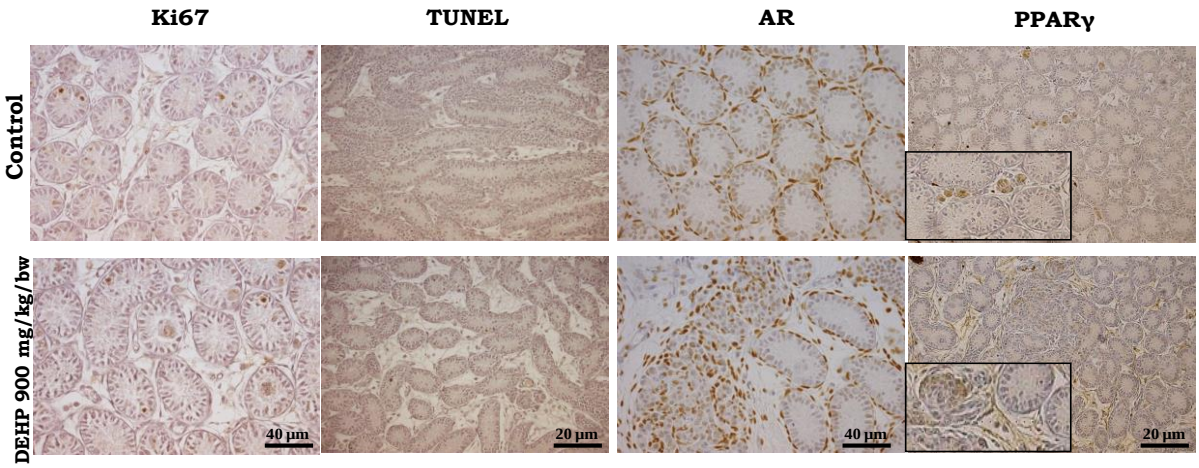
Ki67
TUNEL
AR
PPARγ
Control
DEHP 900 mg/kg/bw
40 μm
20 μm
40 μm
20 μm

Figure capitations

Fig.1: Haematoxylin and eosin staining, immunofluorescence analysis for 3βHSD (specific Leydig cells marker) and double-immunohistochemical staining for SMA (specific marker for peritubular and perivascular cells) and 3βHSD (specific Leydig cells marker) in male rats exposed to 0, 30, 300 or 900 mg DEHP/kg bw/day from GD 7 to GD 21 and from PND 1 to PND 6. Testis of DEHP exposed animals present large Leydig cell clusters and in high dose exposed group note malformed cords.

Table 1: Testicular histopathology at PND 6 in male rat exposed to 0, 30, 300 or 900 mg DEHP/kg bw/day from GD 7 to GD 21 and from PND 1 to PND 6. Percentage of affected males of the total number of males when evaluating one section for testis.

Fig.2: Cord diameters at PND 6 in male rat exposed to 0, 30, 300 or 900 mg DEHP/kg bw/day from GD 7 to GD 21 and from PND 1 to PND 6. DEHP treatment reduces cords diameter in a dose dependent manner. Values are means ± SD. (* *p<0,05*; ** *p<0,01)*

Fig.3: Immunoistochemical staining for ki67 (specific marker of proliferation), TUNEL, immunoistochemical staining for AR (androgen receptor) and PPARγ in male rats exposed to 0 or 900 mg DEHP/kg bw/day from GD 7 to GD 21 and from PND 1 to PND 6. Only few positive ki-67 cells and no apoptotic cells are detected in control or exposed rat group. AR staining detected in peritubular myiod cells and Leydig cells and a weak presence was also detected in Sertoli cells nuclei but no differences between controls and treated groups. PPARγ staining in Leydig cells reduce after DEHP treatment.

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4. Other papers not directly related to PhD project

4.1 Published papers

Nonylphenol effects on human prostate non tumorigenic cells.

M. Forte, M. Di Lorenzo, A. Carrizzo, S. Valiante, C. Vecchione, V. Laforgia, M. De Falco, Toxicology 2016.

HSP90 and pCREB alterations are linked to mancozeb-dependent behavioral and neurodegenerative effects in a marine teleost.

M. Zizza, M. Di Lorenzo, V. Laforgia, E. Furia. G. Sindona, M. Canonaco, R.M. Facciolo, TAAP 2017.

4.2 Submitted papers

Orexin receptor expression is increate during mancozeb-induced feeding impairments and neurodegenerative events in a marine fish.

M. Zizza, M. Di Lorenzo, V. Laforgia, E. Furia. G. Sindona, M. Canonaco, R.M. Facciolo, Neurotoxicology 2017.

Molecular mechanisms of Endocrine Disruptors: interference with endocrine system activity.

M. De Falco, M. Di Lorenzo, V. Laforgia, International book on Endocrine Distruptors, 2017.

4.3 Manuscript in preparation

Nonylphenol acts on prostate cells via estrogen molecular pathway.

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Nonylphenol effects on human prostate non tumorigenic cells

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ABSTRACT

Nonylphenol (NP) is an industrial chemical with estrogenic activity both *in vivo* and *in vitro*; estrogens play a critical role in the development of prostate and may be the cause of some pathological states, including cancer. In this study we examined the effects of NP on human prostate non tumorigenic epithelial cells (PNT1A) investigating on cell proliferation, interaction with estrogen receptors (ERs) and gene expression of genes involved in prostate diseases. We found that NP affects cell proliferation at 10^{-6} M, promoting a cytoplasm-nucleus translocation of ERα and not ERβ, like the natural estrogen 17β-estradiol (E2). Moreover, we showed that NP enhances gene expression of key regulators of cell cycle. Estrogen selective antagonist ICI182780 in part reverted the observed effects of NP. These results confirm the estrogenic activity of NP and suggest that other transduction pathways may be involved in NP action on prostate.

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1. Introduction

Nonylphenol (NP) is generated by the degradation of nonylphenol ethoxilates (NPEs). NPEs are chemicals widely used as non ionic surfactants in the manufacture of rubber and plastic for domestic, industrial and agricultural products (Fiege et al., 2000; Langford, 2002; Vazquez-Duhalt et al., 2005). Due to its high hydrophobicity and low solubility NP accumulates in several environmental matrices, such as seas, rivers, soils, groundwaters and sediments, in a range between 10^{-13} to 10^{-6} M (Berryman et al., 2004; Careghini et al., 2015; Vazquez-Duhalt et al., 2005). It was also found as a contaminant of food and drinking water (Gyllenhammar et al., 2012; Maggioni et al., 2013; Soares et al., 2008). Human exposure to NP may occur by inhalation, cutaneous absorption and ingestion of contaminated food or water (Guenther et al., 2002; Soto et al., 1991). In this regard, NP was found in human amniotic fluid, urine and plasma samples, breast milk, fetal cord serum, placenta and maternal blood, with levels in these tissues generally varying from 10^{-10} to 10^{-9} M (Calafat et al., 2005; Huang et al., 2014). Hovewer, in some cases, concentrations of NP have been reported to be much higher in human samples. In this regards in breast milk of healthy Italian women, Ademollo et al. (Ademollo et al., 2008) detected about 10^{-7} M of NP as well as in urine and in plasma of textile and housekeeping workers were found the same NP levels (Chen et al., 2005). Instead, in maternal cord blood, Chen et al. (Chen et al., 2008) found a concentration of NP of about 10^{-6} M.

NP belongs to the subclass of endocrine disrupting chemicals (EDCs) that mimic the endogenous estrogens, called xenoestrogens (Falconer et al., 2006; Wozniak et al., 2005), that also includes dioxins, polychlorinated biphenyls, hexachlorocyclohexane, octylphenol and bisphenol A (Kuo et al., 2012; Forte et al., 2016). Estrogenic activity of NP has been reported both *in vitro* (de Weert et al., 2008; Soto et al., 1991; White et al., 1994) and *in vivo*, in reproductive and in non reproductive tissues, such as brain (Blom et al., 1998; Laws et al., 2000; Nagel et al., 1999; ter Veld et al., 2008; Xia et al., 2008; De Falco et al., 2014, 2015) and it has been shown that NP interacts with estrogen receptors (ERs), competing with the natural estrogen 17β-estradiol (E2) (Bechi et al., 2006; Kwack et al., 2002; White et al., 1994), although with less specificity (Bechi et al., 2010; Blom et al., 1998; Nagel et al., 1999).

Estrogens predominantly bind two nuclear receptors: the estrogen receptor alpha (ERα) and the estrogen receptor beta

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(ERβ). Both ERα and ERβ bind to the active form of estrogen E2, with similar affinities (Siewit et al., 2010). ERs mediated estrogen signaling in reproductive tissues but also in non-reproductive tissues as the brain, lungs, colon, prostate and cardiovascular system (Shanle and Xu, 2011). In the cell, E2 is able to activate both genomic and non-genomic responses. In the genomic pathway, E2 mediates target gene regulation through binding directly DNA at estrogen response elements (EREs) or indirectly through transcription factors like Sp1 or AP-1 (Kushner et al., 2000; Saville et al., 2000); the non-genomic pathway is not mediated by ERα or ERβ but through the G-protein coupled receptor, GPR30, that localizes in the plasma membrane activating rapid responses such as increased levels of c-AMP (Filardo et al., 2007; Lin et al., 2009; Wang et al., 2010).

Several studies have suggested the role of estrogens in normal and aberrant growth of prostate, alone or in synergy with androgens (Ho et al., 2011) and epidemiological and experimental studies underline a relationship between estrogens/xenoestrogens and pathogenesis of prostate cancer (PCa) (Bostwick et al., 2004; Ho et al., 2006). Neonatal treatment with Bisphenol A (BPA), a well-known xenoestrogen, was reported to induce high-grade prostatic intraepithelial neoplasia in Sprague-Dawley rats (Ho et al., 2006) and to increase cell proliferation of urogenital sinus epithelium (UGE) in the primary prostatic ducts of CD1 mice (Timms et al., 2005). BPA was also found to increase the number of basal epithelial cells in the adult prostate of BALB/c mice (Ogura et al., 2007). Recently, Tarapore et al. (2014) found in prostate cancer patients high BPA urinary levels compared to non prostate cancer patients.

Despite the relationship between estrogen and prostate, the precise functions of the two ER subtypes in this gland remain unclear; several authors have reported differential expression patterns of the two receptors between the epithelial and stromal compartment of the prostate, with ERα localized predominantly in the stroma and ERβ in the epithelium (Fixemer et al., 2003; Leav et al., 2001; Tsurusaki et al., 2003; Weihua and Warner, 2002).

Considered this background and given the human exposure to EDCs, the estrogen-like action of NP is conceivable to influence the normal growth of prostate and to be the cause of some pathological states of this gland, affecting the male reproductive functions. Thus, in this study we evaluated the effects of NP on the proliferation of human non tumorigenic prostate cells (PNT1A), which is responsive to sex hormones (Stephen et al., 2004), the cellular localization of ERα and ERβ after exposure to NP and gene expression of genes involved in pathological states of the prostate such as *cyclin D*, *Ki67*, *p53* and *IL1-β*. We performed the same experiments treating cells with the natural estrogen E2 and with the selective antagonist of estrogen receptors ICI 182,780 (Osborne et al., 2004). This study aims to facilitate the understanding of the mechanisms by which xenoestrogens and estrogens may exert their activity on prostate.

2. Material and methods

2.1. Cell culture

PNT1A cells (a human prostate cell line established by immortalization of adult prostate epithelial cells) were obtained from the European Collection of Cell Culture (ECACC Salisbury, UK). PNT1A cells were grown in red phenol free RPMI-1640 medium (LONZA, Basel, Switzerland), supplemented with 10% dextran-coated charcoal fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 2 mM L-glutamine and antibiotics (100 U/mL penicillin/streptomycin, 10 μg/mL gentamicin) in a humidified incubator at 37 °C and 5% CO_2. When confluent, the cells were detached enzymatically with trypsin-ethylenediamine tetra-acetic acid and subcultured into a new cell culture flasks. The medium was replaced every 2 days. Cells were used for experiments between passages 5–20.

2.2. Chemicals

Nonylphenol (NP), 17β-Estradiol (E2) and selective estrogen antagonist ICI 182,780 (ICI) were purchased from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO) and were dissolved in DMSO (Invitrogen Carlsbad, CA). NP, E2 and ICI were diluted with culture medium at final concentrations from 10^{-12} to 10^{-6} M for NP and E2 and 10^{-5} M for ICI. In all the experiments with the inhibitor, ICI was added 1 h prior to start treatments. Final concentration of DMSO in the medium did not exceed 0.01%.

2.3. MTT assay

The effects of NP or E2 on PNT1A cells proliferation was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-3,5 diphenyl tetrazolium bromide (MTT) test (Sigma Aldrich, St. Louis, MO). Cells were seeded in 200 μL of growth medium (5×10^4 cells/well) in 96-well plates and hormone deprived (1% FBS) for 24 h. Then, NP or E2 was added after dilution to an appropriate concentration (from 10^{-12} M to 10^{-6} M), with or without 10^{-5} M ICI. Control cells were treated with vehicle (DMSO 0.01%). The test was performed for 24 h of incubation. After the incubation period, 10 μL of a MTT solution was added to each well. After 4 h of 37 °C incubation, the culture medium was gently aspirated and replaced by 100 μL of DMSO/isopropanol (1:1) in order to dissolve the formazan crystals. The absorbance of the solubilized dye, which correlates with the number of living cells, was measured with a microplate reader at 570 nm. The test was performed in triplicate.

2.4. Fluorescence microscopy

PNT1A cells were seeded in 4-well chamber slide (Sarstedt, Nürnbrecht, Germany) overnight at a density of 5×10^4/well. After 24 h serum starvation (1% FBS), cells were incubated with 10^{-6} M NP or 10^{-6} M E2, with or without 10^{-5} M ICI for four different times: 15 min, 1 h, 2 h and 6 h. Control group was treated only with vehicle (DMSO 0.01%). Control and treated cells were fixed with methanol for 10 min at RT, permeabilized with 0.25% Triton X-100 for 10 min, washed in PBS, and blocked in 5% normal goat serum (NGS) for 1 h at RT. Then cells were subjected to immunofluorescence protocol using a mouse monoclonal anti-human ERα (Santa Cruz Biotechnology, Santa Cruz, CA, Cat. sc-8005) and a mouse monoclonal anti- human ERβ antibodies (Santa Cruz, Cat. sc-373853), diluted 1:100 in 1% NGS for 24 h at 4 °C. For detection of ERα and ERβ, secondary goat anti-mouse Alex Fluor 488 (Cat. A11001, Invitrogen, Carlsbad, CA), dilution 1:200 in 1% NGS was used. Cell nuclei were counterstained with 0.1 μg/mL Höechst (Invitrogen, Carlsbad, CA, Cat. H3570). Negative control for ERα and ERβ was performed by avoiding incubation with the primary antibodies (Supplementary data Fig. S1). Fluorescent images were taken on an Axioskop (Carl Zeiss, Milano, Italy) epifluorescence microscope using a 40× objective. Axiocam MRc5 and the acquisition software Axiovision 4.7 (Carl Zeiss) were used to capture the images in different channels (Alexa Fluor 488, Höechst 33258). Three independent immunofluorescence experiments were performed for each experimental conditions and different fields were randomly chosen for data analysis. Then, images were processed with the Image J software (developed by Wayne Rasband, National Institutes of Health, USA).

2.5. Protein extraction and separation

Nuclear and cytoplasmatic proteins were extracted from PNT1A cells after two and six hours of treatment with 10^{-6} M NP, 10^{-6} M E2, with or without 10^{-5} M ICI. Control cells were treated with 0.01% of DMSO and western blot was performed for detection of ERα and ERβ. Different buffers were prepared to isolate cytoplasmic/membrane and nuclear proteins: harvest buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M sucurose, 0.1 mM EDTA, 0.5% Triton X-100 and freshly added 1 mM DTT, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 4 mg/mL Aprotinin and 2 mg/mL Pepstatin A), buffer A (10 mM HEPES pH 7–9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and freshly added 1 mM DTT, 1 mM PMSF, 4 mg/mL Aprotinin and 2 mg/mL Pepstatin A) and buffer B (10 mM HEPES pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA 0.1% NP-40 and freshly added 1 mM DTT, 1 mM PMSF, 4 mg/mL Aprotinin and 2 mg/mL Pepstatin A). 10 cm cell dishes with confluent PNT1A cells were placed on ice for 10 min and washed twice with ice cold PBS. Then 100 μL of PBS-EDTA 1 mM was added and cells were scraped and transferred to a microcetrifuge tube. The collected cells were then centrifuged at 3000 rpm for 5 min at 4 °C and then resuspended in cold harvest buffer, incubated on ice for 5 min and subsequently centrifuged at 1000 rpm for 10 min to pellet nuclei. Then the supernatant was transferred into a new tube and centrifuged at 14000 rpm for 15 min, in order to clear the supernatant. This latter contains the cytoplasmic and membrane proteins. Nuclear pellet was then resuspended in buffer A, centrifuged at 1000 rpm, the supernatant was discarded. Then 4 vol of buffer B were added and the tubes were vortex for 15 min at 4 °C to loosen pellet. Finally, a centrifugation for 10 min at 14,000 rpm at 4 °C was performed and the supernatant that contain nuclear extract was transferred into a new tube. Protein concentration was determined with Bradford assay (Biorad).

2.6. Western blot

50 μg of protein extracts for each sample was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk in TBS-Tween for 2 h at room temperature and then incubated with primary antibodies in TBS-Tween and 5% milk overnight. Blocked membranes were then incubated with anti-ERα (1:500) or anti-ERβ (1:500) and with mouse anti-human β-tubulin (1:2000) (Santa Cruz, Cat. sc-5274) or mouse anti-human HDAC2 (1:2000) (Santa Cruz, Cat. sc-55542) overnight and then detected using appropriate horseradish peroxidase-coupled secondary antibody (Santa Cru, Cat. sc-2005) and visualized with enhanced chemiluminescence (Amersham, Thermo Fisher Scientific, Milano, Italy). The purity of nuclear and cytoplasmic fractions was confirmed using anti-HDAC-2 and anti-β-tubulin, respectively. All antibodies have been used to probe the same experimental membrane. In detail, before incubation with another primary antibody, the membranes have been stripped with the stripping solution: 100 mM 2-Mercaptoethanol, 1% SDS, 62.5 mM Tris-HCl pH 6.7 and incubated at 50 °C for 30 min with agitation and, subsequently, the membrane have been re-equilibrated in TBS and then blocked with 5% milk in TBS-Tween for 2 h at room temperature. The rendering of stripping has been tested by evaluating the ECL- signal after treatment with the stripping solution. Only when the signal of the previous antibody was absent, the membrane was incubated with a new antibody. Immunoblotting data were analyzed using ImageJ software to determine optical density (OD) of the bands. The OD reading was normalized on anti-β-tubulin and anti-HDAC2 to account for variations in loading. For each time of treatment (i.e. 2 h, 6 h, 2 h with ICI) were analyzed data of three independent western blotting.

2.7. RNA extraction and RT-qPCR

mRNA expression levels of estrogen target genes were analyzed using real-time PCR. Total RNA from PNT1A control cells and treated for 24 h with 10^{-6} M E2 or 10^{-6} M NP, with or without 10^{-5} M ICI was extracted using Trizol (Life Technologies, Carlsbad, CA). After purification from genomic DNA with TURBO DNA-free™ Kit (Ambion, Life Technologies), the total amount of RNA was quantified with a NanoDrop spectrophotometer. cDNAs were synthesized from 1 μg RNA using the High Capacity cDNA Reverse Transcriptase (Life Technologies) and quantitative PCR (RT- qPCR) was performed by using the 7500 Real-Time PCR System and SYBR® Select Master Mix 2X assay (Applied Biosystem, Foster City, CA, USA). All primers used (Table 1) were designed according to the sequences published on GenBank using Primer Express software version 3.0 and primer efficiencies were tested prior to perform qPCR. The amount of target cDNA was calculated by comparative threshold (Ct) method and expressed by means of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as housekeeping gene, which expression was not affected by the treatment. Three separate experiments (n = 3) were performed for RT-qPCR and each sample was tested in triplicate.

2.8. Statistical analysis

Data reported in graphs are expressed as mean values ±SEM for the indicated number of independent determinations. The statistical significance was calculated by the one way ANOVA with Bonferroni's multiple comparison test, and differences were considered statistically significant when the P value was at least <0.05.

3. Results

3.1. Cell proliferation assay

To determine if NP affects cellular proliferation, PNT1A cells were treated with increasing concentration of NP (from 10^{-12} M to 10^{-6} M) for 24 h of exposure; to assess any similarities, treatment was performed also exposing cells with E2; the same experiments were also carried out in presence of 10^{-5} M ICI. NP stimulated PNT1A cells proliferation at the highest concentration we used (10^{-6} M) (Fig. 1a). At lower concentrations, we did not observe any significant effects when compared to control group. 10^{-5} M ICI inhibited the proliferation induced by 10^{-6} M NP. Similarly, treatment with E2 stimulated PNT1A cells proliferation from 10^{-9} M to 10^{-6} M, with the greatest effect showed at 10^{-6} M (Fig. 1b). E2 induced proliferation is strongly inhibited by adding ICI. Fig. 1c shows as E2 has a greater effect compared to NP on PNT1A cells proliferation.

Table 1
Primers used in qPCR.

Gene	5′-Forward-3′	5′-Reverse-3′
Cyclin D	CGTGGCCTCTAAGATGAAGGA	CGGTGTAGATGCACAGCTTCTC
Cyclin E	GATGACCGGGTTTACCCAAA	CCTCTGGATGGTGCAATAATCC
Ki67	CCCGTGGGAGACGTGGTA	TTCCCGTGACGCTTCCA
p53	TCTGTCCCTTCCCAGAAAACC	CAAGAAGCCCAGACGGAAAC
IL1-β	ACGATGCACCTGTACGATCACT	CACCAAGCTTTTTTGCTGTGAGT
GAPDH	CAAGGCTGTGGGCAAGGT	GGAAGGCCATGCCAGTGA

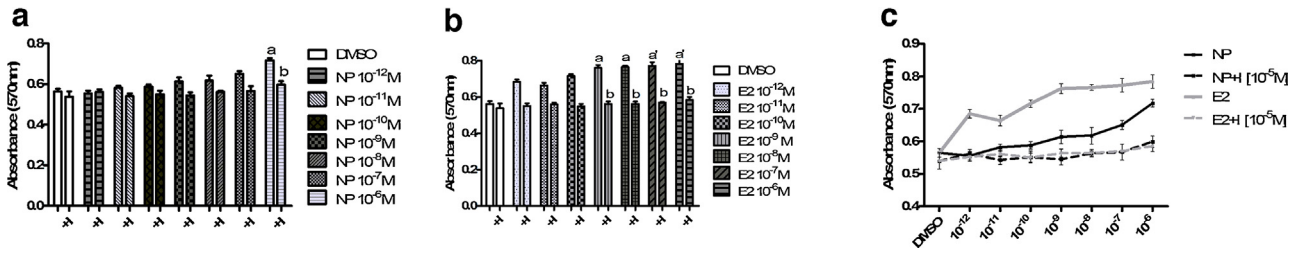

Fig. 1. MTT assay after 24 h of exposure to nonylphenol (NP) and 17β- estradiol (E2) alone or in combination with ICI 182, 780 (+I). NP stimulates PNT1A cells proliferation at 10^{-6} M (a) while E2 at 10^{-9}M, 10^{-8}M, 10^{-7}M and 10^{-6}M (b). 10^{-5} M ICI (I) reverts this effect. E2 shows the greatest effect on proliferation if compared to nonylphenol (**c**). In graph is reported the absorbance measured at 570 nm which correlates with the number of living cells. a, response significantly different than the vehicle control ($P < 0.05$); a', response significantly different than the vehicle control ($P < 0.01$); b, response significantly different than cells without ICI ($P < 0.05$).

3.2. Fluorescence microscopy

3.2.1. Localization of ERα

We investigated on estrogen receptors ERα localization after 10^{-6} M NP and 10^{-6} M E2 treatment, in order to evaluate the interaction between NP and ERs. After 15 min and 1 h ERα remained localized in the cytoplasm after both NP and E2 treatments (data not shown). After 2 h of treatment, NP did not affect ERα cellular localization, that was localized in the cytoplasm, as in control. On the contrary, in E2 treated cells for 2 h, ERα localized predominantly in the nucleus (Fig. 2).

After 6 h of exposure, both in PNT1A cells treated with NP and E2, ERα shifted from the cytoplasm to the nucleus that appeared to be strongly positive, with a weak cytoplasmic fluorescence if compared to control (Fig. 2).

In PNT1A cells pre-treated with ICI ERα was found in the cytoplasm both after 2 and 6 h of treatment with nuclei completely negatives (Fig. 3).

3.2.2. Localization of ERβ

ERβ localization after treatment with 10^{-6} M NP and 10^{-6} M E2 was also investigated; data after 15 min and 1 h (data not shown) as well as after 2 h and 6 h of exposure did not reveal any differences between control and exposed cells. ERβ was localized in the cytoplasm of PNT1A cells and cell nuclei appeared with a weak signal (Fig. 4).

3.3. Western blot analysis

After separation of cytoplasmic and nuclear proteins we performed a translocation study of ERα and ERβ with a western blot analysis, in order to confirm microscopy results after 10^{-6} M NP and E2 exposure. Densitometric analyses were normalized for cytoplasmic and nuclear extracts with β-tubulin (55 Kda) and HDAC2 (55 KDa), respectively. After 2 h of exposure (Fig. 5**)** we found ERα protein (molecular weight 66 KDa) in the cytoplasm of NP treated and non treated PNT1A cells whereas optical density values were significantly lower in E2 treated cells (Fig. 5a,b). Moreover, after 2 h nuclear proteins revealed a signal only in E2 treated cells (Fig. 5a). ERβ (56 KDa) after 2 h of treatment was found only in cytoplasmic fractions (Fig. 5a,c). No signal for HDAC2 and β-tubulin in the cytoplasm and nucleus proteins, respectively, suggest that protein separation was performed correctly.

After 6 h of exposure (Fig. 6**)** we observed a nuclear translocation of ERα both in NP and E2 treated cells (Fig. 6a,b). However, densitometry did not reveal significant differences in nuclear extracts between NP and E2 treated cells (Fig. 6b). In contrast, values were significantly lower in the cytoplasm in treated cells compared to control (Fig. 6b). ERβ was found only in cytoplasmatic fractions (Fig. 6a,c) and there were not significant differences in optic density between treated and non treated cells (Fig. 6c).

Western blot for ERα localization performed in presence of 10^{-5} M ICI after two (Fig. 7a,b) and 6 h of treatment (Fig. 7c,d) revealed ERα exclusively in cytoplasm proteins, with a weak signal in the nuclear extracts after 6 h of exposure (Fig. 7c). No statistically significant differences were showed comparing non treated and treated cells (Fig. 7b,d).

3.4. RT- qPCR analysis

In order to investigate if NP is able to affect gene expression, RT-qPCR analysis of genes involved in cell cycle regulation and in pathological states of prostate were investigated after 24 h of exposure. NP enhanced mRNA levels of *Cyclin D* (Fig. 8a), *Cyclin E* (Fig. 8b), *Ki67* (Fig. 8c) and *IL1-β* (Fig. 8e) while it did not affect *p53* expression (Fig. 8d). Interestingly, ICI reduced gene expression of *Cyclin D* (Fig. 8a) and *Ki67* (Fig. 8c) in PNT1A cells treated with NP, while it did not inhibit gene expression of *Cyclin E* (Fig. 8b) or *IL1-β* (Fig. 8e). E2 significantly affected gene expression up-regulating *Cyclin D* (Fig. 8a) and *Ki67* (Fig. 8c). This induction was strongly inhibited by ICI (Fig. 8a,c).

4. Discussion

EDCs are receiving more and more attention by scientific community, due to their ability to mimic endogenous hormones and altering the metabolism of organisms (De Falco et al., 2014). NP belongs to the family of xenoestrogens and its estrogenic activity is well documented both with *in vitro* (de Weert et al., 2008; Soto et al., 1991; White et al., 1994) and *in vivo* studies (Laws et al., 2000; ter Veld et al., 2008). In this work we seek to investigate the effects of NP on human prostate cells PNT1A, precisely evaluating its estrogenic action in terms of proliferation, interaction with ERs and gene analysis of genes involved in cell cycle regulation and aberrant physiology of prostate. Few studies investigated the effects of NP on prostate, both on cellular and animal models. These findings did not characterize any molecular mechanisms and results often appear to be in conflict. For example, Lee et al. showed that NP is able to reduce the weight of the prostate in rats, in a dose dependent manner (Lee, 1998). In the same study, authors demonstrated that ICI reverted this effect. Similarly, Who et al. obtained the same result but with higher concentration of NP (Woo et al., 2007). Moreover, gestational exposure to NP was reported to affect prostate morphology in F1 rats (Jie et al., 2010). In contrast, other authors failed to demonstrate any adverse effects on rat prostate caused by NP (Inaguma et al., 2004; Odum and Ashby, 2000). These contrasting data may be explained by the time and the way of NP dosage, as well as by the duration of treatment. We conducted our experiments also testing the effects of E2 and ICI.

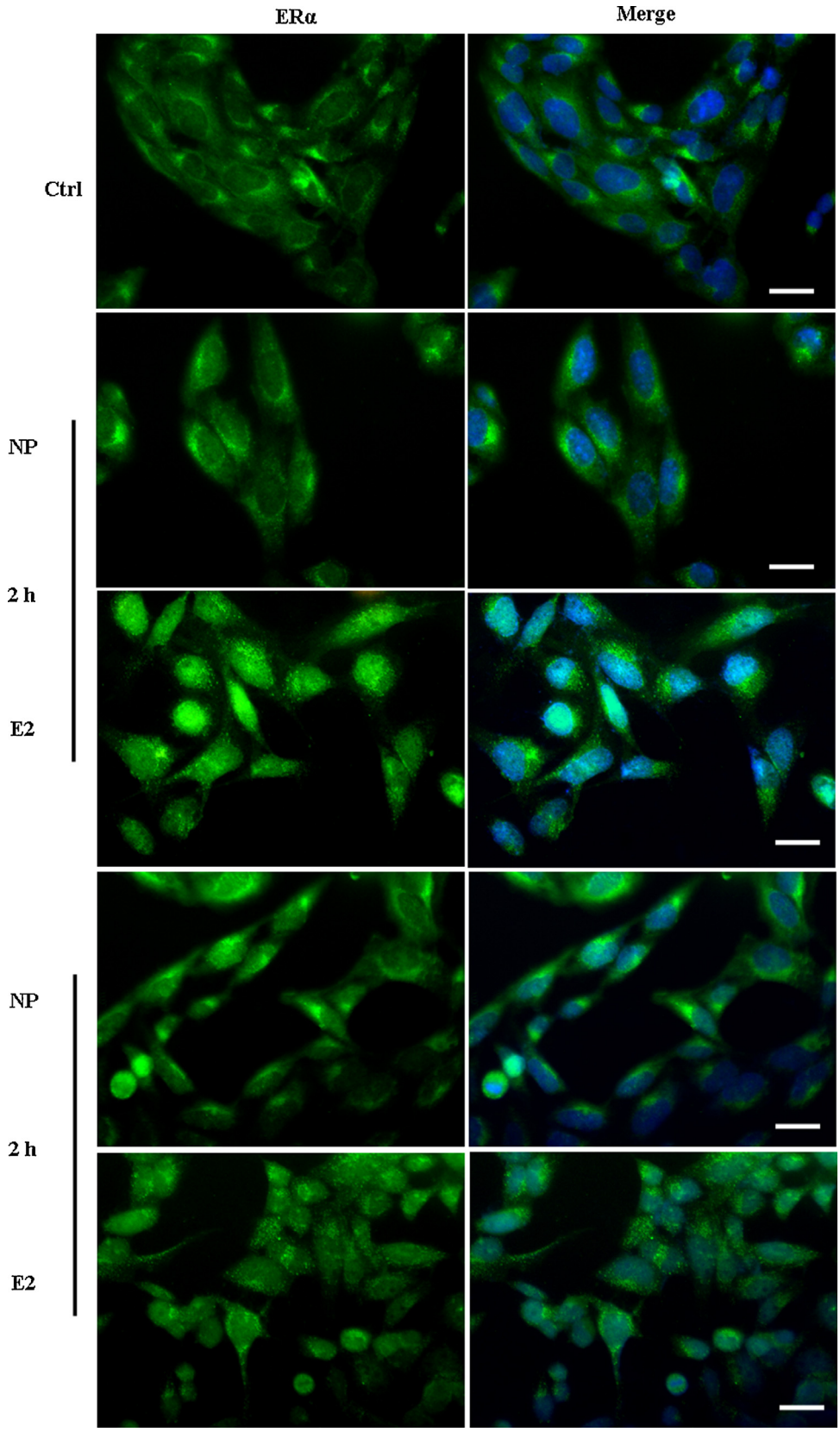

Fig. 2. Localization of ERα after 2 and 6 h of exposure to NP and E2. E2 promotes translocation of ERα to the nucleus at both time of treatment, while NP at 6 h. PNT1A cells were plated in chamber slide under hormone deprived conditions. 24 h later, cells were treated with 10^{-6} M NP or 10^{-6} M E2. ERα (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10 μm.

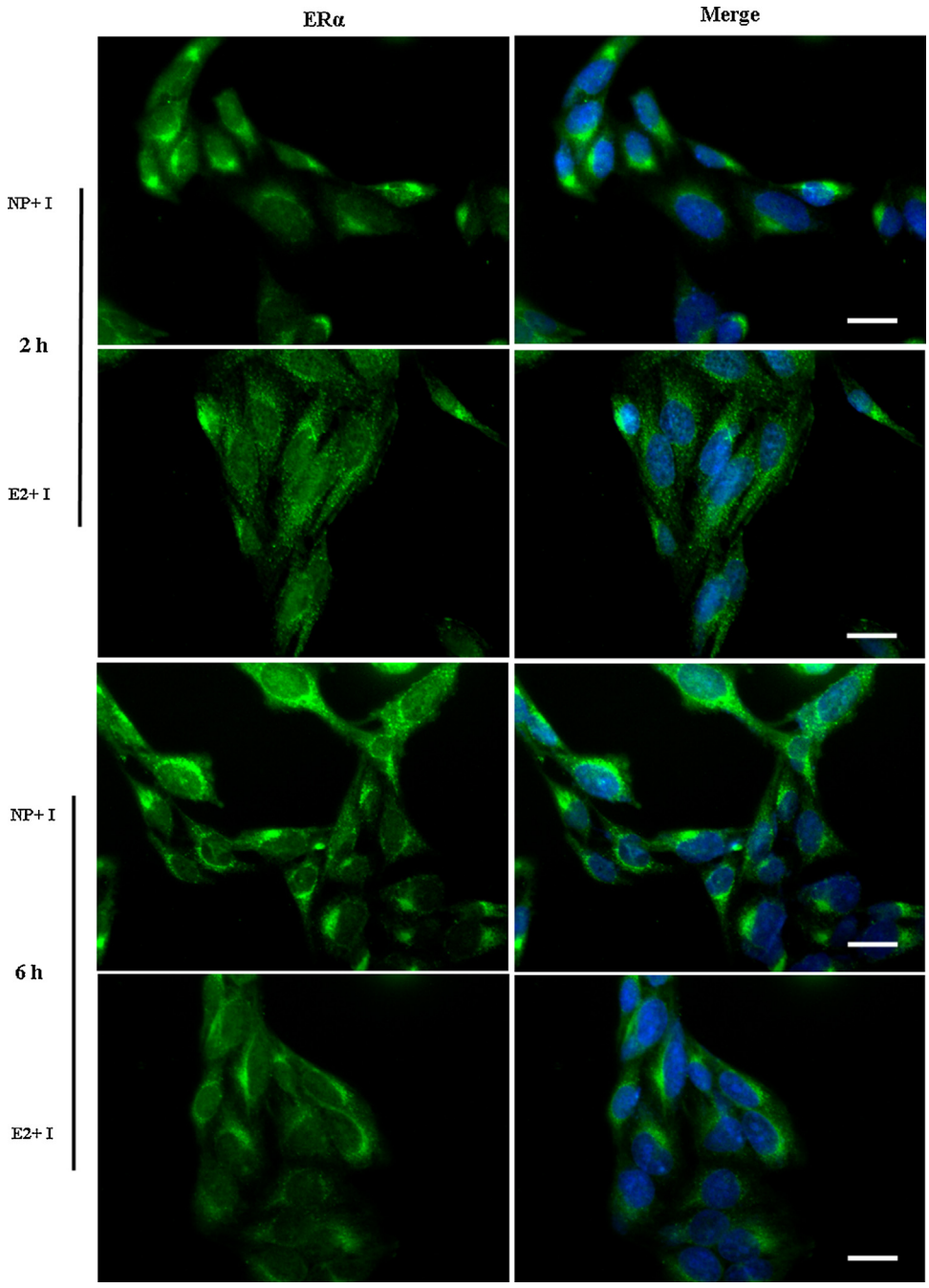

Fig. 3. Localization of ERα after 2 and 6 h of exposure to NP and E2 combined with ICI (+I). ICI inhibits ERα nucleus translocation at both time considered. Cells were pre-treated for 1 h with 10^{-5} M ICI and then treated with 10^{-6} M NP or 10^{-6} M E2. ERα (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10 μm.

We showed that NP stimulated PNT1A cells proliferation after 24 h of exposure at 10^{-6} M as well as E2 did. However, E2 affected PNT1A cells proliferation also at lower concentrations. Interestingly, ICI reverted NP and E2 proliferative stimuli. These results suggest that NP may interfere with normal cell cycle of PNT1A cells as reported by other authors in different cell lines (Choi et al., 2011; Manente et al., 2011). Recently, Gan et al. (Gan et al., 2015) in human prostate epithelial cell line RPWE-1 showed a reduction in cell viability after 24 h exposure to NP. The incongruity between our results and those of Gan et al. can be explained by the different concentrations used. In this study, no effects were reported at 10^{-6} NP and the decrease in cell proliferation appeared to be evident only at high concentration (10^{-5}–10^{-4} M).

With two different approaches, we studied the localization of ERα and ERβ in a time course analysis testing the concentrations (10^{-6} M) that showed the greatest effect on cell proliferation. We demonstrated that E2 induced cytoplasm-nucleus translocation of ERα at both time tested, while NP only after six hour of exposure. Surprisingly, both E2 and NP did not affect ERβ localization. ICI inhibited the ERα translocation observed with NP and E2 alone.

Both proliferation and localization data confirm the estrogenic activity of NP. However, the greatest biological responses showed

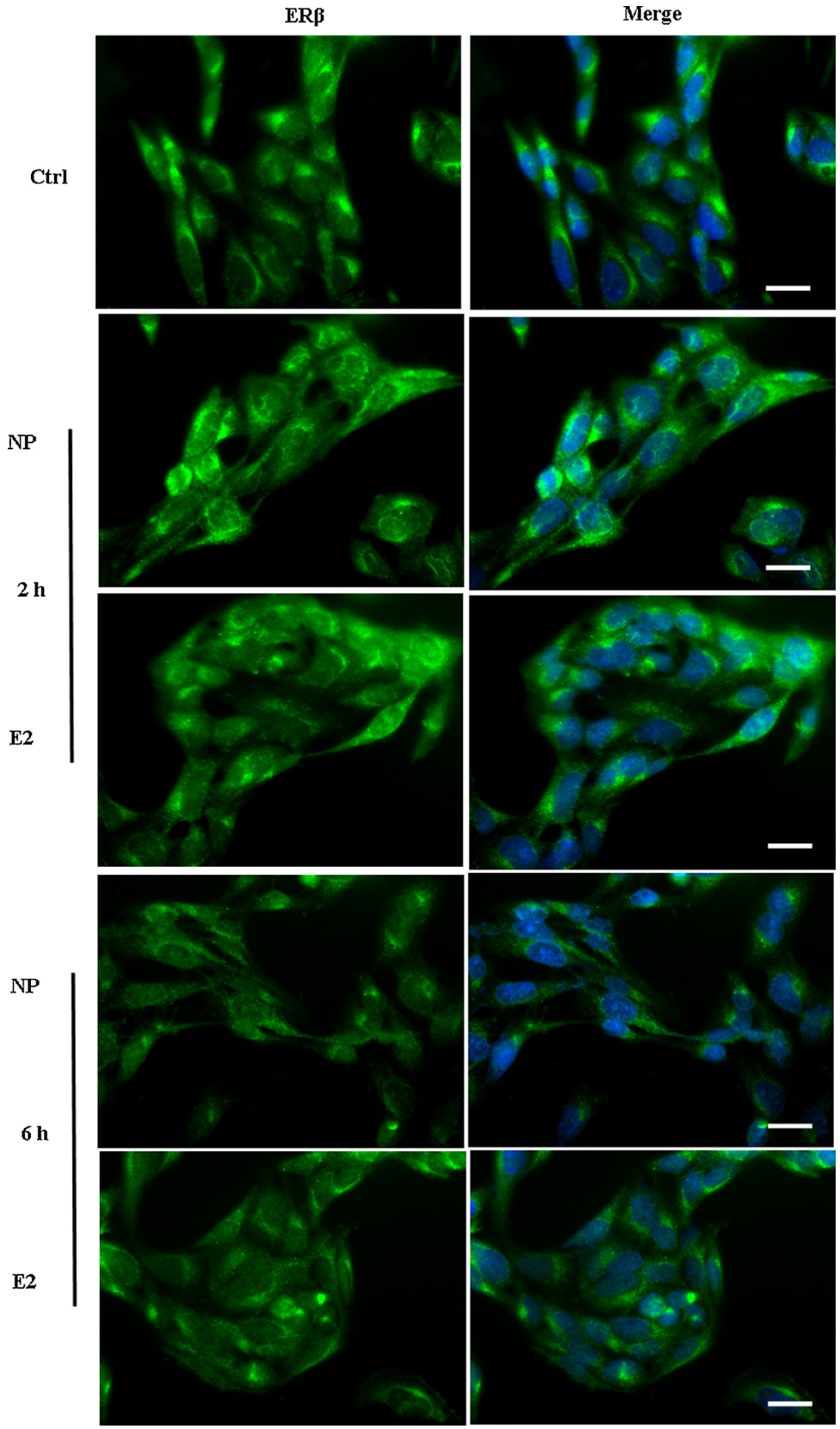

Fig. 4. Localization of ERβ after 2 and 6 h of exposure to NP and E2. In all the images ERβ is localized in cellular cytoplasm. PNT1A cells were plated in chamber slide under hormone deprived conditions. 24 h later, cells were treated with 10^{-6} M NP or 10^{-6} M E2. ERβ (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10 μm.

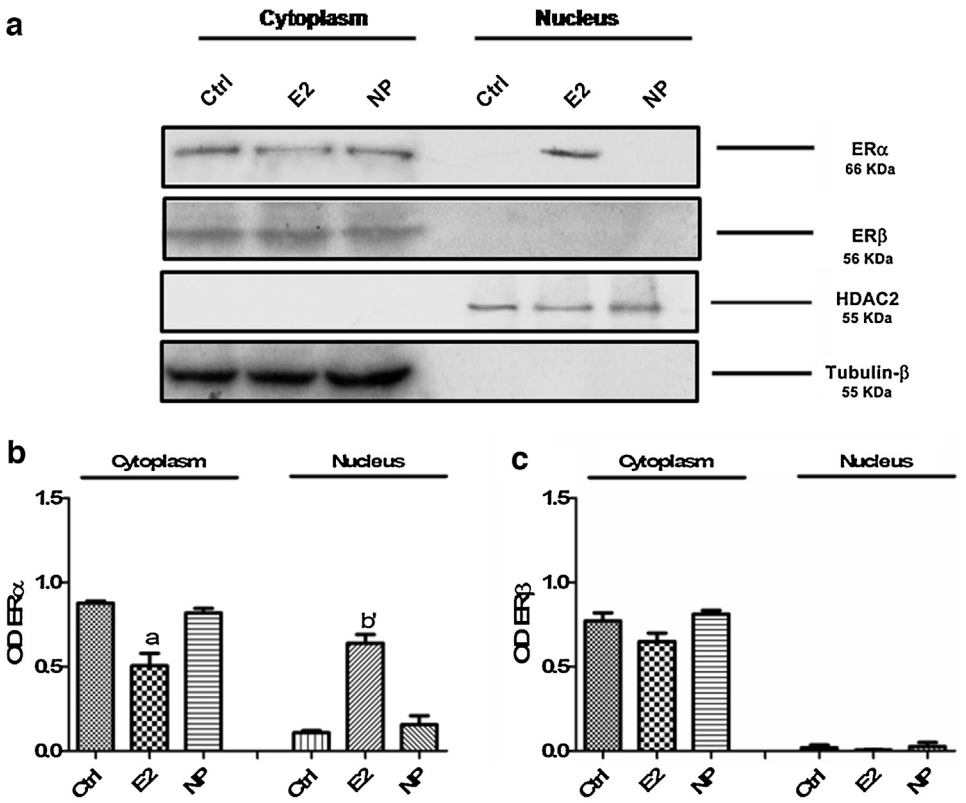

Fig. 5. Western blot analysis and nuclear and cytoplasmic quantification of ERα and ERβ after 2 h of exposure to 10^{-6} M NP and E2. E2 induces nucleus translocation of ERα while ERβ was found only in the cytoplasmatic proteins (a). The graphs represented the Optical density (OD) ratio of ERα and ERβ normalized to the OD of Tubulin β for cytoplasmatic proteins and to the OD of HDAC2 for nuclear proteins (b). (N = 3 separate experiments) a, response significantly different than the cytoplasmatic control ($P < 0.05$); b', response significantly different than the nuclear control ($P < 0.01$)

by E2 can be explained by its best binding affinity with ERs compared to NP (Laws, 2000). Notwithstanding the lowest responses to NP, we can speculate that its proliferative effects, as for E2, is mediated by the interaction with ERα. On this issue, it is well known the role of ERα in cellular proliferation process and carcinogenesis of prostate, while some authors suggested that ERβ seems to be involved in apoptosis of prostate cells (Hartman et al., 2012). For example, it has been reported in knockout ERβ mice prostatic hyperplasia and cancer (Weihua et al., 2001). Moreover, in mice and rats prostate, like in human, it has been shown that ligands that interact with ERβ may reduce proliferation (Ellem and Risbridger, 2009; Omoto et al., 2005; Prins and Korach, 2008).

To assess if the presence of ERα in the nucleus led to the activation of transcription, we analyzed gene expression of E2 gene targets also known to be deregulated in pathological state of the prostate. We demonstrated that NP was able to upregulate *Cyclin D, Cyclin E*, *Ki67* and *IL1-β* gene expression whereas E2 induced upregulation only of *Cyclin D* and *Ki-67*. Moreover, we showed that up-regulation of *Cyclin D* and *Ki67* is mediated by estrogen signaling pathways, while the induction of *Cyclin E* and *IL1-β* involved an estrogen independent pathways, since ICI did not revert this induction. These results of gene expression are in agreement with the showed induced proliferation caused by NP and E2. In this regard, it is well known that *Cyclin D* and *Cyclin E* promoting G1/S phase transition of cell cycle (Kastan and Bartek, 2004) and are often used to screening the carcinogenic potential of EDCs (Diamanti-Kandarakis et al., 2009). Moreover, it has been reported that overexpression of *Cyclin D*, *Ki67*, *Cyclin E* and *IL1β* are a prognostic factors prostate cancer (Aaltomaa et al., 2006; Dey et al., 2013; Sfanos and De Marzo, 2012). In particular, down-regulation of *Cyclin D* and *Cyclin E* has been shown to inhibit tumor progression in different prostate cell lines (Alagbala et al., 2006; Chinni et al., 2001; Lin et al., 2015). In addition, the null effect showed for *p53*, the hallmark of apoptosis, reinforce the idea that NP has a role in promoting prostate cells survival (Gan et al., 2011; Gumulec et al., 2014).

This data, together with the less estrogenic activity of NP in terms of proliferation and interaction with ERα strongly suggest that NP may activate also other transduction pathways, such as the G-protein coupled estrogen receptor GPR30 (Filardo et al., 2007) or the androgen receptor (ARs) (Wang et al., 2010). In this regard, in a recent study, Kim et al. showed that NP induced human tumorigenic prostate cells (LNCaP) proliferation in a pathway mediated by ARs (Kim et al., 2016). In addition to ARs mediate pathways, in epithelial non tumorigenic cells DU145, Gan et al. (Gan et al., 2014) provided evidences about the involvement of GPR30 in NP induced proliferation, when used at concentration from 10^{-8} to 10^{-6} M. Interestingly, according with our data and despite the different prostate model used, in both studies, the concentration that showed the best effects was 10^{-6} M in both the studies.

Considering the exposure level of NP, 10^{-6} M represents an high dose of NP, found rarely in human samples, such as in breast milk and in umbilical cord blood (Chen et al., 2005). Furthermore, it is far from the tolerable daily intake limit values for NP (Woo et al., 2007). Despite these considerations, it should not be underestimated the adverse effects that NP may exert when combined with circulating estrogens or with other EDCs which we are simultaneously exposed. This phenomena, commonly known as

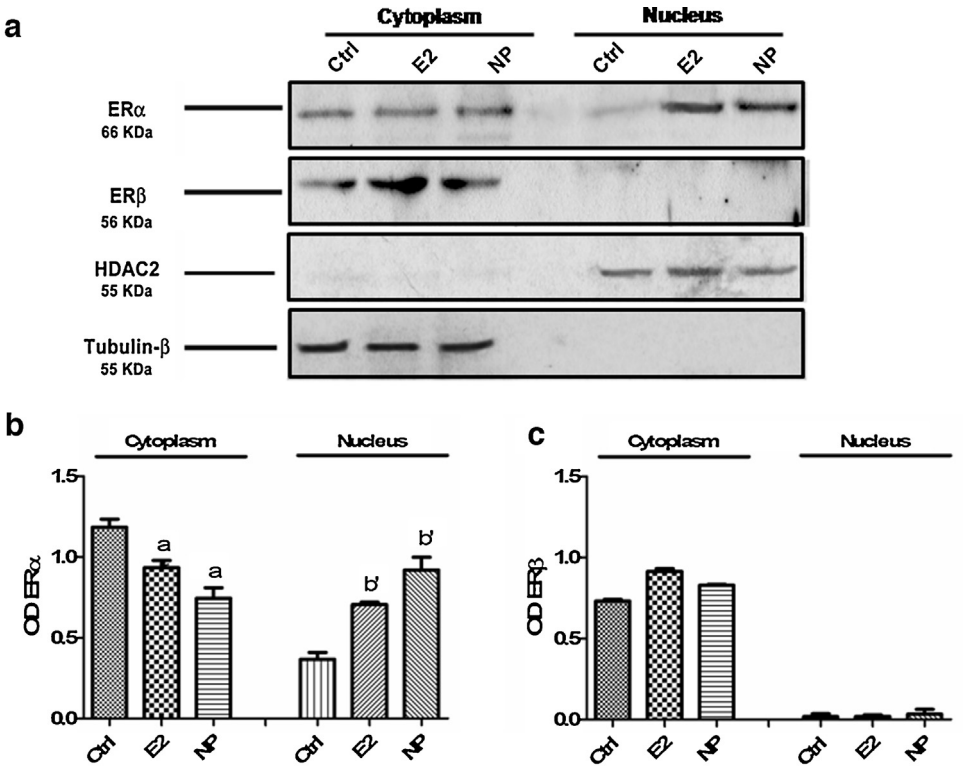

Fig. 6. Western blot analysis and nuclear and cytoplasmic quantification of ERα and ERβ after 6 h of exposure to 10^{-6}M NP and E2. ERα was found in nuclear proteins after E2 and NP treatment while ERβ was found only in the cytoplasmatic proteins (a). The graphs represented the Optical density (OD) ratio of ERα and ERβ normalized to the OD of Tubulin β for cytoplasmic proteins and to the OD of HDAC2 for nuclear proteins (b). (N = 3 separate experiments) a, response significantly different than the cytoplasmatic control ($P < 0.05$); b', response significantly different than the nuclear control ($P < 0.01$).

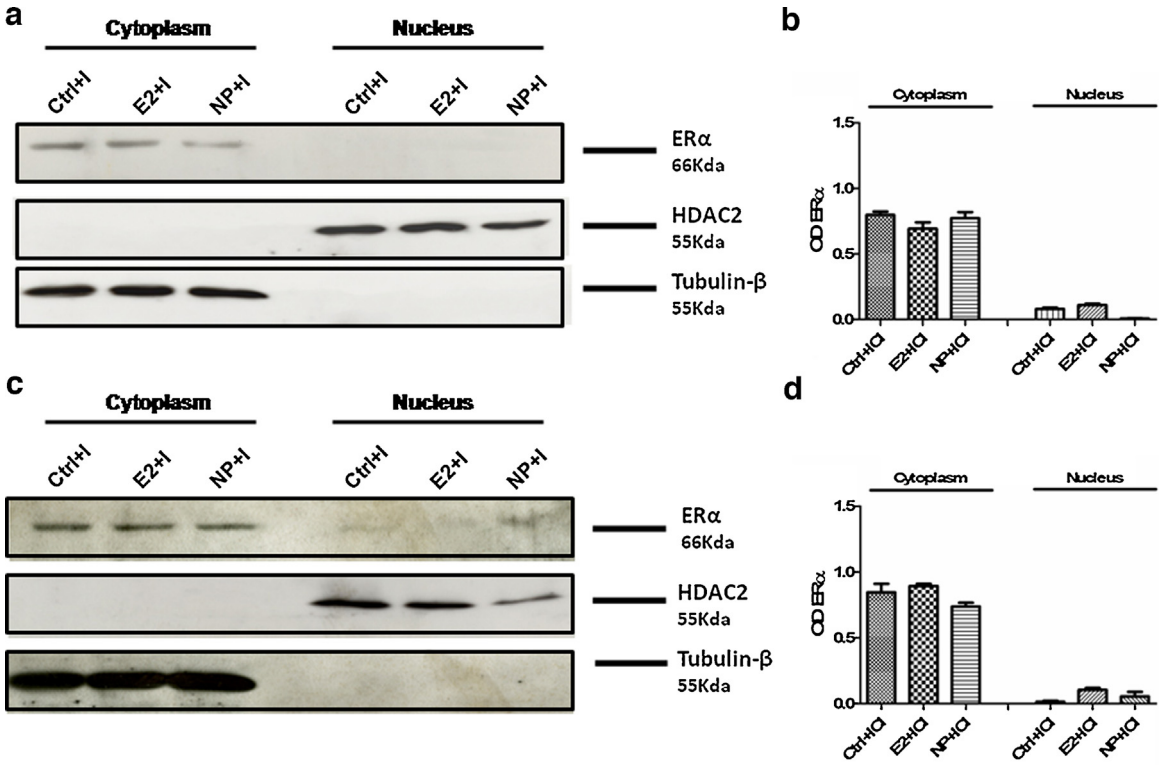

Fig. 7. Western blot analysis and nuclear and cytoplasmic quantification of ERα after pre-treatment with 10^{-5} M ICI 182,780 (+I). ICI inhibits cytoplasm-nucleus translocation of ERα after 2 h of exposure to 10^{-6} M E2 (a–b) and after 6 h of exposure to 10^{-6} M E2 and NP (c–d). The graphs represented the Optical density (OD) ratio of ERα normalized to the OD of Tubulin β for cytoplasmic proteins and to the OD of HDAC2 for nuclear proteins (b, d). (N = 3 separate experiments) no significantly differences.

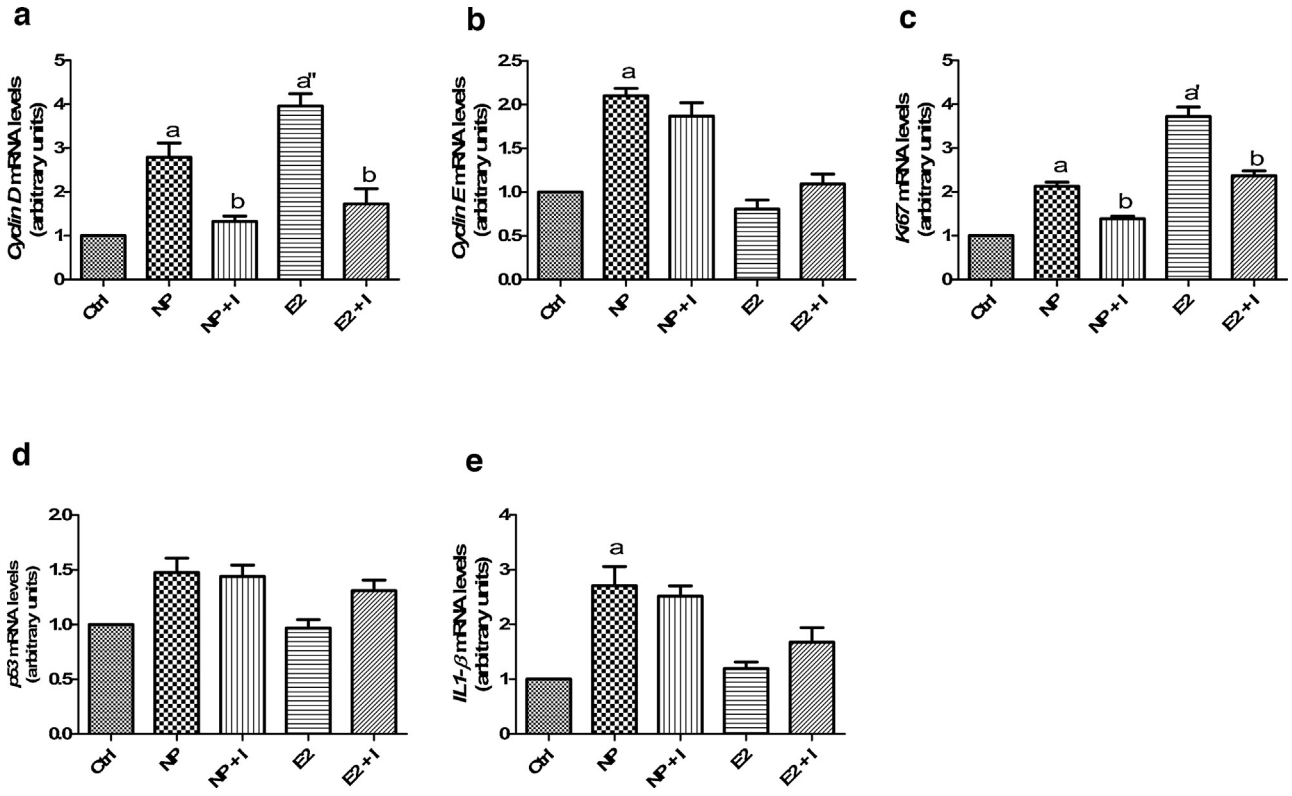

Fig. 8. RT-qPCR analysis after 24 h of exposure to 10^{-6} M NP and E2, alone or in combination with 10^{-5} M ICI (+I). *Cyclin D* (a), *Cyclin E* (b), *Ki67* (c), *p53* (d) and *IL1β* (e) relative mRNA levels were normalized using *GAPDH* as housekeeping gene. (N = 3 separate experiments) a, response significantly different than the vehicle control ($P < 0.05$); a', response significantly different than the vehicle control ($P < 0.01$); a", response significantly different than the vehicle control ($P < 0.001$) b, response significantly different than cells without ICI ($P < 0.05$); b', response significantly different than cells without ICI ($P < 0.01$).

"cocktail effect" is a feature widely accepted for EDCs risk management (Bergman et al., 2012).

In conclusion, we demonstrated that NP acts on PNT1A cells with similar effects if compared to E2, probably mediated by ERα and it may be involved in a deregulation of cell cycle, leading to aberrant proliferation of prostate epithelial cells, which in turn may contribute to pathological states, including cancer. We are also providing data on the dual role of ERs in prostate cells.

Notwithstanding the findings of this study, further evidences remain to be investigated in order to best characterize the risk of NP exposure for prostate diseases. In addition, more cellular and *in vivo* models will be needed. However, our data may help epidemiologists to consider and monitoring the association between NP and prostate pathologies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.05.024.

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HSP90 and pCREB alterations are linked to mancozeb-dependent behavioral and neurodegenerative effects in a marine teleost

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ABSTRACT

The pesticide mancozeb (mz) is recognized as a potent inducer of oxidative stress due to its ability to catalyze the production of reactive oxygen species plus inhibiting mitochondrial respiration thus becoming an environmental risk for neurodegenerative diseases. Despite numerous toxicological studies on mz have been directed to mammals, attention on marine fish is still lacking. Thus, it was our intention to evaluate neurobehavioral activities of ornate wrasses (*Thalassoma pavo*) exposed to 0.2 mg/l of mz after a preliminary screening test (0.07–0.3 mg/l). Treated fish exhibited an evident ($p < 0.001$) latency to reach T-maze arms (>1000%) while exploratory attitudes (total arm entries) diminished (−50%; $p < 0.05$) versus controls during spontaneous exploration tests. Moreover, they showed evident enhancements (+111%) of immobility in the cylinder test. Contextually, strong (−88%; $p < 0.01$) reductions of permanence in light zone of the Light/Dark apparatus along with diminished crossings (−65%) were also detected. Conversely, wrasses displayed evident enhancements (160%) of risk assessment consisting of fast entries in the dark side of this apparatus. From a molecular point of view, a notable activation ($p < 0.005$) of the brain transcription factor pCREB occurred during mz-exposure. Similarly, in situ hybridization supplied increased HSP90 mRNAs in most brain areas such as the lateral part of the dorsal telencephalon (Dl; +68%) and valvula of the cerebellum (VCe; +35%) that also revealed evident argyrophilic signals. Overall, these first indications suggest a possible protective role of the early biomarkers pCREB and HSP90 against fish toxicity.

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1. Introduction

Water pollution represents a serious threat to aquatic organisms and especially fish. Pesticide runoff from agricultural lands are one of the main sources of water contamination, which induces dangerous disturbances to the different physiological facets of fish such as swimming and feeding behaviors (Ullah et al., 2014). Pesticides may easily enter in the fish body through gills, skin and via the food-chain thereby reaching target organs like the brain (Atamaniuk et al., 2014). At this regard, deleterious effects of pesticides are often related to neuronal dysfunctions in both mammals (Lee et al., 2015) and fish (Renick et al., 2016). Consequently the dithiocarbamate fungicides, widely used for preserving different agricultural yields, are considered hazardous toxicants (Rath et al., 2011) due to the alteration of key enzymes such as α-carbonic anhydrase thus accounting for the failure of pH homeostasis, respiration and electrolyte secretion (Kolayli et al., 2011). Among dithiocarbamates, mancozeb (manganese (Mn)/zinc (Zn)-ethylene-bis-dithiocarbamate; mz) is composed of different sub-compounds (Mn, Zn plus ethylene thiourea) that together account for multiple toxic mechanisms operating simultaneously during exposure to this fungicide (Geissen et al., 2010). For this reason, we purposely decided to focus on the effects of the integral compound, which causes wide neuronal damages (Harrison Brody et al., 2013). Indeed, the presence of this molecular complex permits it to catalyze the production of reactive oxygen species (ROS) as well as inhibiting mitochondrial respiration at the brain level (Todt et al., 2016) thus proposing it as an environmental risk for neurodegenerative diseases such as Parkinson's (Pezzoli and Cereda,

Abbreviations: ACS, amino cupric silver stain; CCe, corpus of the cerebellum; DIG, digoxigenin-11-dUTP; Dl, lateral part of the dorsal telencephalon; Dm, medial part of the dorsal telencephalon; HSP90, heat shock protein 90; mz, mancozeb; NDLI, diffuse nucleus of the inferior lobe; NG, nucleus glomerulosus; nIV, trochlear nerve nucleus; OT, optic tectum; pCREB, cAMP response element-binding protein; RS, superior reticular nucleus; TLo, torus longitudinalis; VCe, valvula of the cerebellum; Vl, lateral part of the ventral telencephalon; VTel, ventral telencephalon; Vv, ventral part of the ventral telencephalon.

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2013). At present, indications are beginning to consider mz as an important endocrine disruptor (Thienpont et al., 2011), an oxidative stressor of gills and blood (Kubrak et al., 2012), plus being an inductor of oxidative damage to lipids and proteins in brain, liver and kidney of fish (Atamaniuk et al., 2014).

Based on the above considerations, together with the lack of toxicological studies on marine fish contaminated by mz, the present study aimed to investigate neuronal and behavioral effects of this fungicide on the marine teleost ornate wrasse (*Thalassoma pavo*) exposed to 0.2 mg/l of mz. This concentration was chosen on the basis of a preliminary screening test of sublethal concentrations (0.07, 0.14, 0.2, 0.3 mg/l) handled in our laboratory. These doses are in line with those used in other studies (Jarrard et al., 2004) along with environmentally relevant concentrations detected in waterbodies near agricultural fields (Shenoy et al., 2009) and in view of the recommended mz concentrations being a thousand times higher for crop treatment (Atamaniuk et al., 2014). Despite the frequent application of mz throughout the world, indications regarding its environmentally relevant concentrations in seawater are still lacking, perhaps because this fungicide is often considered a compound with a low toxicity even if recent evidences are beginning to indicate a high risk imposed to fish due to water contamination (Marques et al., 2016). In any case, it is considered a marine pollutant as reported by the Pesticide Properties Database of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/424.htm) and it is known that the runoff of mz from the river or soil near coasts can easily reach the sea especially since Italy is geographically surrounded by the Mediterranean sea.

Hence, the different behavioral parameters (Light/Dark Test, Spontaneous Exploration Test in a T-maze and Cylinder Test) along with specific molecular markers were investigated during cellular impairments following exposure to this fungicide. For the present work, the phosphorylated and active form of CREB (cAMP response element-binding protein, pCREB) was preferred since it is an important transcription linker between a number of neurotransmitters and downstream gene expression thus favoring neuronal survival and proliferation (Dworkin et al., 2007) together with antianxiety-like conditions (Chakravarty et al., 2013). At the same time, HSP90 was also evaluated due to its well-known role on environmental stress (Wang et al., 2016; Zizza et al., 2016) in which they mitigate deleterious effects of protein misfolding in a similar manner to neurodegenerative diseases (Marino Gammazza et al., 2016). Indications deriving from the present study may provide novel bearings concerning the activation of protective mechanisms against mz-dependent toxicity on fish neurobiological activities.

2. Materials and methods

Before treating fish, it was necessary to determine the solubility parameter of mz due to the lack of indications in seawater. For this part, experiments were conducted according to previous procedures (Furia et al., 2011; Naccarato et al., 2016).

2.1. Analytical procedure

Mz was not quantified directly but the samples were treated to quantify Mn as previously reported (Pariseau et al., 2009). With this aim, mz (1.5 mg) was dissolved in 1.5 l of seawater; the mixture was vortexed at least for 2 h and then filtered with an highly retentive filter paper (Whatman 42). The filtrate was diluted (1:100) with filtered seawater. This solution was then analyzed by adding the reagent HNO_3 (65%; Suprapur; Merck, Darmstadt, Germany) via Elan DRC-e ICP-MS instrument (Perkin-Elmer SCIEX, Canada) using 55 Mn isotope and seawater as a blank. Quantitative analysis was performed designing an eight-point calibration curve (calibration range: 0.1–1000 μg/l) in which the calibration solutions were prepared by diluting Merck XXI and Perkin Elmer 2 multi-element standards solutions to 10 mg/l (Ultrapure water; Milli-Q plus system, Millipore, Bedford, MA). Total Mn concentration (519 μg/l) corresponds to the highest solubility of mz in seawater. The quantification limit of the analyte is 0.03 μg/l. We had a satisfactory quantitative recovery for Mn and thus we avoided using isotope dilution (Mn does not have stable isotopes to use for isotope dilution).

2.2. Animals and treatments

Adult female specimens of *Thalassoma pavo* (7–13 g body weight; 8–11 cm body length: n = 30), which was already used for the evaluation of neurotoxic effects (Zizza et al., 2013, 2014), were obtained from a local supplier. They were acclimated in our laboratory for at least 1 week in 80 l tanks under a natural photoperiod in aerated and filtered seawater. During acclimation, fish were fed once a day with small pieces of frozen prawns corresponding to 2% of the biomass in the tank (Facciolo et al., 2010). Water quality parameters i.e. salinity (35%), density (1.027–1.028 g/cm^3), hardness (100 mg $CaCO_3$/l) and dissolved oxygen (8–8.6 mg/l) as well as temperature (20–22 °C) plus pH (7.5–8.0) were daily checked to assure that they remained within these ranges. Animal maintenance and experimental procedures complied with the legislative law n°116 (27-01-1992) and with European Directive (2010/63/EU) for the correct use of laboratory animals. Efforts were made to minimize animal suffering and reduce number of fish used.

After acclimation, fish were exposed for 96 h to mz (Sigma, Milan-Italy) dissolved in seawater to reach the nominal sub-lethal concentration of 0.2 mg/l (n = 15) and compared to controls (n = 15), which were not exposed to the pesticide. This concentration fell within the solubility parameters of mz in seawater according to indications obtained by a ICP-MS analytical procedure. It resulted to be the most effective non-lethal dose capable of inducing behavioral effects (data not shown) among the different mz concentrations (0.07, 0.14, 0.2, 0.3 mg/l) as well as falling within the same range used by others (Jarrard et al., 2004). A static renewal exposure system was applied, with the pesticide concentration being renewed in seawater every 24 h, according to standard procedure guidelines (American Society for Testing Material, 2014). This type of exposure system, together with a basic pH and a relatively high temperature, guarantees a constant pesticide concentration within 24 h since degradation of mz occurs after a much later time, i.e. 39 h (López-Fernández et al., 2017). Tanks were only equipped with an aerator without any chemical filters to avoid modifications of the pesticide concentration. Water parameters were constantly checked to ensure that they remained within the ranges reported above. During exposure, fish were fed as above according to our previous toxicological studies (Giusi et al., 2008; Zizza et al., 2014).

2.3. Behavioral assessment

2.3.1. Spontaneous exploration test in a T-maze. A T-shaped glass tank consisting of a start compartment of 40 × 40 cm, a passage lane (40 × 20 cm) and two arms of the same length (20 × 20 cm) was used to assess the effects of mz on spontaneous exploration. In the present study, this type of maze represents an novel arena to assess fish exploration according to a previous study (Grossman et al., 2010). Following a 96 h exposure to the fungicide, all fish were individually tested. Each fish was placed in the start compartment and observed for 6 min using a digital camera positioned at the top. The following parameters were evaluated:

- *latency time to reach arms*: the time (s) to reach arms from the start compartment;
- *total arm entries*: the total number of entries in the maze arms;
- *time spent in arms*: the total time (s) spent in the maze arms.

2.3.2. Cylinder test. Each animal was introduced alone in a cylinder apparatus, which consisted of a 6 l glass cylinder (16 cm diameter and 20 cm height) maximally filled with seawater and divided in two

equal virtual portions as reported by Grossman et al. (2010). Tests (6 min) were recorded by two digital cameras, one at the top and the second one opposite to the cylinder. Such an apparatus was used as a novel tank to examine effects of mz on the following end-points: time (s) spent at the bottom of the tank, time (s) spent along the cylinder walls, time (s) spent in a immobile state and time (s) spent in different movements (motor activity).

2.3.3. Light/dark preference test. Light/Dark preference test was carried out at the end of the exposure session, in order to assess anxiety-like behaviors (Maximino et al., 2011). The Light/Dark apparatus used for the test consisted of a rectangular glass tank (25 × 20 × 40 cm h × d × l) subdivided in two equal compartments without any physical barrier between them, as previously reported (Zizza et al., 2016). The light part of the apparatus consisted of the transparent walls of the aquarium with a diffused light located above. The dark compartment was shielded from the light source with an opaque black lid along with opaque black walls and bottom. Also in this case, all animals were individually introduced into the Light/Dark apparatus. Each behavioral test, which lasted 6 min, was filmed and the following end-points were evaluated:

- *light permanence*: the total time (s) spent in the light compartment;
- *crossings*: the number of transitions between the two compartments;
- *risk assessment*: the number of fast entries in the dark side of the apparatus or a partial entry within the white compartment (Maximino et al., 2011).

All behavioral data were analyzed using the software EthoLog 2.2.5 (Visual Basic; Brazil) and values were reported as mean activity ± standard error of mean (SEM).

At the end of the behavioral session and before molecular procedures, fish were checked for sexual identification by morphological observations of the ovary that did not show any sign of ovarian atresia that could have indicated an initial transition to the testicular growth (Liu et al., 2017).

2.4. Neurodegenerative analysis

A neurodegenerative analysis was carried out by applying Amino Cupric Silver (ACS) technique to verify neuronal damages caused by mz. This technique has been widely used for the detection of both necrotic and apoptotic events in fish via the formation of silver precipitated granules (argyrophilic reaction) in neuronal fields where neurodegeneration occurred (Zizza et al., 2016). With this purpose, fish (n = 5) exposed to the fungicide for 96 h plus controls (n = 5) were first anesthetized in ice water and then sacrificed by spinal transection. Brains were removed, frozen on dried ice and stored at −20 °C. Subsequently, they were mounted on a freezing stage of a sliding cryostat (Microm-HM505E; Zeiss, Wallford, Germany) to obtain a series of representative sections (30 μm) for ACS protocol as previously described (Zizza et al., 2014). Afterwards, sections were kept in a rapid fixer solution for 5 min and counterstained with 0.5% neutral red solution (Carlo Erba, Milan-Italy) for 25 min, dehydrated in ethanol (50–100%) plus xylene, and mounted with DPX (*p*-xylene-bis[*N*-pyridinium bromide]; Sigma) for observations at a bright-field Dialux EB 20 microscope (Leitz, Stuttgart, Germany).

2.5. Western blot

Brain tissues of exposed-fish (n = 5) plus controls (n = 5) were homogenized and lysed on ice with RIPA lysis buffer containing protease and a mixture of phosphatase inhibitors (Santa Cruz, Biotechnology, Milan-Italy) for 30 min. Homogenates were centrifuged at 12000 rpm for 20 min at 4 °C. Total protein quantities were determined by using BCA protein assay kit (PIERCE, Milan-Italy). 50 μg of proteins of each sample were boiled for 5 min in SDS buffer [50 mM Tris-HCl (pH 6.8), 2 g/100 ml SDS, 10% (v/v) glycerol, 0.1 g/100 ml Bromophenolblue], separated on 10% SDS-PAGE and transferred to a PVDF membrane for blotting (Trans-Blot® Semi-Dry Transfer Cell, Biorad). Membranes were incubated for 1 h at room temperature with a blocking buffer (TBS, 0.05% Tween-20 and 5% BSA). After blocking, membranes were incubated overnight at 4 °C with Rabbit anti-pCREB antibody or rabbit anti-β-actin antibody (Santa Cruz Biotechnology) diluted 1:200 in TBS-T containing 2% BSA. The membranes were washed four times for 5 min in TBS, 0.05% Tween-20 before a 1 h incubation in a buffer (TBS, 0.05% Tween-20 and 2% BSA) containing horseradish peroxidase-linked anti-rabbit IgG (Santa Cruz Biotechnology) at 1:4000 dilution. The membranes were washed four times and specific protein bands were detected with chemiluminescence (ECL, Santa Cruz, Milan-Italy) using C-DiGit Chemiluminescent Western Blot Scanner (LI-COR). Western blots were analyzed using Image Studio Software to determine optical densities (OD) of the bands. OD readings were normalized to β-actin values to account for variations in loading.

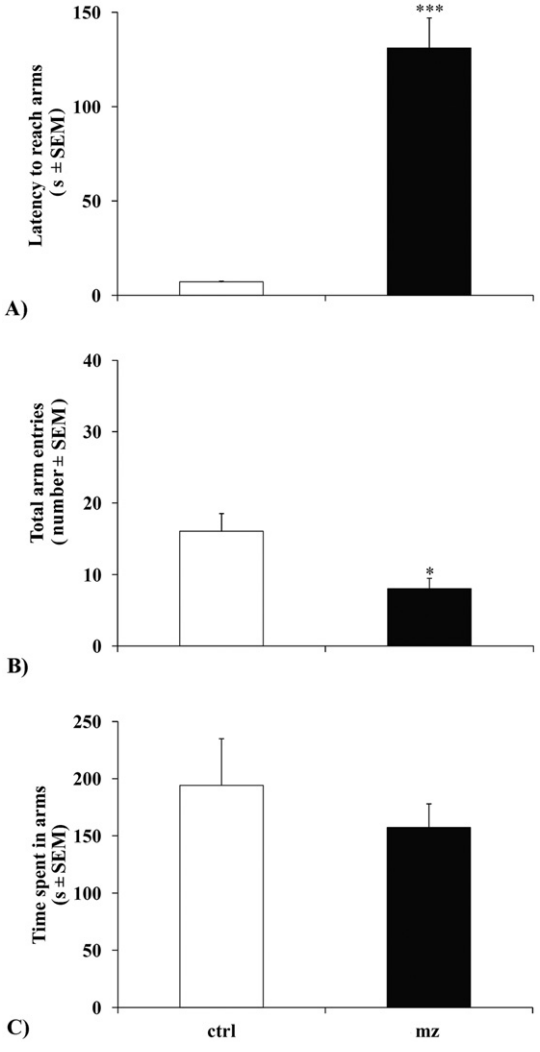


Fig. 1. The effects of mz on spontaneous exploration of *Thalassoma pavo* in a T-shaped apparatus. Latency time to reach arms (s ± SEM; A), total arm entries (number ± SEM; B) and time spent in arms (s ± SEM; C) were tested during a 6 min behavioral session. The above behavioral activities were evaluated in fish exposed to mz (0.2 mg/l) for 96 h (n = 15) and compared to controls (ctrl; n = 15) by using unpaired Student's *t*-test. $^{*}p < 0.05$, $^{***}p < 0.001$.

2.6. Transcriptional analysis

To evaluate the influence of mz on brain HSP90 transcription, an in situ hybridization analysis was performed by using a specific antisense oligonucleotide DNA probe. Such a probe of 43 nucleotides was 5′-CACAAAGAGGGTGATGGGGTATCCGATGAACTGAGAGTGCTTT-3′ previously designed (Giusi et al., 2008) on the basis of HSP90 partial nucleotide sequence of *Thalassoma pavo* (GenBank cod. EF392848) labeled at the 3′-tailing with digoxigenin-11-dUTP (DIG, Roche Diagnostics, Monza-Italy) and compared to a sense oligonucleotide probe. With this intention, fish exposed for 96 h with mz (n = 5) and controls (n = 5) were sacrificed as described above. Subsequently, brains were rapidly removed, stored at −40 °C and mounted on a cryostat freezing stage (Microm-HM505E; Zeiss) to obtain a series of coronal sections (14 μm) that were incubated with a 100 ng of HSP90 antisense probe overnight at 50 °C in a humidified chamber as described in another study (Facciolo et al., 2012). Immunological detection using an anti-digoxigenin antibody (1:100) was obtained as previously reported (Zizza et al., 2014). Hybridization signals (expressed as OD ± SEM), observed at a bright-field Dialux EB 20 microscope (Leitz) were determined in duplicates on each brain antimere of 6 brain sections for anterior plus posterior brain slides. Expression levels of HSP90 mRNA were obtained by using an Image Software of the National Institutes of Health (Scion Image 2.0), in which an internal standard was used for OD calibration. Background level was estimated and included in all final calculations. The different encephalic nuclei were identified using perciformes atlases (Cerdá-Reverter et al., 2001a,b, 2008).

2.7. Statistical analysis

Statistical differences between mz-exposed fish with respect to controls were evaluated for all experimental data by using an unpaired Student *t*-test with a significant level of $p < 0.05$. The determination of number of animals of the present study was carried out using a free online statistical program (http://stat.ubc.ca/~rollin/stats/ssize/n2.html; Department of Statistics of the University of British Columbia-Canada) in which a 95% power corresponded to a sample size of at least 5 and 15 individuals for molecular/neurodegenerative and behavioral analyses, respectively, when a 2-sided 5% level of significance was used.

3. Results

3.1. Effects of mancozeb on behavior

3.1.1. Spontaneous exploration test. Fish treated with mz exhibited significantly differentiated exploration activities as indicated by their very strong ($p < 0.001$) increased latency to reach arms (>1000%) with respect to controls that instead demonstrated immediate and rapid movements from the start compartment (Fig. 1A). Contextually, exploratory attitudes in mz-exposed fish diminished as pointed out by a moderate reduction of total arm entries (−50%; $p < 0.05$) while controls displayed a more consistent number of arm entries (Fig. 1B). Conversely, the total time spent in arms did not significantly change with respect to un-treated animals (Fig. 1C).

3.1.2. Cylinder test. During the cylinder test, neither mz-exposed fish nor controls exhibited any type of transition toward the upper portion of the apparatus (Fig. 2A). Indeed, the two experimental groups spent the entire time at the bottom of the tank without reaching the upper zone. However, it was worthy to note that fish exposed to mz spent a conspicuous part of the test along the walls of the cylinder as demonstrated by the strong increase ($p < 0.01$; +76%) of such an endpoint with respect to controls (Fig. 2B). Accordingly, an evident enhancement (+111%) of immobility was observed after mz exposure with respect to controls (Fig. 2C). At the same time, the fungicide induced a moderate reduction (−56%) of motor activity compared to untreated fish (Fig. 2D).

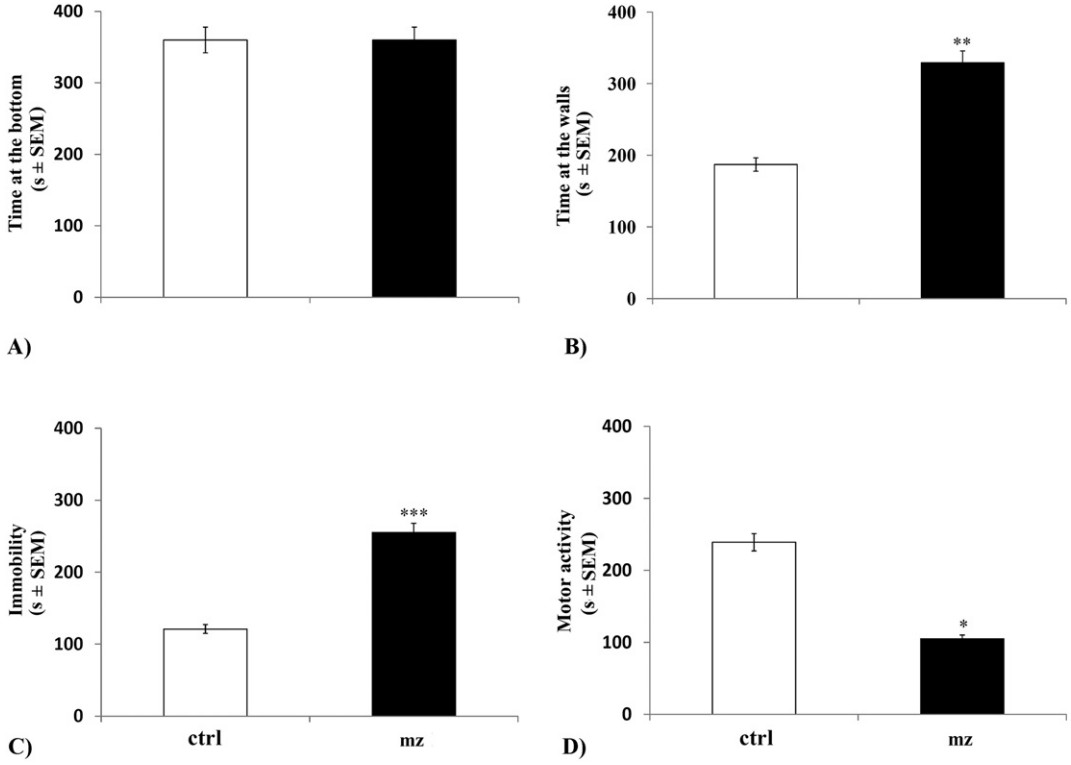


Fig. 2. The effects of mz during a cylinder test in the ornate wrasses. Data were expressed as total time spent (s ± SEM) at the bottom (A), along the cylinder wall (B), in an immobility state (C) and during motor activity (D) by fish exposed to 0.2 mg/l of mz (n = 15) with respect to controls (ctrl; n = 15) for a 6 min behavioral session in a cylinder tank. Statistical differences were evaluated by using unpaired Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

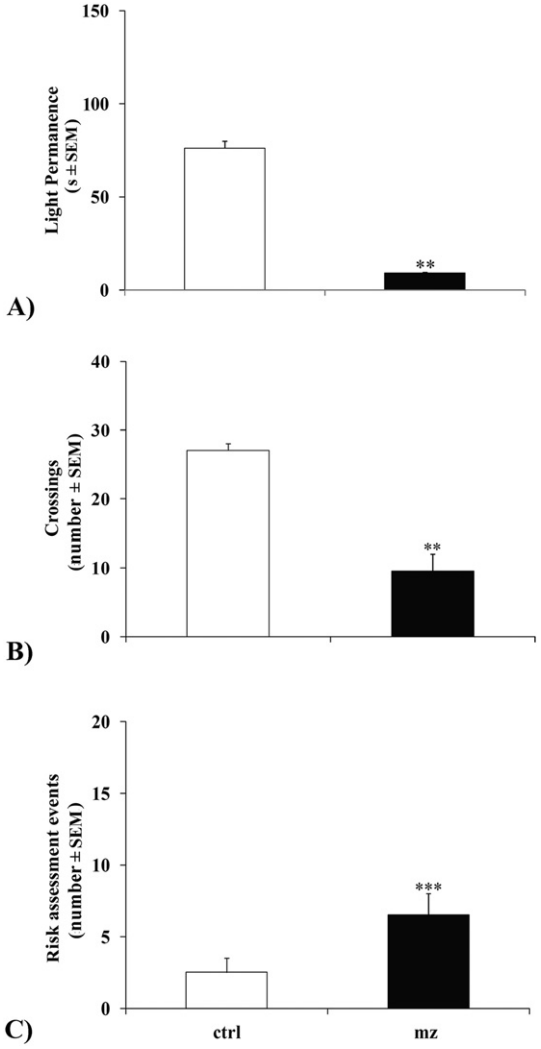


Fig. 3. The effects of mz on anxiety-like behaviors of *Thalassoma pavo*. Light permanence (s ± SEM; A), the number of crossings (number ± SEM; B) and of risk assessment events (number ± SEM; C) were tested during a 6 min behavioral session in a Light/Dark apparatus. The above behavioral activities were evaluated in fish exposed to mz (0.2 mg/l) for 96 h (n = 15) and compared to controls (ctrl; n = 15) by using unpaired Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.1.3. Light/dark preference test. Light/dark test highlighted an anxiety-like behavior in fish exposed to the pesticide. Indeed, treated fish displayed a strong (−88%) reduction of permanence in the light zone of the apparatus compared to controls (Fig. 3A). Moreover, the number of crossings strongly diminished (−65%) in fish exposed to mz thus indicating reduced explorative attitudes with respect to controls (Fig. 3B). Conversely, a very strong enhancement (160%) of the number of risk assessment events occurred in the pesticide-treated group versus controls (Fig. 3C).

3.2. Neurodegenerative effects of mancozeb

The behavioral impairments observed in treated fish coincided with several damaged cells displaying heavily stained granules within the numerous neuronal fields following exposure to mz as revealed by ACS analysis of the anterior and medio-posterior areas (check schemes). The argyrophilic reaction was evident in the lateral part of the dorsal telencephalon (Dl; Fig. 4A), in the medial part of the dorsal telencephalon (Dm; Fig. 4B) and in the ventral telencephalon (VTel; Fig. 4C) of mz-exposed specimens with respect to few rare dark granules of controls as shown in the respective brain areas (Fig. 4A′–C′). As far as diencephalon was concerned, the diffuse nucleus of the inferior lobe (NDLI; Fig. 5A) also resulted to be heavily damaged. Similarly, an evident argyrophilic signal was reported in the different cellular layers of the optic tectum (OT; Fig. 5B), in the valvula of the cerebellum (VCe; Fig. 5C) and in the superior reticular nucleus (RS Fig. 5D) compared with the respective brain areas (Fig. 5A′–D′) of controls.

3.3. Neuromolecular effects of mancozeb

From a molecular point of view, a notable activation of the transcription factor pCREB occurred during mz-exposure. Indeed, OD levels of pCREB band (Fig. 6A), which were normalized by using values deriving from densitometric evaluation of β-actin bands (Fig. 6B), were very significantly ($p < 0.005$) enhanced in treated fish rather than the respective pCREB band of controls. At the same time, in situ hybridization revealed an activation of HSP90 transcriptional responses as displayed by very dense antisense signals in the representative brain section of exposed-fish (Fig. 7A) and controls (Fig. 7B) compared with sense signals (Fig. 7C). In particular, a notable increase of mRNA levels was found in Dl (+68%), whereas in other telencephalic fields such as Dm (+30%), the ventral part of the ventral telencephalon (Vv; +33%) and the lateral part of ventral telencephalon (Vl; +41%), only moderate transcript enhancements were observed (Fig. 7D). Similarly, moderate HSP90 up-regulation events occurred in some extra-telencephalic sites such as NG (+30%), OT (+30%), VCe (+35%), the trochlear nerve nucleus (nIV; + 31%) and RS (+30%).

4. Discussion

This work provided first evidences about behavioral and neuronal effects of the dithiocarbamate mz in a marine fish, in which the molecular elements pCREB and HSP90 constitute crucial factors for the activation of neuroprotective measures against pesticides. At the behavioral level, motor performances were notably impaired by mz since spontaneous exploration activity revealed significant alterations of almost all parameters and namely the latency to reach arms or total arm entries. Such explorative alterations occurred together with other motor deficits, which included increase of fish immobility and reduction of time spent moving when fish were tested in the cylinder apparatus. Similarly, these behavioral disturbances were also detected in *Caenorhabditis elegans* after exposure to mz as suggested by this fungicide disrupting its swim to crawl locomotor transition (Harrison Brody et al., 2013), which may very likely be linked to mitochondrial dysfunctions and increased production of ROS (Todt et al., 2016). In addition, motor impairments related to an altered synaptic transmission in the developing cerebellar cortex were also observed in mice prenatally exposed to mz (Miranda-Contreras et al., 2005). In line with the above findings, recent works have reported the ability of Maneb to account for failure of motor activity and motor coordination in rats (Tinakoua et al., 2015) thus suggesting such behaviors as a major target of Mn-containing dithiocarbamates. Among the behavioral difficulties reported in the present study, it was interesting to note the onset of anxiety-like behaviors in mz-treated wrasses. Indeed, the Light/Dark test revealed not only a great reduction of the permanence in the light compartment of the apparatus but also an evident enhancement of risk assessments, which corroborate an additional element of anxiogenic performances as previously indicated in fish exposed to copper and mercury (Maximino et al., 2011; Zizza et al., 2016).

It is known that exposure of fish to pesticides is often related to severe behavioral deficits (Bonansea et al., 2016) deriving in many cases from damages of specific brain areas (Pereira and de Campos Júnior, 2015). Even for this fungicide, its elevated toxic potentiality seems to be directed at the neuronal level especially since Mn and Zn, which are part of the molecular complex, are neurotoxic themselves (Apaydin et al., 2016; Eom et al., 2016). Indeed, the behavioral

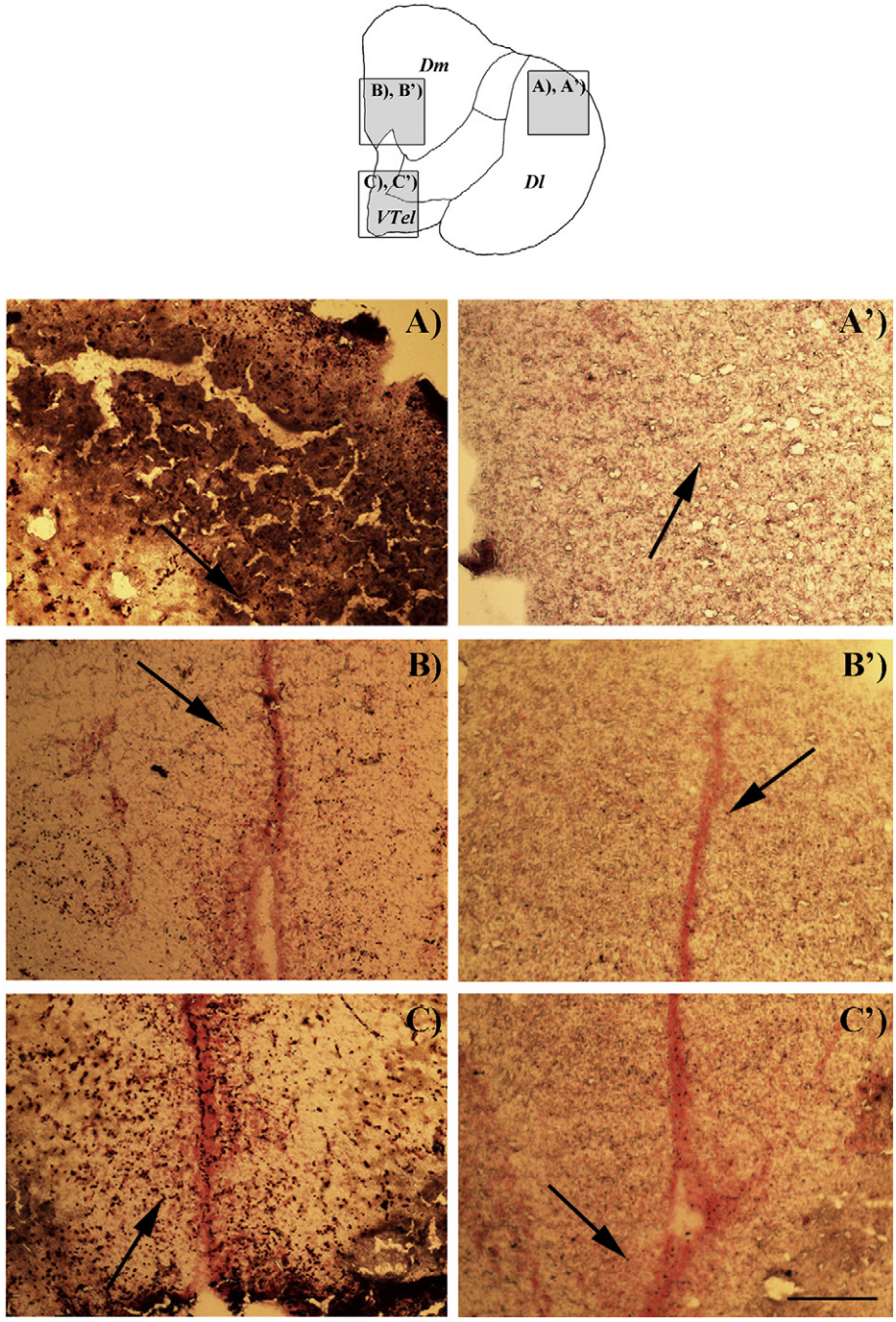
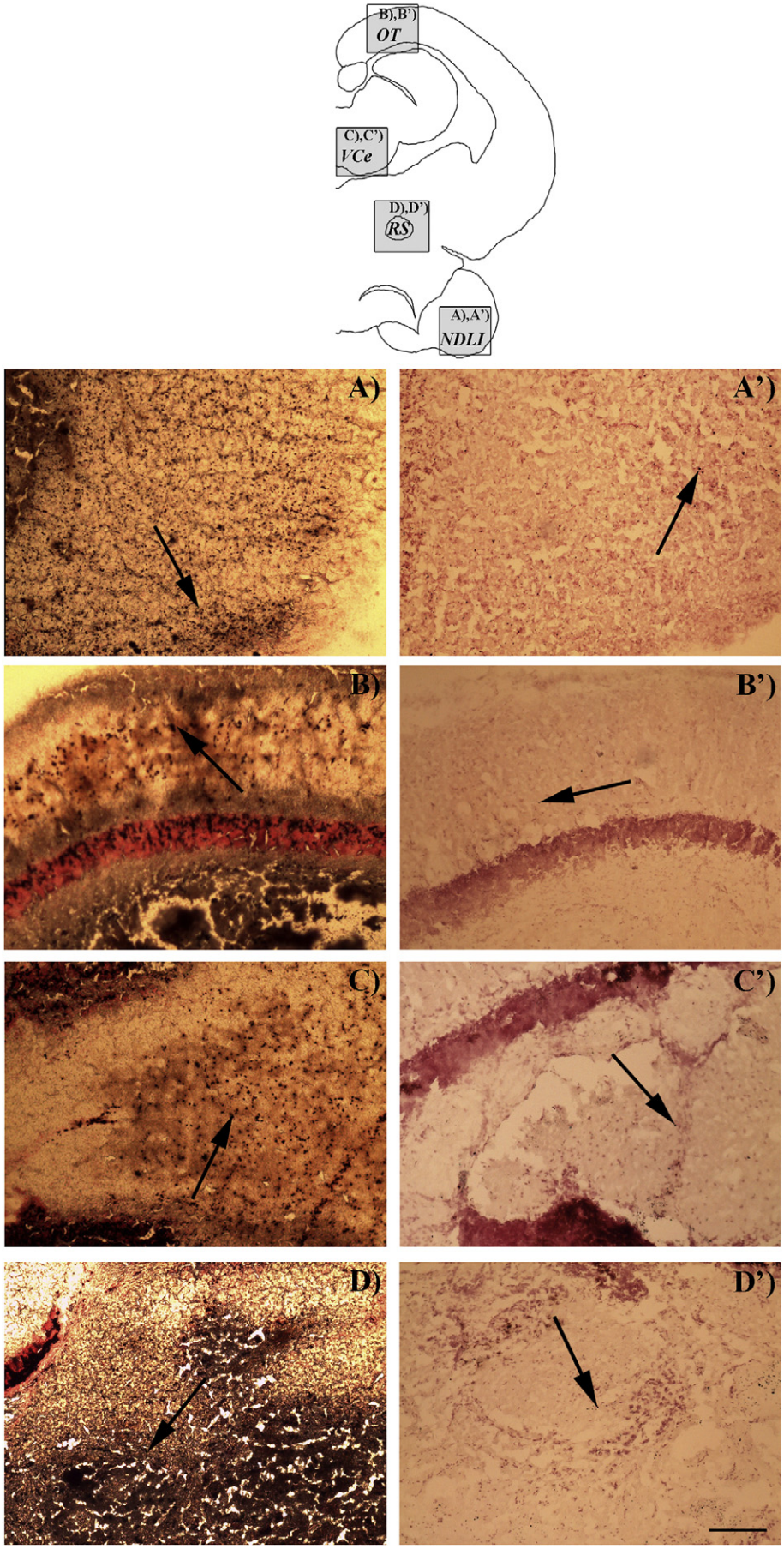


Fig. 4. Neurodegeneration in the telencephalic areas of *Thalassoma pavo* following mz exposure. ACS photomicrographs (and the relative scheme) of some brain sections of fish exposed to 0.2 mg/l of mz (n = 5) displayed argyrophilic signals (arrows) consisting of dark granules in Dl (A), Dm (B), VTel (C) with respect to only few granules observed in the respective areas (A′, B′ C′) of controls (n = 5). Scale bar: 100 μm.

alterations of our wrasses tend to preferentially point to encephalic damages in motor-related regions such as telencephalon, OT, cerebellum and RS as shown by their intense argyrophilic reaction after mz exposure. In particular, the latter nucleus belonging to the reticular formation is the largest source of descending signals to the spinal cord that are involved in initiation and directional control over the fast escape behavior in teleosts (Babin et al., 2014). Following this line, it has been also demonstrated that motor disturbances may be due to degeneration of certain categories of cells such as the astrocytes of the nigro-striatal circuit known to actively control motor functions (Tatsumi et al., 2016). In this context, it may very well be that astrocytes play a major role during toxic reactions especially if we consider their protective role against neurotoxicants via a notable release of ATP in age-related neurodegeneration (Kubik and Philbert, 2015). Furthermore, it appears that mz and other Mn-containing dithiocarbamates (Maneb) may induce additive toxic effects on enhanced nuclear factor

Fig. 5. Neurodegeneration in the medio-posterior brain areas of *Thalassoma pavo* following mz exposure. ACS photomicrographs (and the relative scheme) of some brain sections of fish exposed to 0.2 mg/l of mz (n = 5) displayed argyrophilic signals (arrows) consisting of dark granules in NDLI (A), OT (B), VCe (C) and RS (D) with respect to only few granules observed in the respective areas (A′, B′, C′, D′) of controls (n = 5). Scale bar: 100 μm.


B),B')
OT
C),C')
VCe
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RS
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A)
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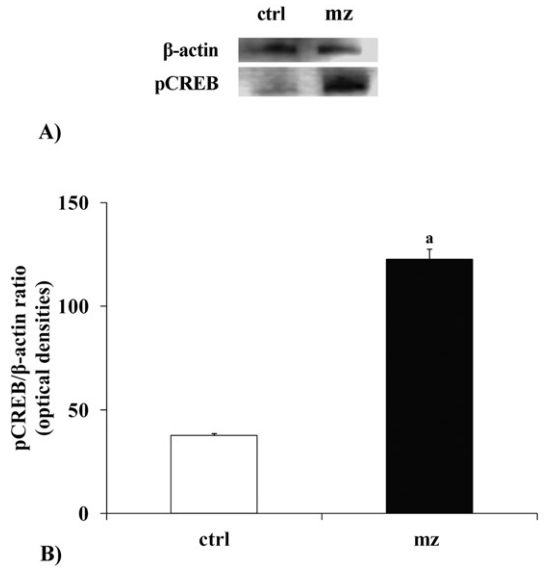

Fig. 6. The effects of mz on pCREB expression in *Thalassoma pavo* brain. Data were reported as optical densities of pCREB bands that were normalized by using β-actin values (A) in fish exposed to 0.2 mg/l of mz (n = 5) and compared to controls (ctrl; n = 5; B). Statistical differences were evaluated by using unpaired Student *t*-test. $^{a}p < 0.005$.

(NF-κB)-dependent dopaminergic cell damages triggered by sub-toxic doses of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPP(+), an active metabolite of the Parkinsonian mimetic MPTP (Williams et al., 2013).

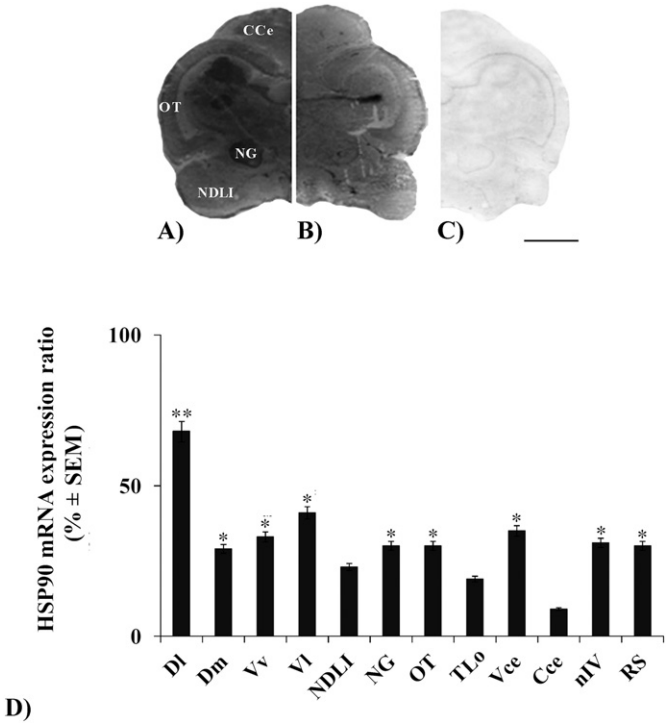

Fig. 7. HSP90 transcriptional changes in some encephalic nuclei of *Thalassoma pavo* following exposure to mz. Representative medio-posterior brain sections labeled with 100 ng of HSP90 antisense probes of treated (A) and control (B) fish were compared to the sense probe (C). Scale bar: 1.25 mm. Data were reported as expression ratio (% ± SEM) of HSP90 mRNAs in some brain sites of fish exposed to 0.2 mg/l of mz for 96 h (n = 5) with respect to controls (ctrl; n = 5). Statistical differences were evaluated by using unpaired Student *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$. Abbreviations: CCe, corpus of the cerebellum; Dl, lateral part of the dorsal telencephalon; Dm, medial part of the dorsal telencephalon; NDLI, diffuse nucleus of the inferior lobe; NG, nucleus glomerulosus; nIV, trochlear nerve nucleus; OT, optic tectum; RS, superior reticular nucleus; TLo, torus longitudinalis; VCe, valvula of the cerebellum; Vl, lateral part of the ventral telencephalon; Vv, ventral part of the ventral telencephalon.

It was noteworthy that a marked activation of pCREB was reported during exposure to this fungicide, which may underlie a neuroprotective role in the fish brain exposed to contaminants. Such a feature tends to go in a similar direction of the abundant expression of this transcription factor being responsible for the activation of synaptic plasticity together with neuronal survival (Kitagawa et al., 2012). Moreover, and of greater importance is that its activation following the phosphorylation of serine 133 induces gene expression of survival factors thus yielding neurons resistant to subsequent severe ischemia. Recently, increased expression levels of such a protein have been associated with enhanced hippocampal neurogenesis induced by environmental enrichment in adult rats (Zhang et al., 2016). In the case of fish, the abundance of pCREB in all known neurogenic regions seems to be responsible for the triggering of cell proliferation and modulation of embryonic brain development as reported for zebrafish (Dworkin et al., 2007). It is thus tempting to speculate that a conspicuous presence of pCREB in mz-exposed wrasses, by stimulating neurogenesis, assures tolerance against toxic conditions along with neuroprotective ability toward the repairing of brain damages.

In a same manner, it is plausible that the up-regulation of HSP90 mRNA, detected in many encephalic nuclei after mz-exposure, may constitute a part of the pro-survival program activated by pCREB. Such a feature is strongly supported on the one hand by HSP90/Akt/CREB pathways upregulating the glial cell line-derived neurotrophic factor, a protein used for the treatment of neurodegenerative disorders such as Parkinson's disease (Cen et al., 2006) and on the other hand by CREB-dependent transactivation of HSP70.3 above all during heat-shock/ischemia-like conditions (Sasi et al., 2014). This should not be so surprising since studies confirm that high levels of HSPs are involved with protective mechanisms against different stressful conditions (Mahanty et al., 2017), including hypoxia (Giusi et al., 2012). In addition, our recent findings demonstrated that high HSP90 mRNAs are precocious elements activating protective events in the brain of both marine and freshwater fish especially after a recovery period from exposure to some heavy metals like copper (Zizza et al., 2014, 2016). At the same time, it has been reported that pesticides are also responsible for the activation of HSP90 in other teleosts (Peng et al., 2015; Xing et al., 2015) thus proposing this chaperone as a crucial biomarker of environmental contamination.

Taken together, these first results on mz-dependent motor deficits and anxiety-like states of a marine fish point to interesting molecular and behavioral responses adopted by wrasses during toxic conditions. In particular, the contemporary activation of pCREB and HSP90 during behavioral alterations strengthen them as early indicators of aquatic contamination that may assure cellular tolerance with eventual repairing of brain damages following exposure to mz. Hence, the present study would surely benefit from evidences on cross-talking ability of the above molecular factors with some neuropeptidergic systems, which will be considered in a future work (MS in preparation). This type of condition may be achieved by their interactions with key neuropeptides like orexin (ORX) as previously demonstrated (Sokołowska et al., 2014), very likely in concert with other neuroreceptor circuits (Facciolo et al., 2011; Crudo et al., 2013). The fact that the ORXergic system may exert a key role on encephalic neurogenic events in fish during pesticide toxicity is turning out to be of major concern given that ORXs play a determinant role during stressful responses against metal contamination (Zizza et al., 2011, 2014, 2017). We are still at the beginning but our results plus recent findings on a genetic hazard to fish contaminated by mz may encourage biomonitoring programs of aquatic ecosystems and regulatory policies regarding the utilization of this agrochemical (Marques et al., 2016).

Transparency document

The Transparency document associated to this article can be found, in the online version.

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Keywords: manganese/zinc-ethylene-bis-dithiocarbamate; feeding latency; food intake; orexin receptor; hypothalamic degeneration.

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Abstract: Food intake ensures energy resources sufficient for basic metabolism, immune system and reproductive investment. It is already known that food-seeking performances, which are crucially controlled by orexins (ORXs), may be under the influence of environmental factors including pollutants. Among these, mancozeb (mz) is becoming an environmental risk for neurodegenerative diseases. Due to few studies on marine fish exposed to mz, it was our intention to correlate feeding latency, food intake and feeding duration to potential neurodegenerative processes in key hypothalamic sites and expression changes of the ORX neuroreceptor (ORXR) in the ornate wrasses (Thalassoma pavo). Hence, fish exposed for 96h to 0.2 mg/l of mz (deriving from a 0.07, 0.14, 0.2, 0.3 mg/l screening test) displayed a moderate reduction ($p<0.05$) of food intake compared to controls as early as 24h, which became more evident ($p<0.01$) at 72h. After 96h food intake was only moderately reduced. Moreover, significant enhancements of feeding latency were reported at 24h up to 72h ($p<0.001$) and even feeding duration was enhanced up to 72h ($p<0.001$) by then becoming moderately increased at 96h. Additionally, a reduction (-80%; $p<0.01$) of body weight was also detected at the end of exposure. Likewise, a notable ($p<0.001$) activation of ORXR protein occurred together with mRNA upregulations in hypothalamic areas such as the diffuse nucleus of the inferior lobe (+48%) that also exhibited evident degenerative neuronal fields. Overall, these results highlight an ORX role as a vital component of the neuroprotective program under environmental conditions that can interfere with feeding behaviors.

Highlights

- **Food intake was reduced by 0.2 mg/l of mancozeb**
- **Mancozeb accounted for enhancements of feeding latency and duration**
- **A reduction of fish body weight occurred after 96h of mancozeb exposure**
- **Neurodegeneration was detected in hypothalamic sites after mancozeb exposure**
- **Mancozeb induced increases of orexin receptor mRNA and protein in fish brain**

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Orexin receptor expression is increased during mancozeb-induced feeding impairments and neurodegenerative events in a marine fish

Running title:

Mancozeb effects on feeding and ORX neuroreceptor in fish

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Abstract

Food intake ensures energy resources sufficient for basic metabolism, immune system and reproductive investment. It is already known that food-seeking performances, which are crucially controlled by orexins (ORXs), may be under the influence of environmental factors including pollutants. Among these, mancozeb (mz) is becoming an environmental risk for neurodegenerative diseases. Due to few studies on marine fish exposed to mz, it was our intention to correlate feeding latency, food intake and feeding duration to potential neurodegenerative processes in key hypothalamic sites and expression changes of the ORX neuroreceptor (ORXR) in the ornate wrasses (*Thalassoma pavo*). Hence, fish exposed for 96h to 0.2 mg/l of mz (deriving from a 0.07, 0.14, 0.2, 0.3 mg/l screening test) displayed a moderate reduction ($p<0.05$) of food intake compared to controls as early as 24h, which became more evident ($p<0.01$) at 72h. After 96h food intake was only moderately reduced. Moreover, significant enhancements of feeding latency were reported at 24h up to 72h ($p<0.001$) and even feeding duration was enhanced up to 72h ($p<0.001$) by then becoming moderately increased at 96h. Additionally, a reduction (-80%; $p<0.01$) of body weight was also detected at the end of exposure. Likewise, a notable ($p<0.001$) activation of ORXR protein occurred together with mRNA upregulations in hypothalamic areas such as the diffuse nucleus of the inferior lobe (+48%) that also exhibited evident degenerative neuronal fields. Overall, these results highlight an ORX role as a vital component of the neuroprotective program under environmental conditions that can interfere with feeding behaviors.

Keywords

manganese/zinc-ethylene-bis-dithiocarbamate; feeding latency; food intake; orexin receptor; hypothalamic degeneration.

1. Introduction

Food intake and energy metabolism result to be vital for the survival of an organism since these processes ensure optimal allocation of energy resources to cover the basic maintenance of metabolism and immune system, somatic growth and reproductive investment (Rønnestad et al., 2017). External factors, such as temperature and photoperiod, stress, predators, and food availability, as well as internal factors, such as genetics, life stage, gut filling, and stored energy tend to play a major role on the success of feeding habits. In this context the regulation of food intake is mainly elicited by the hypothalamus, a key brain hub containing neurons that express neuropeptides involved in the regulation of feeding and energy homeostasis via the integration of peripheral signals (Volkoff, 2014). Among orexigenic neuropeptides, great attention has been recently directed to orexins (ORXs) for their crucial role in appetite stimulation (Facciolo *et al.*, 2011; Gao and Hermes, 2015) but even for the ability to influence reward-based feeding behaviors from fish to mammals (Facciolo et al., 2012; Sakurai, 2014). As reported above food-seeking performances may be influenced by a plethora of environmental factors including dangerous pollutants that represent a serious threat for the various behaviors (Giusi et al., 2010). This is particularly true for fish that are continuously exposed to hazardous toxins, such as agricultural chemicals that may produce consequences on different fish organs (Atamaniuk et al., 2014; Ren et al., 2016) and indirect effects to entire fish communities (Giaquinto et al., 2017).

Contextually, studies have shown that carbamates may induce different toxic effects on aquatic vertebrates such as reduction of swimming speed together with the inhibition of biochemical parameters during both adulthood (Shuman-Goodier and Propper, 2016) and early-life stages (Andrade et al., 2016). Among carbamates, mancozeb (manganese (Mn)/zinc (Zn) -ethylene-bis-dithiocarbamate; mz), a fungicide belonging to ethylene-bis-dithiocarbamate compounds (EBCD), is composed of different sub-compounds (Mn, Zn plus ethylene thiourea) that together account for multiple toxic mechanisms operating simultaneously during its exposure (Geissen et al., 2010). Indeed, it is known to be a potent endocrine disruptor (Thienpont et al., 2011), as well as an

inductor of oxidative damage to lipids/proteins in different organs of fish (Atamaniuk et al., 2014) and a neurotoxicant by inducing both behavioral deficits and neuronal vulnerability (Harrison Brody et al., 2013). Such a pesticide is particularly dangerous for aquatic life since it may reach the sea from the nearby agricultural lands or by means of contaminated rivers. For this reason, mz is recognized as a marine pollutant by the Pesticide Properties Database of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/424.htm). In this context, our recent study highlighted it as an inductor of anxiety-like states and motor disturbances linked to neuronal alterations of the marine fish *Thalassoma pavo* (Zizza et al., 2017a). At date, no toxicological studies are conducted on any other marine fish contaminated by mz especially regarding neuronal effects associated to a vital physiological activity such as feeding.

On this basis, it was our intention to examine effects of a 96h exposure of mz (0.2 mg/l) on feeding-related performances (i.e. feeding latency, food intake and feeding duration) of the ornate wrasses (*Thalassoma pavo*). This concentration was chosen on the basis of a preliminary screening test of sub-lethal concentrations (0.07, 0.14, 0.2, 0.3 mg/l) handled in our laboratory. These doses are in line with those used in other studies (Jarrard et al., 2004) along with environmentally relevant concentrations detected in waterbodies near agricultural fields (Shenoy et al., 2009). At the same time, potential neurodegenerative processes in key hypothalamic sites and expression changes of the ORX neuroreceptor (ORXR) of such a fish species were also investigated by applying both *in situ* hybridization and western blotting methods. Results deriving from behavioral and neuromolecular evaluations may provide first evidences about the ability of such a fungicide to influence fish feeding behaviors and contextually highlighting neuronal responses of the ORXergic neuroreceptor system that studies have indicated as a key biomarker of stressful conditions (Pavlidis et al., 2015) including neurotoxic pollutants (Zizza et al., 2013, 2014).

2. Materials and Methods

2.1 Animal Housing

Before starting treatments, adult female specimens of *Thalassoma pavo* (body weight 7-13 g, body length 8-11 cm), obtained from a local supplier, were acclimated for at least 1 week in 80 l aquaria under a natural photoperiod in aerated and filtered seawater. During acclimation fish were fed once a day with small pieces of frozen prawns corresponding to 2% of the biomass in the tank. Water quality parameters i.e. salinity (35%) density (1.027-1.028 g/cm^3) hardness (100 mg $CaCO_3$/l) and dissolved oxygen (8-8.6 mg/l) as well as temperature (20-22 °C) plus pH (7.5-8.0) were daily monitored to verify that they remained within the appropriate ranges. Animal maintenance and experimental procedures complied with the legislative law n°116 (27-01-1992) and with European Directive (2010/63/EU) for the correct use of laboratory animals. Efforts were made to minimize animal suffering and reduce number of fish used.

2.2 Mancozeb exposure

The determination of the most effective sub-lethal concentration was based on a screening of different mz (Sigma, Milan-Italy) concentrations (0.07, 0.14, 0.2, 0.3 mg/l) within an 96h period in which food intake was used as a behavioral endpoint. From this screening, fish were exposed for 96h to the nominal non-lethal concentration of 0.2 mg/l dissolved in seawater (n=15) and compared to untreated controls (n=15). Such a concentration derived from solubility indications of mz in seawater obtained by an ICP-MS analytical procedure in the same fish species that displayed evident motor alterations (Zizza et al., 2017a) as well as falling within the same range used by others (Jarrard et al., 2004).

A *static renewal exposure procedure* was chosen, in which daily renewal of the fungicide concentration in seawater, was conducted according to standard procedure guidelines (American Society for Testing Material, 2014). This type of exposure system, together with a basic pH and a relatively high temperature, guarantees a constant pesticide concentration within 24h since degradation of mz occurs at a later time, i.e. 39h (López-Fernández et al., 2017). Chemical filters were not used to prevent reduction of mz concentration and aquaria were only equipped with

aerator to ensure an optimal oxygen concentration. Water parameters were monitored even during treatment to ensure that they remained within adequate ranges. During exposure animals were fed as above according to our previous toxicological studies (Giusi et al., 2008; Zizza et al., 2014)

2.3 Feeding performances

The following parameters were observed 1h, 24h, 48h, 72h and 96h after a mz exposure:

- **Food intake:** daily, residual food was recovered, dried, weighed and compared to the initial quantities supplied to fish in order to evaluate the quantity of food consumed as previously reported (Facciolo et al., 2011).
- **Feeding latency:** time needed for fish to approach food (Kuz'mina, 2011) within a 15 min interval observation session after having assured that all fish reached food sources.
- **Feeding duration:** total time spent executing feeding maneuvers including both complete and incomplete feeding acts during 1h observation (Abbott and Volkoff, 2011).

In addition, fish body weights were measured at the begin and at the end of mz exposure (96h) and compared with those of controls. The feeding parameters were obtained using a digital camera (SONY, DSC-W310) and video were analyzed by the behavioral Software Etholog 2.2.5 (Visual basic; Brazil). Values were expressed as mean activity ± standard error of mean (SEM) versus their controls. At the end of the behavioral session and before molecular procedures, fish were checked for sexual identification by morphological observations of the ovaries that did not show any sign of atresia that could have indicated an initial transition to the testicular growth (Liu et al., 2016).

2.4 Neurodegenerative analysis

A neurodegenerative analysis was applied, using Amino Cupric Silver (ACS) technique, to assess if mz-mediated feeding disturbances coincided with neuronal damages in critical hypothalamic areas that control feeding-related functions. Teleosts receiving mz for 96h (n=5) plus untreated fish (n=5)

were sacrificed at the end of the behavioral session and brains removed, frozen on dried ice and stored at -20°C. Subsequently, brains were mounted on a freezing stage of a sliding cryostat (Microm-HM505E; Zeiss, Wallford, Germany) to obtain a series of 30 μm sections for ACS protocol. Briefly, washed brain sections underwent the different ACS phases as previously described (Zizza et al., 2014). The sections were then transferred to a rapid fixer solution (5 min) and counterstained with 0.5% neutral red (Carlo Erba, Milan, Italy) for 25 min, dehydrated in ethanol (50-100%) plus in xylene, and mounted with DPX (*p*-xylene-bis[*N*-pyridinium bromide]; Sigma) for microscope (Leitz, Stuttgart, Germany) observation along with acquisition of the photomicrographs.

2.5 In situ hybridization method

In situ hybridization was performed to check transcriptional variation of ORXR due to mz exposure. For this aim a specific oligonucleotidic antisense DNA probe, previously designed (Facciolo et al., 2009) on the basis of ORXR partial nucleotide sequence of *Thalassoma pavo* (GenBank cod. EF547365.1) was used. Such a probe was labeled at the 3'-tailing with digoxigenin-11-dUTP (DIG, Roche Diagnostics, Monza - Italy). At the end of the behavioral session fish exposed for 96h to mz (n=5) plus controls (n=5) were sacrificed as described above. Subsequently, brains were rapidly removed, stored at −20°C and mounted on a cryostat freezing stage (Microm-HM505E; Zeiss) to obtain a series of coronal sections of 14 μm appropriate for the *in situ* hybridization protocol. Sections were incubated with a 100-ng of ORXR antisense probe overnight at 50 °C in a humidified chamber. Immunological detection using an anti-digoxigenin antibody (1:100) was obtained as previously reported (Zizza et al., 2014). Hybridization signals, measured as optical densitities (OD ± SEM), observed in a bright-field Dialux EB 20 microscope (Leitz) were determined in duplicates on each brain antimere of 6 brain sections for anterior plus posterior brain slides for at least three different experiments as previously described (Zizza et al., 2016). Expression levels of ORXR mRNA were obtained by using an Image software of the National

Institutes of Health (Scion Image 2.0), in which an internal standard was used for OD calibration. Background level was estimated and included in all final calculations. The different encephalic nuclei were identified using perciformes atlases (Cerdà-Reverter et al., 2001a,b; 2008).

2.6 Protein extraction and western blot analysis

Brain tissues of exposed (n=5) fish as well as controls (n=5) were homogenizated and lysed for 30 min on ice using RIPA lysis buffer containing a mixture of phosphatase and protease inhibitors (Santa Cruz Biotechnology, Milan-Italy). Homogenates were centrifuged at 12000 g for 20 min at 4 °C and total protein amounts of samples were measured by the BCA protein assay reagent kit (PIERCE, Milan- Italy).

50 µg of proteins of each sample were boiled for 5 min in SDS buffer [50 mM Tris-HCl (pH 6.8), 2 g 100 ml^{-1} SDS, 10% (v/v) glycerol, 0.1 g 100 ml^{-1} Bromophenolblue], separated on 10% SDS-PAGE and transferred to a PVDF membrane for blotting (Trans-Blot® Semi-Dry Transfer Cell, Biorad) as previously reported (Forte et al., 2016; Zizza et al., 2017a). Subsequently, membranes were incubated for 1 h at room temperature with a blocking buffer (TBS, 0.05% Tween-20 and 5% milk). After blocking, followed an overnight incubation of membranes with rabbit anti-ORX_1R (Abcam, ab68718) or rabbit anti-β-actin (Santa Cruz Biotechnology, Milan - Italy) antibodies diluted 1:200 in TBS-T containing 2% milk at 4° C. The membranes were washed four times for 10 min in TBS, 0.05% Tween-20 before a 1h incubation with goat anti-rabbit IgG (HRP) (1:3000; Abcam ab-6721) secondary antibody diluited in TBS-T containing 2% milk. The membranes were washed four times and specific protein bands were detected with chemiluminescence (ECL, Santa Cruz, Milan - Italy) using C-DiGit Chemiluminescent Western Blot Scanner (LI-COR). Western blots were analyzed using Image Studio Software to determine OD of the bands. OD reading was normalized to β-actin to account for variations in loading. All experiments were performed in biological and technical triplicates.

227 *2.7 Statistical analysis*

228 Statistical differences between mz-exposed fish with respect to controls were evaluated, in the case
229 of the screening for food intake, by using a two-way repeated measures for ANOVA with
230 differences being established by Scheffe's *post hoc* comparison test when there was a significant p
231 value <0.05. Moreover, a one-way repeated measures for ANOVA with differences being
232 established by Scheffe's *post hoc* comparison test when there was a significant p value <0.05 was
233 carried out for both feeding latency and duration. For all other experimental data, an unpaired
234 Student's t-test with a significant level of $p<0.05$ was handled. The determination of the number of
235 animals for the present study was conducted using a free online statistical program
236 (http://stat.ubc.ca/~rollin/stats/ssize/n2.html; Department of Statistics of the University of British
237 Columbia-Canada) in which a 95% power corresponded to a sample size of at least 5 and 15
238 individuals for molecular/neurodegenerative and behavioral analyses, respectively, when a 2-sided
239 5% level of significance was used.

240

241 **3. Results**

242 *3.1 Effects on feeding behavior*

243 In order to determine the most effective sub-lethal mz exposure, a food intake screening of different
244 concentrations (0.07, 0.14, 0.2, 0.3 mg/l) within an 96h period was handled. From the time course
245 graph (Fig. 1), it appeared that only 0.2 and 0.3 mg/l significantly influenced food consumption
246 ($F_{(4,21)} = 2.85$; $p<0.05$). Taking into consideration that the latter concentration led to death in some
247 treated fish, even though in an order of <10%, we decided to use 0.2 mg/l since it was able to
248 induce evident behavioral effects without causing death. With this concentration, no significant
249 variation was detected after 1h of exposure in mz-treated fish with respect to controls. Indeed, a
250 moderate reduction ($p<0.05$) of food intake occurred after 24h and remained constant after 48h of
251 treatment. Such an effect resulted notably significant ($p<0.01$) at 72h, when a strong decrease of the
252 percentage of ingested food was observed in treated fish compared to controls that instead

consumed almost all their food. Conversely, after 96h of mz exposure food intake was only moderately reduced. During the entire feeding observations no cannibalism was registered.

- please insert figure 1 here-

Moreover, exposure to 0.2 mg/l of mz determined significant enhancements of feeding latency during the entire period of exposure ($F_{(1,24)} = 4.27$; $p<0.05$). Indeed, such an effect appeared at 24h by causing a very strong increase ($p<0.001$) of time spent to reach food with respect to controls that instead went immediately toward food sources (Fig. 2). Interestingly enough, after 48 and 72h of exposure this alteration was maintained within the same significant extent ($p<0.001$), despite a numerically lower variation with respect to 24h. Only after 96h, it seemed as though the latency time would be reduced, even if maintaining a notable enhancement ($p<0.01$) of time spent to reach food.

- please insert figure 2 here-

Contextually, it was also interesting to note that treated fish spent more time in feeding maneuvers with respect to controls ($F_{(1,24)} = 4.31$; $p<0.05$). Indeed, feeding maneuvers resulted to be notably enhanced ($p<0.01$) at 24 and 48h, which very strongly further increased ($p<0.001$) at 72h (Fig. 3). On the other hand, such a trend was inverted thus achieving only a moderate increase ($p<0.05$) when compared with controls at 96h. Additionally, a significant reduction (-80%; $p<0.01$) of body weight was detected in treated fish after 96h of mz exposure with respect to controls that instead gained weight during the same experimental period (Fig. 4).

- please insert figure 3 and 4 here-

3.2 Neurodegenerative phenomena

Following the application of ACS methods it was possible to observe notable degenerative phenomena induced by the fungicide in some diencephalic neuronal fields of treated fish after 96h of exposure. Indeed, a marked argyrophilic reaction was reported in the periventricular nuclei of the hypothalamus. In particular, the anterior tuberal nucleus (NAT) displayed a conspicuous number of

degenerated elements (Fig. 5A). At the same time, even more degenerative neuronal fields were observed in the lateral part of the diffuse nucleus of the inferior lobe (NDLIl) and nucleus of the lateral recess (NRL) (Fig. 5B, C) along with in the nucleus glomerulosus (NG) and in the commissural preglomerular nucleus (NPGc; Fig. 5D, E) rather than controls that displayed no sign of neurodegeneration since only a few, if any, damaged cells were visible (Fig. 5F).

- please insert figure 5 here-

3.3 Effects on ORXR expression

Interestingly, feeding alterations were linked to an evident increase of ORXR expression at both transcriptional and protein levels. In particular, from *in situ* hybridization analysis an overall up-regulation of ORXR mRNA was reported in mz-treated fish compared to controls as shown by antisense signals in the brain section of control (Fig. 6A) and treated fish (Fig. 6B). More specifically, increased transcriptional levels ($p<0.01$) were detected in the medial part of the dorsal telencephalon (Dm, +60%) and in the optic tectum (OT; +81%; Fig. 6C). On the other hand, only moderate up-regulations ($p<0.05$) were observed in the lateral part of the dorsal telencephalon (Dl, +48%), in the ventral part of the ventral telencephalon (Vv, +32%), in NDLI (+48%), NRL (+30%) NAT (+38%), NG (+38%), NPGc (+31%) as well as in the corpus (Cce, +40%) and valvula (Vce, +31%) of the cerebellum.

The increased expression of the above mRNA appeared to tightly coincide with an evident neuronal increase of ORXR protein after mz exposure. Indeed, from the densitometric evaluation (OD) of western blot bands (Fig. 7A), a very strong ($p<0.001$; Fig. 7B) enhancement of such a receptor was reported in mz-treated fish when compared with controls after normalization with β-actin bands.

- please insert figures 6 and 7 here-

4. Discussion

The present work examined, for the first time, feeding alterations evoked by EBCD mz in the marine fish *Thalassoma pavo*, which contextually highlighted an involvement of the ORXergic

system in the adaptive measures against neuronal damages in feeding-related encephalic sites. In particular, during an exposure of 96h, this pesticide exerted potent inhibitory effects on feeding activities thereby heavily disturbing all of the considered behavioral parameters. Such effects are in line with feeding impairments reported in this same fish following treatments with other toxic agents (Zizza et al., 2014) including pesticides (Giusi et al., 2010). In the present study notable enhancements of feeding latency, accompanied by significant reductions of food intake, were observed. One explanation for such effects may be due to toxic actions of mz at the olfactory level thus causing a delay in food perception and a consequent reduction of the amount of food consumption. In particular, it seems that exposure to mz accounts for a concentration-dependent reduction of the electro-olfactogram amplitude in response to the odorant L-serine in the Coho salmon (Jarrard et al., 2004). This hypothesis finds further support in numerous evidences suggesting that damages of the olfactory epithelium are one of the main cause of feeding alterations induced by pesticides (Lal et al., 2013). Analogous effects were also reported after treatment with the herbicide atrazine affecting chemosensory responses to odors and thus ability of crayfish to localize food sources (Belanger et al., 2016). Similarly, even exposure to copper tends to impair the ability of fish to respond to amino acids (Baldwin et al., 2011) with an altered latency period for eating and food consumption (Kuz'mina, 2011).

Interestingly enough, search for food (expressed in terms of feeding duration) was notably increased in mz-exposed fish. Even in this case, such a result may be attributed to olfactory toxicity conditions that promote a fasting state, despite their incapacity to efficiently recognize and locate food sources. Aside these effects, fish exhibited difficulties in executing food-seeking maneuvers together with clear signs of loss of equilibrium during swimming gait that is in good agreement with disturbed locomotor activity (spontaneous exploration and evident immobility states) not only after 96h of mz exposure (Zizza et al., 2017a) but also after a shorter period (Harrison Brody et al., 2013). Motor dysfunctions constituting the basis of altered feeding capabilities should not be so surprising since exposure to environmental pollutants has been frequently linked to impaired

predator-prey interactions (Monde et al., 2016). Indeed, predator avoidance is very often altered by sub-lethal exposure to toxicants as pointed out by reduced prey survival and increased susceptibility behaviors toward predation (Scott and Sloman, 2004). In this context, feeding alterations due to motor deficits may represent an additional convincing condition since the Vce and medial nucleus of reticular formation, which control motor functions, were severely damaged by mz (Zizza et al., 2017a). Accordingly, a marked degeneration in different diencephalic nuclei, such as NDLIl, NG and NPGc, strengthened the harmful neurotoxic effects of this fungicide as also reported by others (Harrison Brody et al., 2013). In particular, damages of the multisensory system NDLI, which is noted for receiving gustatory and visual inputs (also via NG) plus showing a reciprocal connection with the reticular formation (Ahrens and Wullimans, 2002), together with the feeding-related preglomerular complex (Kato et al., 2012) did not allow our fish to correctly orient toward food items. Moreover, even degeneration of NRL and NAT neuronal fields tends to interfere with normal neuropeptide and neurotransmitter activity, considered pivotal for the regulation of feeding and locomotor performances (Canosa et al., 2011).

The fungicide showed to be highly responsible for the enhancement of both ORXR mRNA and protein that in our opinion may be viewed as a compensation against mz inhibition of food-intake. It is already known that during brief periods of fasting an elevated production of prepro-ORX mRNA occurs in the hypothalamus of fish (Chen et al., 2011). At the same time, brain mRNA expression of ORX was also reported to be higher in fasted dourado at both feeding time and one-hour post feeding (Volkoff et al., 2016), which is in line with that observed in other fish species (Rønnestad et al., 2017; Volkoff, 2014). To our knowledge, this is a first study highlighting fasting as an inductor of ORXR up-regulation in fish, since perhaps an activation of the receptor, sequential to an increase of the neuropeptide, may result in initiating food intake as a consequence of food anticipatory activity, which is predicted by the endogenous clock of our fish (López-Olmeda et al., 2012). Interestingly, the fact that increased expression of ORXR also occurs in neuron-ablated larvae of zebrafish tends to underlie a compensatory reaction suggesting not only that ORXRs are

360 intact but also more sensitive to ORXergic signals (Elbaz et al., 2012). Moreover, the up-regulation
361 of the receptor transcript plus neurodegenerative phenomena following $CuCl_2$ (Zizza et al., 2014)
362 and lead (Zizza et al., 2013) exposure were previously reported in the same fish species displaying a
363 reduction of feeding episodes. It may be also plausible that an enhanced production of ORXR is
364 part of a neuroprotective program activated against chemical toxicity for both metals and mz. Such
365 a neuroprotective role of the ORXergic system is in good agreement with other studies
366 demonstrating the ability of ORX-A to diminish palmitic acid-induced cell death like that in
367 hypothalamic cells of mice via reduced caspase 3/7 apoptosis, stabilization of Bcl-2 gene expression
368 and a diminished Bax/Bcl-2 gene expression ratio (Duffy et al., 2016). Furthermore, ORX-A
369 treatment attenuated MPP(+)-induced cell injury in a cellular model of Parkinson's disease (Feng et
370 al., 2014), along with inducing significant reductions of infarct size and macrophage/microglial
371 infiltration in mice subjected to transient middle cerebral artery occlusion (Xiong et al., 2013).
372 According to these results, a recent study also demonstrated that ORX-A elicits, via type 1 receptor,
373 significant neuroprotective effects against 6-hydroxydopamine-induced SH-SY5Y cell damage as
374 an *in vitro* model of Parkinson's disease (Pasban-Aliabadi et al., 2017). On the other hand, even
375 ORX-B is able to exert protective effects for dopamine neurons through the activation of $ORXR_2$
376 (Guerreiro et al., 2015).

377 At this regard, we have recently demonstrated that ORX-A administration is able to rescue
378 some neurobehavioral alterations caused by both short-term and chronic exposure to heavy metals
379 in ornate wrasses (Zizza et al., 2013, 2017b). Following this line, it has been observed that a
380 neuronal response of the ORXergic system overlaps activation of the neuroprotective factor CREB,
381 after mz exposure of the same fish species (Zizza et al., 2017a). In this context, other studies
382 contributed to demonstrate the tight relationship between the above neuropeptidergic system and
383 CREB phosphorylation (Fukushima et al., 2015; Zhang et al., 2015). Indeed, it has been shown that
384 activation of ORX_2R by ORXs administration coincided with long-term phosphorylation of CREB
385 in CHO cells over-expressing such a receptor subtype (Guo and Feng, 2012). Accordingly, long-

lasting synaptic plasticity in ORX-producing neurons of the lateral hypothalamus occurred together with CREB activation suggesting that CREB-mediated pathways may supply a significant contribution to synaptic potentiation in these cells (Rao et al., 2013). In addition, under chemical hypoxia ORXs are able to potentially increase neuronal viability and preserve cortical neurons against oxidative stress via Akt signaling that also involves CREB activation (Sokołowska et al., 2014). Given these evidences plus oxidative stress being one of the main causes of mz-induced neuronal damages (Domico et al., 2007), protective measures in our teleost exposed to mz may be very likely promoted by the ORXergic system via CREB activation. Similarly, the concomitant up-regulation of HSP90 mRNA, after mz-exposure (Zizza et al., 2017a), may be viewed as a part of the pro-survival program activated by pCREB. This should not be so surprising since high levels of HSPs have been included within protective mechanisms against stressful conditions (Mahanty et al., 2017), including hypoxia (Giusi et al., 2012). In addition, it has been widely shown that pesticides are responsible for the activation of HSP90 in other teleosts (Peng et al., 2015; Xing et al., 2015) thus attributing a protective role to such a chaperone that together with ORX-A (Zizza et al., 2017b) may determine a greater resistance of brain cells against oxidative stress (Sokołowska et al., 2014).

Taken together these findings underlie mz-dependent feeding impairments in *Thalassoma pavo*, which may begin to expand our considerations regarding the deleterious effects of this neurotoxin on fish plus highlighting an ORX response against mz neurotoxicity. The latter aspect constitutes an important step from both molecular and toxicological point of view thereby adding novel indications on the ORXergic neurophysiological role, especially during fasting state. Moreover, ORX system plus CREB and HSPs, together with other major neuroreceptor circuits (Crudo et al., 2013; Facciolo et al., 2011), may be considered a vital component of the neuroprotective program operating against chemical toxicity by promoting adaptive and protective processes under such environmental conditions that pose a serious risk for fish.

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Figure legends

Fig. 1

Effects of different mz sub-lethal concentrations on food intake of the ornate wrasses. Food intake was daily measured during 96h of exposure to different mz concentrations (0.07-0.3 mg/l) and reported as percentage (%) ± SEM of food ingested by treated fish (n=15) compared to controls (n=15). Statistical differences were evaluated by using two-way repeated measures ANOVA followed by Scheffe's *post hoc* test when $p < 0.05$. *$p < 0.05$; **$p < 0.01$.

Fig. 2

Mz effects on feeding latency of the ornate wrasses. This parameter was daily measured during 96h of mz exposure (0.2 mg/l) and reported as time (s ± SEM) spent by treated fish (n=15) to reach food with respect to controls (n=15) as described in the Materials and Methods section. Statistical differences were evaluated by using one-way repeated measures ANOVA followed by Scheffe's *post hoc* test when $p < 0.05$. **$p< 0.01$; ***$p<0.001$.

Fig. 3

Mz effects on feeding duration in *Thalassoma pavo*. This parameter was daily evaluated during 96 h of mz exposure (0.2 mg/l) and reported as time (s ± SEM) spent by both treated (n=15) and controls (n=15) fish executing typical food-seeking maneuvers as described in the Materials and Methods section. Statistical differences were evaluated by using one-way repeated measures ANOVA followed by Scheffe's *post hoc* test when $p<0.05$. *$p< 0.05$; **$p<0.01$; ***$p<0.001$.

Fig. 4

Mz effects on fish body weight of the ornate wrasses. Data were expressed as body weight variation (g ± SEM) from the beginning of mz exposure up to 96h in treated fish (n=15) compared to controls (n=15). Statistical differences were evaluated by using unpaired Student's t-test: ** $p<0.01$.

Fig. 5

Neurodegeneration induced by mz exposure in some diencephalic nuclei of *Thalassoma pavo*. Representative photomicrographs of brain sections stained with ACS displaying argyrophilic dark granules (arrows) in NAT (A), NDLIl (B), NRL (C), NG (D), NPGc (E) of treated fish (n=5) compared to NDLIl (F) as representative area of untreated fish (n=5). Scale bar 100 μm.

Fig. 6

Mz effects on ORXR mRNA transcription in some brain nuclei of *Thalassoma pavo*. Representative medio-posterior brain sections labeled with 100 ng of antisense probes of control (A) and (B) treated fish. Scale bar: 2 mm. Data were reported as expression ratio (% ± SEM) of ORXR mRNA in some encephalic nuclei of treated fish (n=5) with respect to controls (n=5; C). Statistical differences were evaluated by using unpaired Student's t-test. *$p<0.05$; **$p<0.01$. Abbreviations: Cce, corpus of the cerebellum; Dl, lateral part of the dorsal telencephalon; Dm, medial part of the dorsal telencephalon; NAT, anterior tuberal nucleus; NDLI, diffuse nucleus of the inferior lobe; NG, nucleus glomerulosus; NPGc, commissural preglomerular nucleus; NRL, nucleus of the lateral recess; OT, optic tectum; TLo, torus longitudinalis; Vce, valvula of the cerebellum; Vl, lateral part of the ventral telencephalon; Vv, ventral part of the ventral telencephalon.

Fig. 7

Mz effects on ORXR protein expression in *Thalassoma pavo* brain. Data were reported as optical densities ratio of ORXR western blotting bands normalized by using β-actin values (A) in treated fish (n=5) compared to controls (ctrl; n=5; B). Statistical differences were evaluated by using unpaired Student's t-test: $^{***}p<0.001$.

Figure 1

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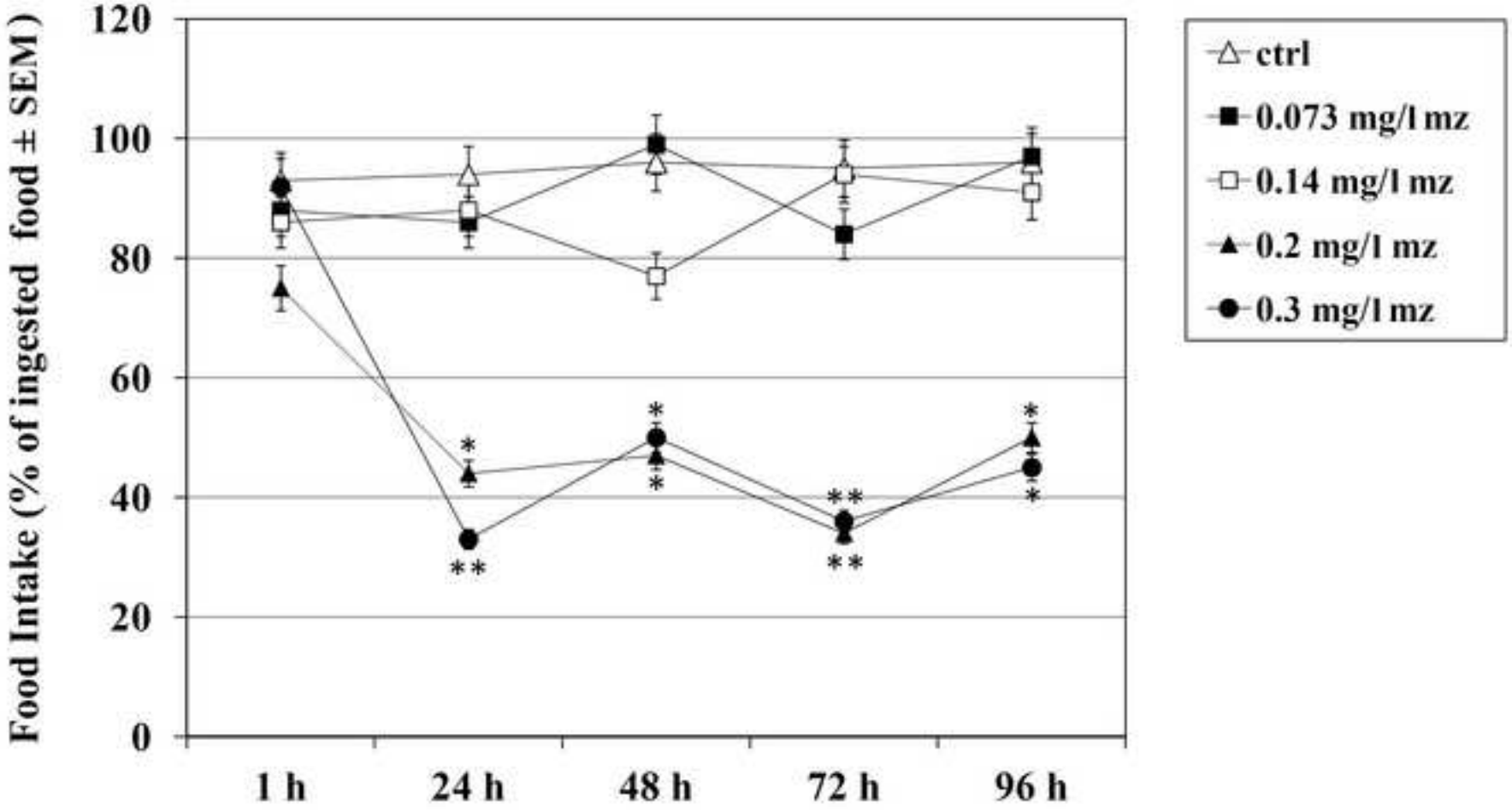

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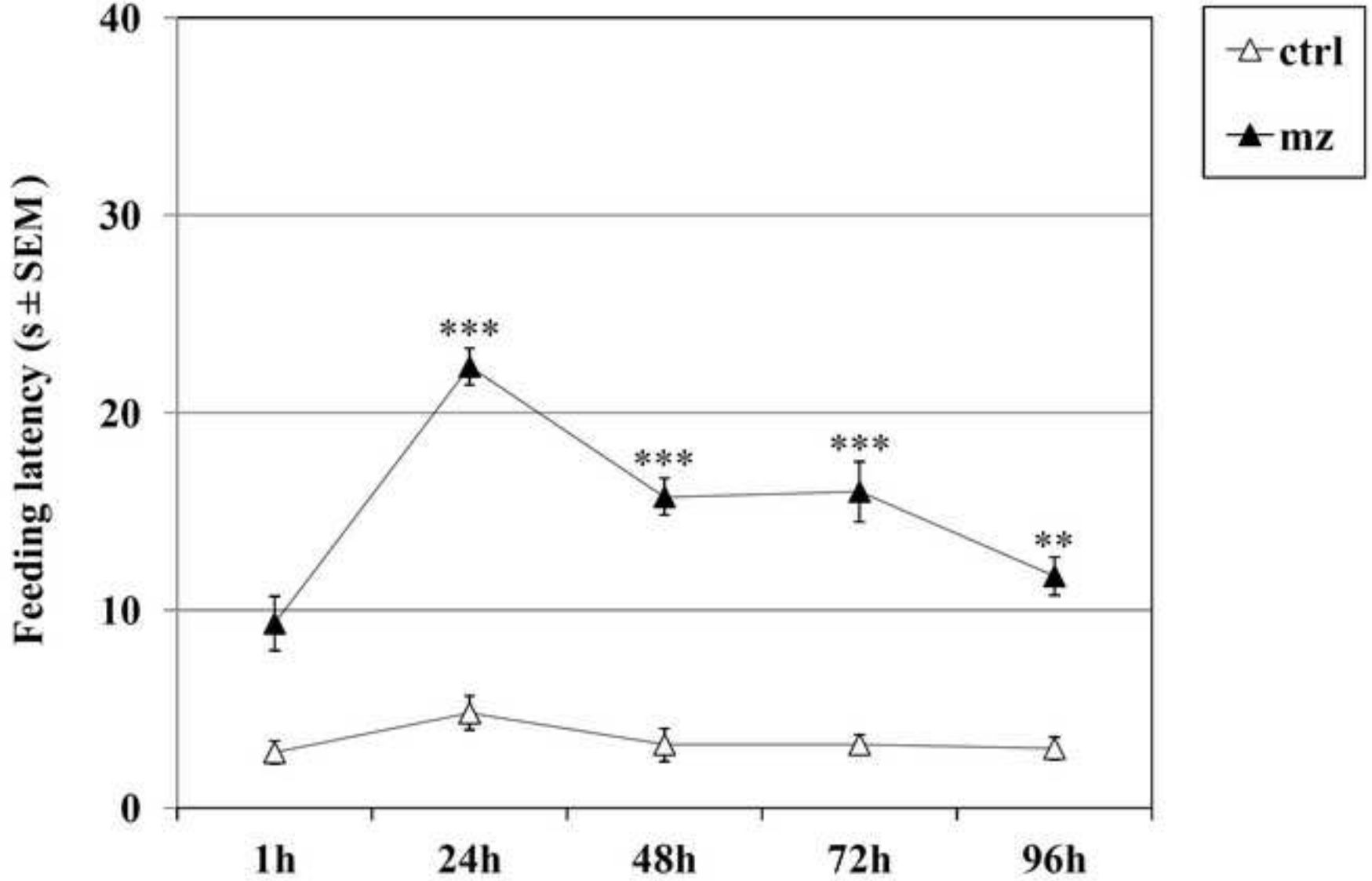

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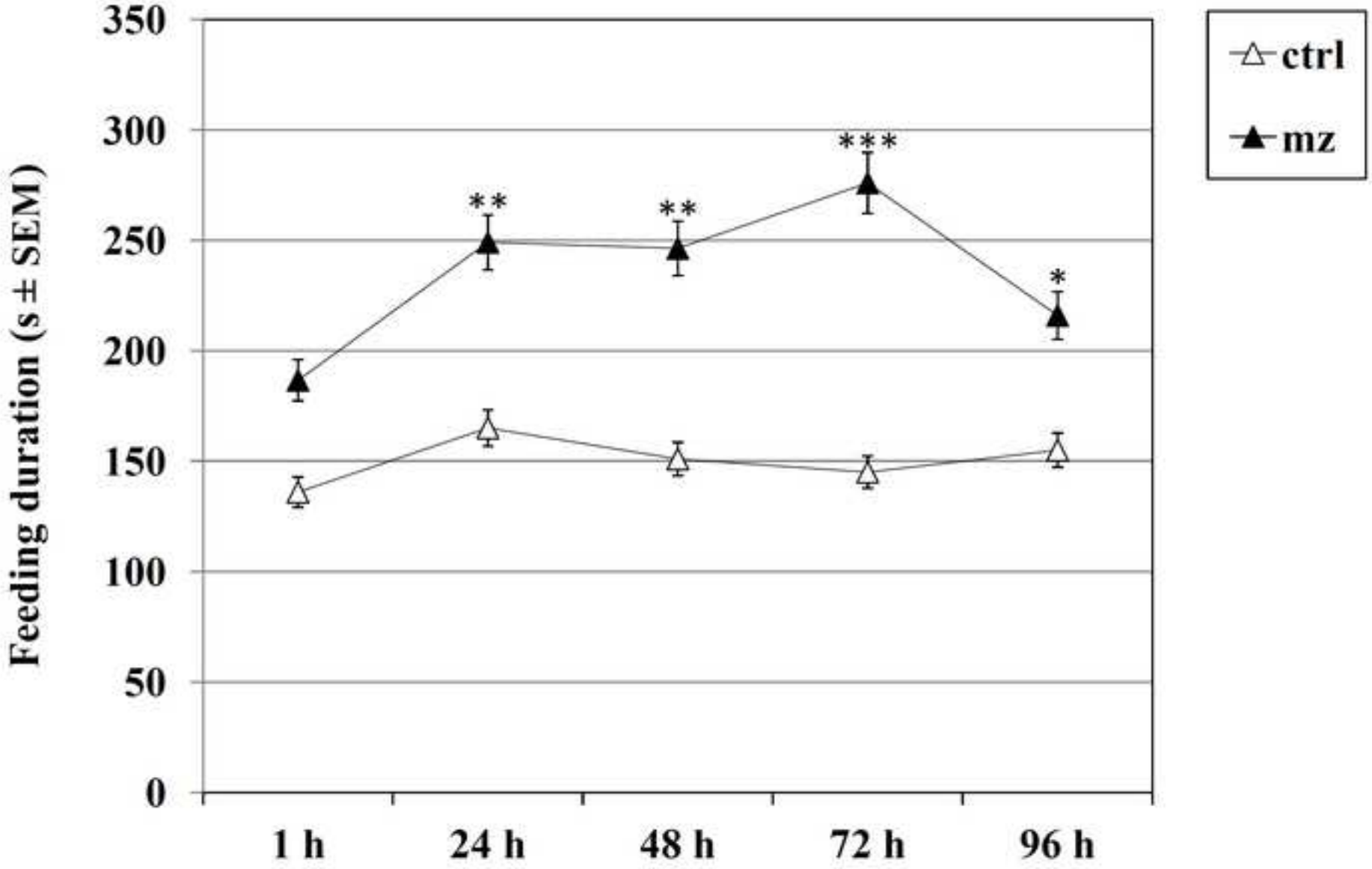

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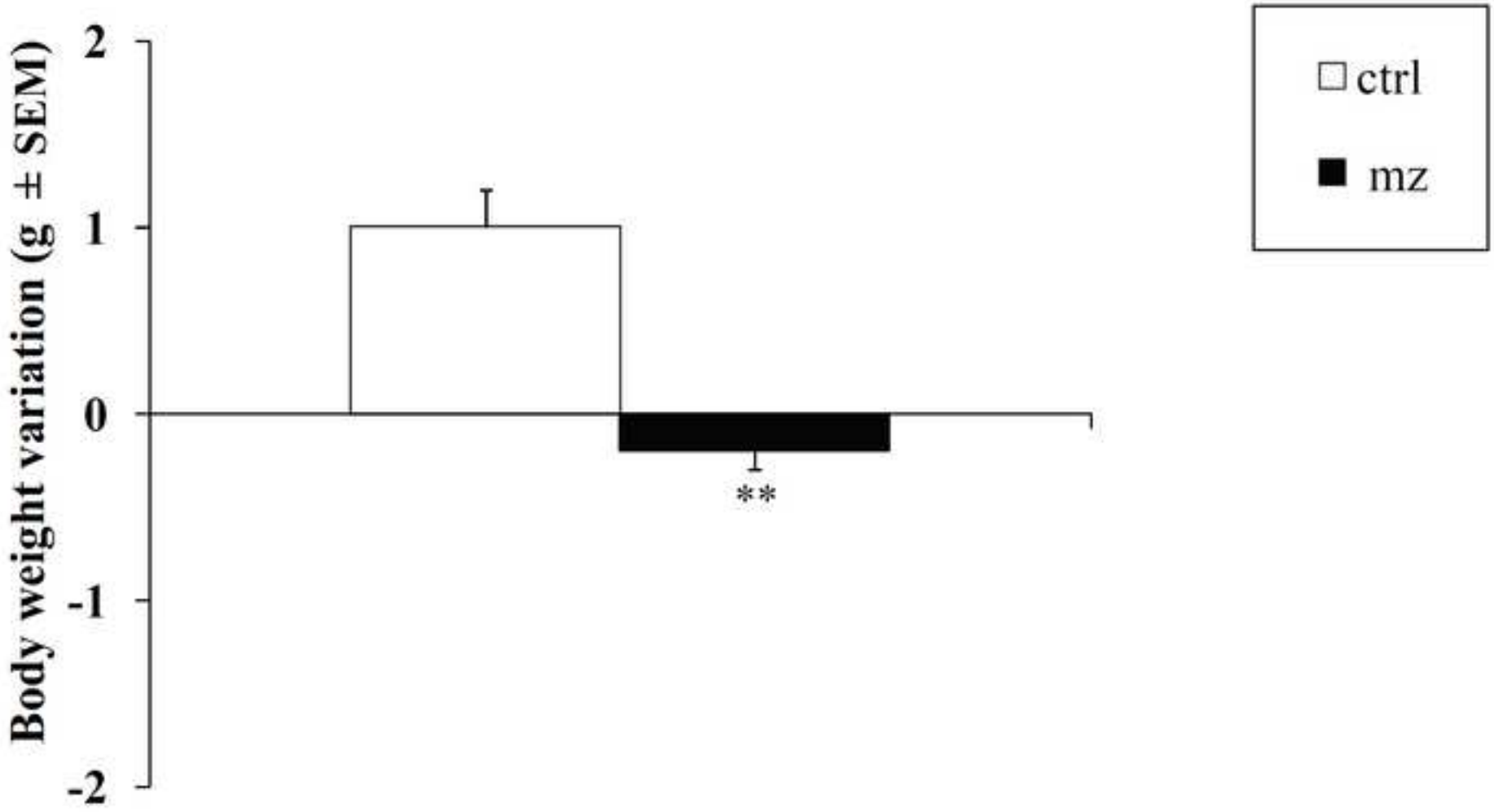

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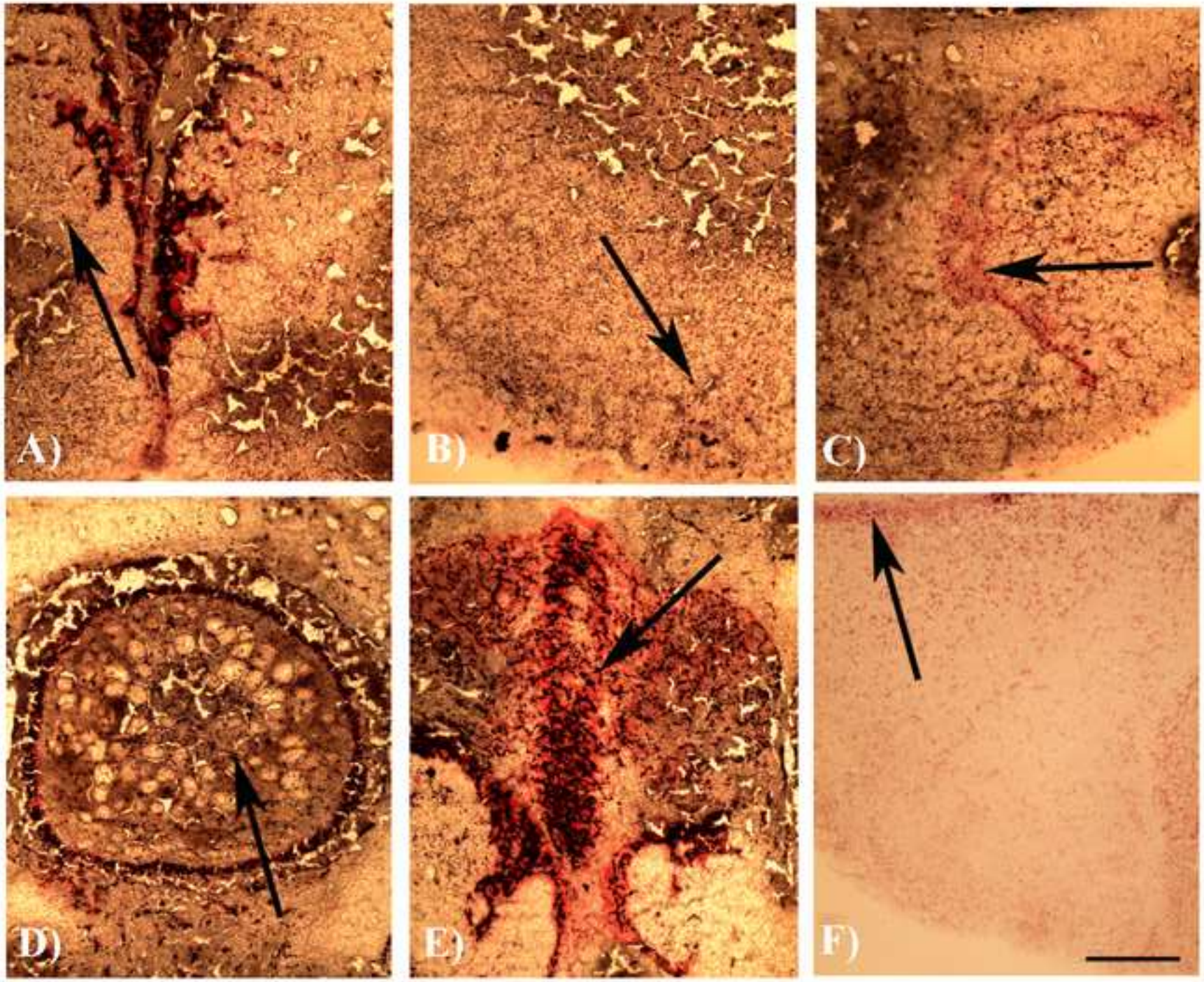

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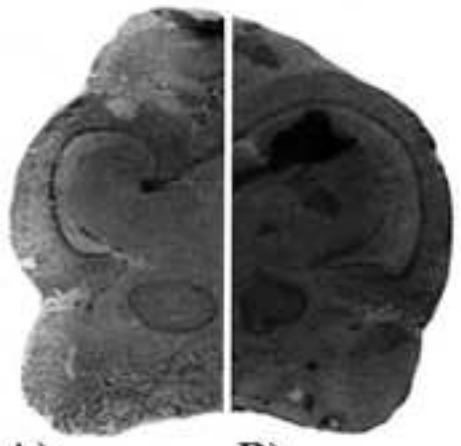

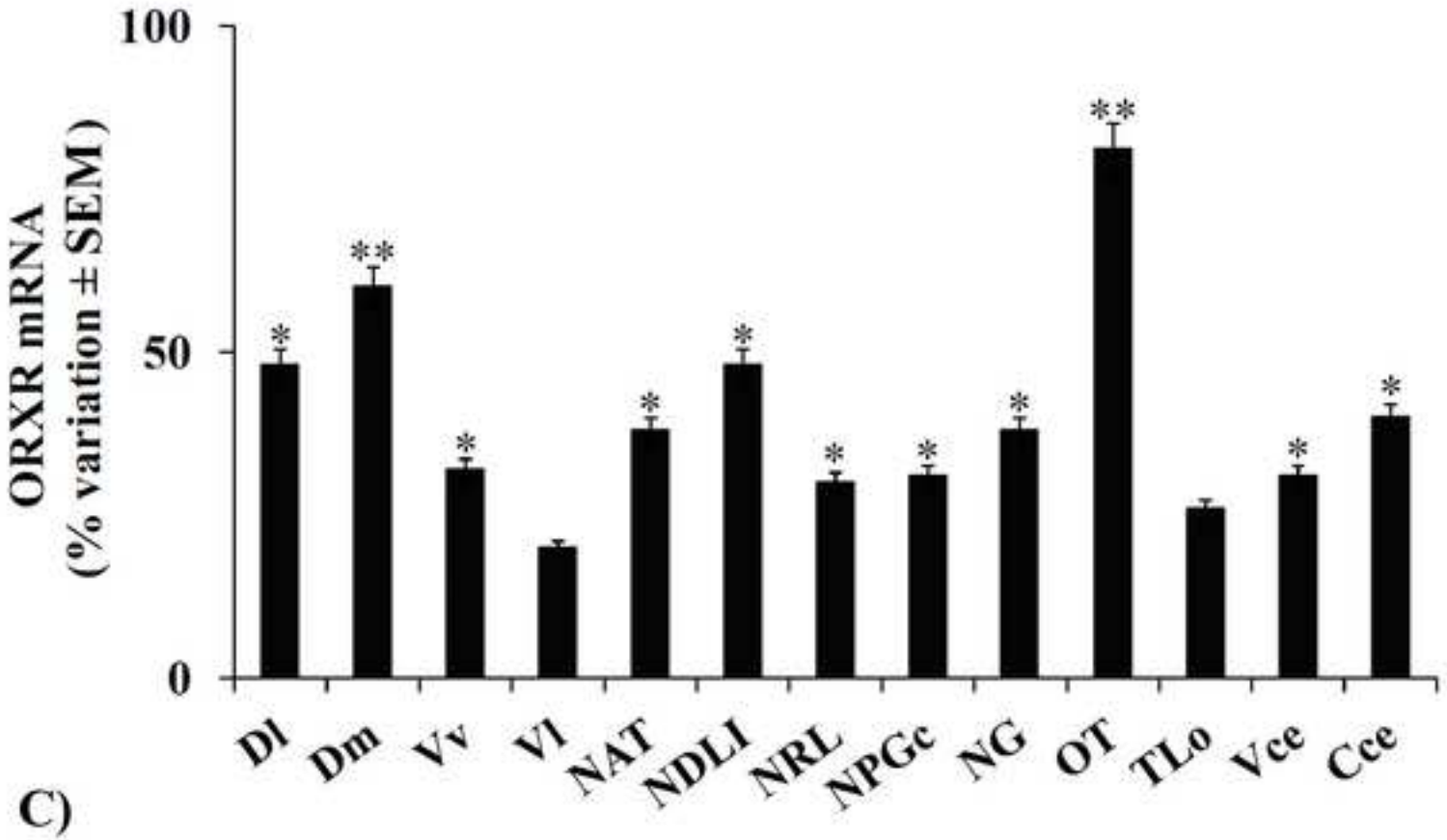

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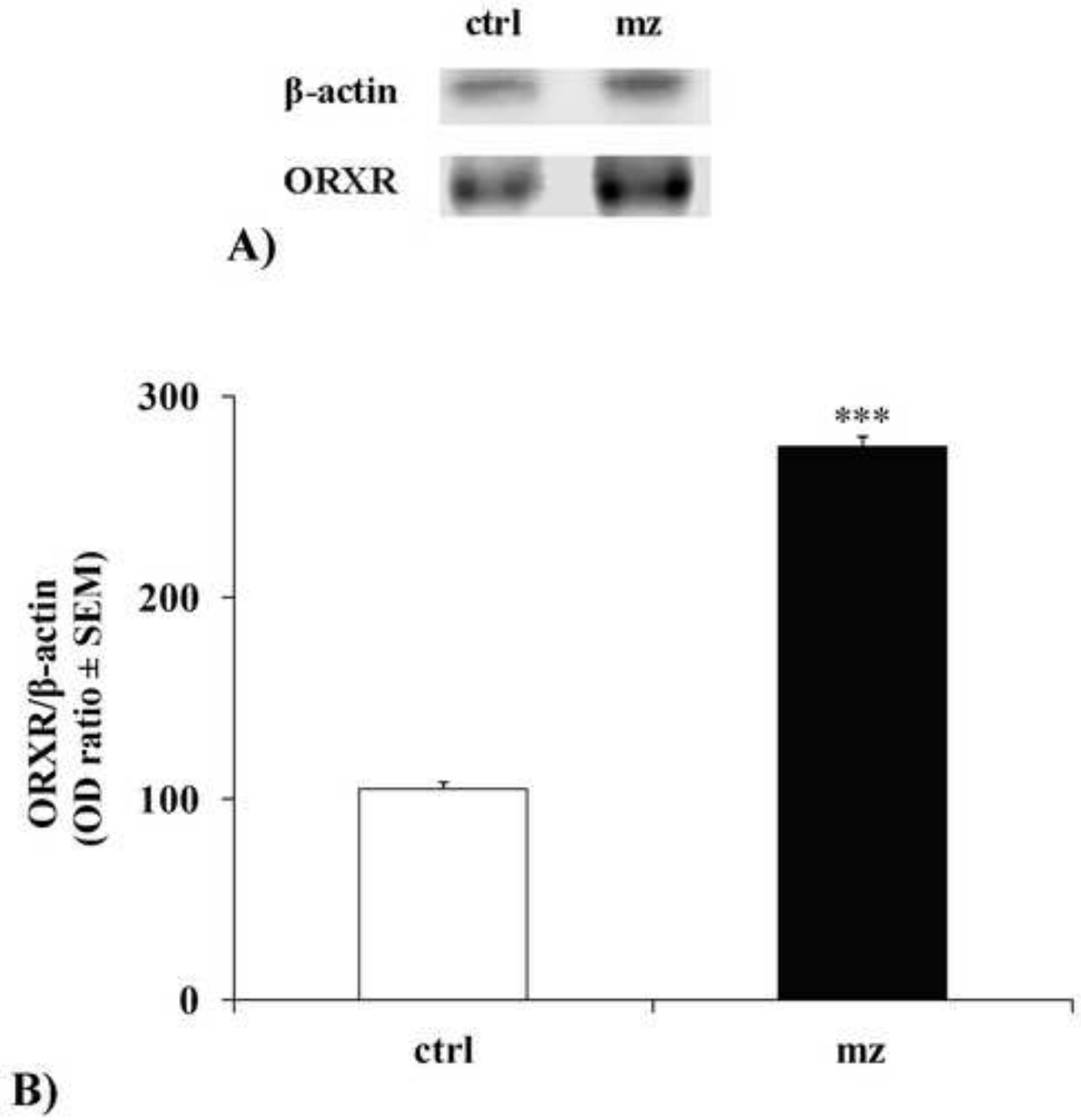

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Endocrine disruptors and their effect on Environment and Human Health, 2017: 000-000 ISBN: 978-81-308-0000-0 Editors: Mita Damiano Gustavo

2. Molecular mechanisms of endocrine disruptors: Interference with the endocrine system activity

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Abstract. Endocrine disrupting chemicals (EDCs) are an heterogeneous group of compounds able to interfere with hormonal functions by mimicking the endogenous hormones. This feature makes them able to interact with different cellular and molecular targets that affect all the biological functions of organisms. Many EDCs have a structural similarity with several endogenous hormones and this allows them to interact physically with specific receptors even though with different binding affinities each time. In this review we have collected some of the various and manifold molecular mechanisms activated by EDCs. Of these, the receptor-mediated pathway prevails; it is based on the interaction with estrogen receptors (ERs). However, this is not the only way they can use to determine endocrine interference. Several *in vitro* and *in vivo* studies have shown the existence of non-receptor and non-genomic pathways that are much faster and trigger a number of signal transduction pathways that control multiple cellular functions such as proliferation, differentiation

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and motility. Finally, several EDCs affect the hypothalamus-pituitary axis and the hormonal systems involved in the thyroid and the adrenal glands. Their wide presence in the environment and the multiple exposure paths to which we are constantly subjected, make EDCs a very wide health problem. Determining the specific molecular mechanisms that they are able to activate is an important step in trying to reduce the risk associated with their presence in our daily lives.

Introduction

The endocrine system is formed by different glands producing several hormones which are able to regulate metabolism, growth, development and reproduction in humans and animals (Witorsch, 2002). Endocrine Disruptor Chemicals (EDCs) are exogenous compounds, of natural or synthetic origin, that are able to interfere with different hormonal pathways such as production, transport, metabolism and action of natural hormones. EDCs can act as agonists and/or antagonists of natural hormones. EDCs are characterized by multiple mechanisms: they can function in genomic, epigenomic or non-genomic manner. In this way, EDCs negatively influence homeostasis, reproduction and developmental processes (Sun et al. 2016). EDCs are ubiquitously present in the environment because they are used in different industrial (industrial chemicals, plastic packaging components) and agricultural (pesticides, fungicides, insecticides, herbicides) purposes (Nappi et al. 2016). Other than, EDCs are used for the preparation of detergents, cosmetics, sun lotion for personal care and in the manufacturing of toys. Moreover, they can be presents in the environment as natural compounds like phytoestrogens. EDC classification is complicated but in general they can be divided in short-lived pollutants and persistent organic pollutants (POPs) (Giulivo et al. 2016). The first category includes phtalates and bisphenol A, whereas POPs include the organochorine pesticides dichlorodiphenyltrichloroethane (DDT) and other industrial products as dioxins, flame retardants (Giulivo et al. 2016). Due to their whole diffusion in each matrix (sediments, soils, water, atmosphere), human exposure to EDCs is unavoidable and can mainly occur through the food chain, by the consumption of contaminated tap water and food, by dermal exposure and/or by inhalation of volatile compounds and airborne fine and ultrafine particulate matter (Nappi et al. 2016). Another important EDC peculiarity is their lipophilic nature that allows their persistence in the environment other than biomagnification and bioaccumulation processes. Morevoer, it is important to consider that animals and humans are exposed to complex

mixtures of EDCs. These compounds can have a great complexity of mechanism of action, because their can contemporary act on multiple signaling pathways and targets (Nappi et al. 2016).

Other the way of exposure, particularly important is the consideration of the time of in order to evaluate the next impact of EDCs on biological systems. Specifically, the fetal life represents a period of special attention, since important processes such as organogenesis and tissue differentiation must occur through a series of well-regulated molecular, biochemical and cellular events (Prusinski et al. 2016). Any perturbation of only one of developmental key point can cause adverse effect in the "tomorrow" person. It has been demonstrated that environmental exposures during specific "window of susceptibility" can permanently reprogram normal physiology of polluted organisms. In this view, prenatal and early postnatal developmental processes are more susceptible to EDC action since each minimum change of timing and/or activation/inhibition pathways can alter all the other cellular events. However, humans are continously exposed to EDCs daily during all the life, hence all stages of body physiology are potential targets of endocrine disruption. Moreover, EDCs differ from other environmental pollutants since they are able to function at small doses but inducing subtle changes at cellular and tissue levels that finally evoke pathophysiological effects (Prusinski et al. 2016). At today, all human body systems are negatively influenced by EDCs: cardiovascular system (Roseboom 2012), nervous system (Nesan and Kurrasch 2016), reproductive system (Crews and McLachlan 2006, Maqbool et al. 2016), digestive system (Janesick and Blumberg 2016; Nappi et al. 2016) and obviusly endocrine system. Moreover, EDCs are linked to carcinogenes, teratogenesis and transgenerational inheritance of phenotype (Bernal and Jirtle 2010; Prusinski et al. 2016). In the last twenty years, the study of EDCs have completely revolutionized the concept of teratological compounds, in fact from substances inducing structural abnormalities at birth they have been transformed in molecules and/or mixtures of chemicals involved in the developmental origin of adult diseases (McLachlan 2016). Among all the well known substances acting as teratological compounds, a pioneer of EDCs that has contributed to the modification of endocrine disruption view in the teratology, was diethylstilbestrol (DES) that for the first time had been demonstrated as the cause of trasnplacental carcinogenesis: DES took by the mother during pregnancy was able to induce cancer later in the life of the daughters (McLachlan 2016).

Multiple compounds for multiple molecular mechanisms

As written above, EDCs can have many differents molecular behaviour and they can act on multiple cellular targets. However, the more important EDC targets are the nuclear receptors such as estrogen (ER), progesterone (PR) and androgen (AR) receptors, steroid (mineralcorticoid, glucocorticoid) receptors, thyroid receptors (TR) and peroxisome proliferator-activated receptors (PPAR) (Wuttke et al. 2010; Yang et al. 2015; Giulivo et al. 2016;). Due to the broad involvement of these receptors in many different cell and tissue functions, it is evident the attention needed to EDC role in biological interference at several points. Recent studies have demonstrated that EDCs can also act with membrane receptors like estrogen receptor GPER or other ER splice variants. Moreover, EDCs interfere with enzyme activity such as hormone metabolizing enzymes like aromatase (Sanderson, 2006), 5-reductase (Kalfa et al. 2009), 3-hydroxysteroid dehydrogenase (Ye et al. 2011) and 11-hydroxysteroid dehydrogenases (Odermatt et al. 2006; Guo et al. 2012; Giulivo et al. 2016).

Among EDCs there are numerous compounds that exert an estrogenic effects. It is well known that estrogen is a female hormone but its role is not only to regulate female reproductive cycle but also to influence non reproductive organs regulating lipid metabolism, protein synthesis and behaviour (Kiyama and Wada-Kiyama 2015). So, estrogenic chemicals that mimick this endogenous female hormone are able to interfere with normal body homeostasis through different mechanisms. In fact, estrogenic signaling can be divided in intracellular and extracellular mechanism. The intracellular pathway involves genomic activation such as transcription of specific target genes, and non-genomic pathway through the activation of transduction signals mediated by membrane receptors (Kiyama and Wada-Kiyama 2015). The extracellular pathway, on the contrary, involves other hormones, growth factors and cytokines (Kiyama and Wada-Kiyama 2015). It is very difficult to identify all chemicals that act as estrogenic compounds since other EDCs can have multiple effects including estrogenic actions (For a list of several estrogenic chemicals, see Kiyama and Wada-Kiyama 2015 review). Considering the estrogenic compounds, it is important to specify that they have contradictory effects since they can behave as estrogen or anti-estrogen, agonist or antagonist of ERs. Often, it has been pointed out a biphasic activity, better depending of the dose they show an estrogenic or antiestrogenic activity. Anyway, they can show different cellular pathways (Kiyama and Wada-Kiyama 2015). This mechanism of action is often shared among different EDCs.

In order to describe a view of different molecular mechanisms, we have subdivided their targets in genomic, non genomic and non-receptorial pathways.

Genomic pathway

The genomic pathway is the main target of EDCs. This pathway starts with the binding of chemicals with the nuclear estrogen receptors (ERs). There are two different ERs: ERα and ERβ, both involved as transducer. They are encoded by different genes, respectively *ESR1* located at 6q25.1 and *ESR2* located at 14q23.2-q23.3 on human chromosomes. Even so, the receptor proteins share a common structural organization based on the presence of three functional domains: the A/B domain at the N-terminal region, involved in the transcriptional activation of ER target genes; the C domain responsible for the receptor dimerization and DNA binding; the E/F domain at the C-terminal region involved in the ligand binding, nuclear translocation and transactivation of target gene expression (Nilsson et al. 2001; Kiyama and Wada-Kiyama 2015). Both *ESR1* and *ESR2* show splice variants that are responsible of differences in the expression at cell or tissue level, in the specificity and/or affinity for a ligand, in the localization and function in the cells (Taylor et al. 2010; Kiyama and Wada-Kiyama 2015). Co-regulators and other transcriptional factors, such as Sp1 and AP1, are often needed for the transactivation of target genes (Kiyama and Wada-Kiyama 2015). The complex (endogenous or not) ligand - ER binds the DNA at specific site acting as transcription factor in order to up-regulate or down-regulate the transcription of target genes (generally bringing the Estrogen-Responsive-Elements) (Kiyama and Wada-Kiyama 2015). The change in ER conformation depending on the ligand bound, renders ERs more or less prone to the transcriptional coactivators or corepressors recognition (Acconcia et al. 2015). Very large amount of estrogenic chemicals (fungicides, herbicides, insecticides, several pharmaceutical estrogens, plasticizer, pollutants) use ERs as dealer to induce endocrine interference (Kiyama and Wada-Kiyama 2015). Among these, BPA has structural features that confer it the ability to bind to the both ERα and ERβ (Bolli et al. 2008; Bolli et al. 2010; Acconcia et al. 2015). BPA binding to ERs produces a displacement of α-helices of LBD of ERα due to a not proper accommodation in the hormone-binding site; so BPA can function as ERα agonist. On the contrary BPA is not able to bind the LBD of ERβ, acting as antagonist (Ascenzi et al. 2006; Acconcia et al. 2015). These

differences in the binding of the two ERs induce a varied regulatory activity on gene expression (Acconcia et al. 2015). Many other EDCs are able to bind ERs. It has been demonstrated that nonylphenol (NP) is able to induce cytoplasm to nucleus translocation of ERα but not ERβ in human epithelial prostate cells (Forte et al. 2016). This translocation induced ERα activation of transcription of specific genes such as cyclin D1 and ki67 that allow cell proliferation (Forte et al. 2016). Recently, another nuclear receptor family has been identified as part of estrogen signaling. This family includes some nuclear estrogen-related receptor such as ERRα, ERRβ and ERRγ. These "alternative" receptors act as ligand-dependent transcription factors but their natural ligand is still unknown. Several compounds prefer ERR pathways such as genistein and resveratrol, as well as chlordane, diethylstilbestrol and toxaphene that are ERR antagonists. In some case it is possible a crosstalk between ER and ERRs as demonstrated for resveratrol (Kiyama and Wada-Kiyama 2015). That's why nuclear receptor pathway is more complex and interlaced.

Non-genomic pathway

Estrogen or xenoestrogens can also bind to the membrane receptors stimulating signaling cascade through different protein involvement. Generally, the non-genomic pathway is very fast and rapidly occurs. Canonical ERα and ERβ can translocate to the membrane after the modification. Here, they bind to caveolin-1 after palmitoylation, after that they are translocated to the membrane and anchored as a dimer (Soltysik and Czekaj 2013; Kiyama and Wada-Kiyama 2015). It has been shown that this pathway is used by the cells to rapidly respond to hypothalamic stimulation (Micevych and Kelly 2012; Kiyama and Wada-Kiyama 2015). Endogenous estradiol (E2) activates ERα-mediated extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and phosphatidyl-inositol-3-kinase/AKT (PI3K/AKT) pathways, as well as the ERβ-mediated p38/MAPK signaling (Acconcia and Marino 2011; Acconcia et al. 2015). It has been demonstrated that xenoestrogenic compounds such as BPA can activate ERK/MAPK and AKT phosphorylation (Bolli et al. 2008; Marino et al. 2012; Acconcia et al. 2015). Another EDC such as Arsenic (As) is able to interfere with estrogen signaling pathways (Watson and Yager 2007; Bae-Jump et al. 2008; Chatterjee and Chatterji 2010; Sun et al. 2016). Particularly, it has been shown that As interacts with the MAPK pathway (mitogen-activated protein kinase), which plays a crucial role in different cell functions such as cell growth, differentiation, survival, and death

(Chatterjee and Chetterji 2010; Sun et al. 2016). Moreover, it has been demonstrated that As suppresses the interaction of ERs with some transcription factors like Sp-1, AP-1 and NF-κB (Watson and Yager 2007; Sun et al. 2016).

Different membrane ERs (mERs) have already been identified such as G-protein coupled estrogen receptor (GPER). The mERs GPER, previously known as G-protein-coupled receptor 30 (GPR30), is encoded by the *GPER* gene located at chromosome 7p22.3. GPER is a 7-membran-spanning protein highly expressed in the hypothalamus, pituitary gland, adrenal medulla, renal pelvis and ovary (Hazell et al. 2009; Soltysik and Czekaj 2013; Kiyama and Wada-Kiyama 2015). GPER shows a high affinity for the endogenous estrogens and other hormones such as aldosterone (Kiyama and Wada-Kiyama 2015). GPER can be located at the membrane of endoplasmic reticulum, Golgi apparatus and can be also present in the nucleus (Soltysik and Czekaj 2013; Kiyama and Wada-Kiyama 2015). GPER activation induces rapid non-genomic signaling (Kiyama and Wada-Kiyama 2015). Different xenoestrogenic compounds have been demonstrated bind to the GPER such as BPA, diethylstilbestrol, genistein, NP and many others (Kiyama and Wada-Kiyama 2015). These compunds act as agonists to the GPER. Moreover, it has been demonstrated that GPER in involved in the signaling pathways mediated by other receptors like serotonin 1A receptor (Li et al. 2013c; Kiyama and Wada-Kiyama 2015). Particularly, it acts inhibiting serotonin 1A receptor (Xu et al. 2009; McAllister et al. 2012; Akama et al. 2013; Kiyama and Wada-Kiyama 2015). Moreover, GPER crosstalks with other signaling pathways involved in different cell functions such as proliferation (Ma et al. 2014), migration (Li et al. 2014a), collagen expression (Li et al. 2013a), NO synthesis (Rowlands et al. 2011), and inflammatory response (Luo et al. 2012; Santolla et al. 2014; Kiyama and Wada-Kiyama 2015). It seems evident that due to involvement of GPER in many different cell pathways involved in any stage of proliferation, differentiation and migration, that all the chemicals able to bind this receptor can deeply interfere with many cell and tissue important processes.

Other receptors, such as estrogen-related receptors (ERRs) that are variants of ERα and ERβ (for example ER-X and ER-α36) have been recently identified showing the broad complexity of estrogen responsive signaling (Kiyama and Wada-Kiyama 2015). Specifically, ER-X is a ERα splice variant; it is a 62-63 kDa membrane protein (Soltysik and Czekaj 2013; Kiyama and Wada-Kiyama 2015). Binding of estrogen to ER-X is associated in particular to the brain, uterus and heart functions. It has been

demonstrated that after binding, ER-X activate MAPK and ERK signaling (Toran-Allerand et al. 2002; Toran-Allerand et al. 2005; Ullrich et al. 2008; Kiyama and Wada-Kiyama 2015). Another ERα splice variant is ER-α36 is located at the membrane. This receptor lacks both AF-1 and AF-2 domais but present DNA-binding domain and partial ligand domain. It has been shown that ER-α36 is able to inibit both ERα and ERβ in a dominant-negative manner. This ability allows it to be involved in different carcinogenesis pathways such as testosterone carcinogenesis (Lin et al. 2009) and breast cancer (Rao et al. 2011; Kiyama and Wada-Kiyama 2015). Different cascade proteins can activated by ER-α36 such as MAPK/ERK, Akt, and c-SRC (Kang et al. 2011; Zhang et al. 2014c; Wang et al. 2013b; Kiyama and Wada-Kiyama 2015).

Both genomic and non-genomic pathways can be considered as direct signaling mechanisms that differently can induce many functional outcomes such as apoptosis, cell growth, differentiation, inflammation and carcinogenesis (Kiyama and Wada-Kiyama 2015). However, each pathway can influence other cellular outcomes by crosstalk and/or bypassing. Moreover, the direct signaling mechanisms also induce the secretion of autocrine/paracrine or endocrine factors enlarging the range of targets involved.

Epigenetic pathways

EDCs can also act through epigenetic mechanisms. Epigenetic mechanisms are particularly important to address the potential health effects of lower-level exposures within the general population; moreover it is useful to explain how EDC exposure during the development can cause adverse effects in the adult (Prusinski et al. 2016). It has been shown, for example, that genistein is able to induce epigenetic changes of non-genomic estrogen receptor (ER) signaling through the activation of the PI3K/AKT pathway (Prusinski et al. 2016). This phosphorylates histone methyltransferase Enhancer of Zeste Homolog 2 (EZH2), a potent epigenetic regulator of gene expression (Sandovici et al. 2013). Finally, this epigenetic pathway increases the overall expression of estrogen-responsive genes (Cook et al. 2005; Prusinski et al. 2016). Likewise, also BPA increases EZH2 that in turn increases histone H3 trimethylation at lysine 27 (Doherty et al. 2010; Santangeli et al. 2017). Furthermore, BPA was demonstrated to alter methyltransferase 1 and 3A which are epigenetic regulators of expression of genes encoding estrogen receptors (Kundakovic et al. 2013; Santangeli et al. 2017). BPA induces hypermethylation of estrogen promoter region in rat

testis (Doshi et al. 2011; Santangeli et al. 2017). Other studies have shown that BPA strongly increases the expression of the secretaglobin gene, *Scgb2a1*, by way of the increased enrichment of acetylated H3K9 and hypomethylation of DNA for a CpG island upstream of the transcription start site of *Scgb2a1* (Wong et al. 2015; Prusinski et al. 2016). SCGB2A1 is being an interesting marker of carcinogenesis, since its gene and protein overexpression is linked to endometrial, breast and lung cancers (Li and Richardson 2009; Prusinski et al. 2016).

Non receptorial pathways

Recently, different studies highlight other non nuclear receptor mediated pathways involved in estrogenic and xenoestrogenic actions. In fact, it is well known that natural hormones and xenoestrogens can also act by enzymatic and binding protein pathways (Mueller and Korach 2001; Sheikh et al. 2017). Particularly, sex steroids interact with plasma sex hormone-binding globulin (SHBG) that is a circulatory protein secreted by liver important in maintaining the balance between bioavalable and not avalaible hormones (Anderson 1974; Sheikh et al. 2017). Particularly, it has been demonstrated that the free portion of steroid hormones represents a small percentage, about 1-3% of the total steroids. Despite this low amount represents the bioactive portion for the target tissues (Hammond, 2011; Laurent and Vanderschueren 2014; Sheikh et al. 2017). The SHBG binds both androgens and estrogens with nanomolar affinity. SHBG has two subunits each containing two laminin G-like domains: the N-terminal domain presents the steroid-binding pocket and calcium and zinc binding sites; the C-terminal domain shows residues for glycosylation (Hammond 2011; Sheikh et al. 2017). It regulates hormonal free portion able to bind receptors that is important in clinical practice. Moreover, SHBG influences hormonal metabolic clearance (Hammond 2011; Sheikh et al. 2017). Alteration of SHBG function and/or amount has been associated with various human diseases such as ovarian dysfunctions, male and female infertility, endometrial cancer, diabetes, cardiovascular diseases (Cherkasov et al. 2005; Sheikh et al. 2017). Many different EDCs can bind SHBG, among these some alkylphenols such as BPA, NP, octylphenol (OP) have been shown binding ability (Dechaud et al., 1999; Jury et al., 2000; Cherkasov et al., 2008; Hong et al., 2015; Sheikh et al. 2017). Recently, it has been demonstrated that BPA, NP and OP have high structural similarity with endogenous hormones and for this they can strongly bind SHBG (Sheikh et al. 2017). Among three alkylphenols Sheikh et colleagues (2017) have seen that NP is the more potent endocrine disruptor of androgen and

estrogen signaling since it shows the most high binding affinity with SHBG (Sheikh et al. 2017). Binding of xenoestrogens to SGBG displaces endogenous testosterone and estradiol from SHBG steroid pocket in native plasma from men and women (Dechaud et al. 1999; Sheikh et al. 2017). In an other study, 125 structurally diverse compounds have been tested in competitive binding assay for SHBG and it has been shown that BPA, OP, and NP have a potential competing function (Hong et al. 2015; Sheikh et al. 2017). Xenoestrogenic binding to SHBG induce a lower clearance rate that allows a major accumulation of EDCs in the body. Moreover, in children during the prepubertal period (Apter et al., 1984; Belgorosky and Rivarola, 1986) and in women during pregnancy (Anderson 1974), SHBG levels are higher whereas testosterone and estradiol concentrations remain lower. Under such conditions, BPA, NP and OP ability to bing SHBG may affect the metabolism and tissue availability of natural steroids (Sheikh et al. 2017). This "new" EDC molecular target of endocrine disruption open a broad scenario of interference with steroid homeostasis in the human body.

EDCs and hypothalamus-pituitary axis

Thyroid gland

Another important field to consider in EDC pollution is the involvement and activation of other hormonal pathways, such as thyroid and adrenal glands. EDCs can impair thyroid system through different mechanisms: disruption of TH serum transporters such as transthyretin (TTR) and thyroxine-binding globulin (TBG), aberrant binding to TH nuclear receptors, or disruption of TH-metabolizing enzymes such as deiodinases (DIO) and sulfotransferase (SULT) (Aufmkolk et al. 1986; Meerts et al. 2000; Schmutzler et al. 2004; Kitamura et al. 2008; Kojima et al. 2009; Szabo et al. 2009; Butt et al. 2011; Butt and Stapleton 2013; Leonetti et al. 2016). Particularly, it has been demonstrated that different EDCs are able to negatively influence thyroid hormone levels (Zhou et al. 2002; Sciarrillo et al. 2010; Noyes et al. 2013; de Coch et al. 2014; Eisenreich and Rowe 2014; Yost et al. 2016). Several studies have demonstrated that prenatal exposure to thyroid hormone endocrine disruptors affect birth weight, can cause preterm births, other than affect the glucose and lipid metabolism (Molehin et al. 2016; Shah-Kulkarni et al. 2016). Infact, THs are known to be particularly important for fetal growth and development (Costa et al. 2014; Leonetti et al. 2016). Different interference pathways can be activated in alteration of thyroid functions.

For example, it has been hypothesized that perfluorinated compounds (PFCs) may increase the hepatic production of TBG (Knox et al. 2011). Other PFCs like perfluorooctane sulfonate (PFOS) may increase the thyroidal conversion of T4 to T3 via type 1 deiodinase (Yu et al. 2009; Shah-Kulkarni et al. 2016). Polybrominated diphenylethers (PBDEs) and other halogenated compounds (Butt et al. 2011; Butt and Stapleton 2013; Leonetti et al. 2016) are able to inhibit the activities of TH-metabolizing enzymes so impacting the placental TH concentrations and fetal TH delivery (Leonetti et al. 2016). Other compounds such as As alters thyroid hormone production. Particularly, it has been shown that after consuming diet containing As for 15 weeks, plasma levels of T3 and T4 are decreased while the ratios of T4/T3 are increased (Meltzer et al. 2002; Sun et al. 2016). Moreover, Ciarrocca et al. (2012) have shown an increase of the levels of thyroid stimulating hormone (TSH) and thyroglobulin and a decrease of free T4 and T3 contents (Ciarrocca et al. 2012; Sun et al. 2016). Opposite effects were seen in rats where feeding food containing As increases T3 levels and decreases T4/T3 ratios (Glattre et al. 1995). As thyreotoxicity is related to alteration of TR-related gene expression. Particularly, AsIII inhibites the activity of thyroid peroxidase, a major enzyme involved in the synthesis of T4 and T3 (Palazzolo and Jansen 2008; Sun et al. 2016). Thyroid disruption has been demonstrated also in different animal models. It has been shown that PBDEs are able to disrupt thyroid hormone signaling in *Xenopus laevis* tadpole (Yost et al. 2016). Specifically, Yost and colleagues have demonstrated that 2,2',4,4'-tetrabomodiphenyl ether (BDE-47) specifically alterate transcriptomic expression of thyroid hormone-related genes (Yost et al. 2016). BDE-47 reduces expression of *trα, trβ* and *tshβ* and decreases thyroid hormone plasma levels (Yost et al. 2016). Moreover, BDE-47 influences also thyroid hormone transport; in fact it suppresses expression of two thyroid hormone transporters *mct8* and *oatp1c1* (Yost et al. 2016). In fish, AsIII significantly increases the levels of T4 (Sun et al. 2015; Sun et al. 2016). On the contrary, in *Podarcis sicula* lizards, NP induced a significant decrease of TSH, T4 and T3 plasma levels and affected histological features of lizard thyroid (Sciarrillo et al. 2010).

Adrenal gland

Another important organ in the control of endocrine homeostasis is the adrenal gland. Adrenal gland plays a role in the body response mechanisms

to stress, maintaining the homeostasis of the organism (De Falco et al. 2014). Despite its relevance in the body physiology, relatively few studies have investigated the possible/existing links between endocrine disruptors and the HPA axis (De Falco et al. 2007; De Falco et al. 2010). Recently, it has been demonstrated that As significantly increases ACTH and corticosterone levels in rodents (Jana et al. 2006; Sun et al. 2016). Particularly, it has been shown that As is able to reduce levels of corticotropin-releasing factor receptor 1 and to potentiate binding between serotonin and serotonin 5-hydroxytriptamine receptor (Martinez et al. 2008). Moreover, As acts on glucocorticoid receptors (GRs). Specifically, it has been demonstrated that AsIII can modify GR activity blocking steroid binding to GRs (Lopez et al. 1990; Kaltreider et al. 2001; Sun et al. 2016). Recently, Ahir et al. (2013) have demonstrated that AsIII has a biphasic effect on GR function depending of its dose. At low dose, AsIII enhances glucocorticoid induction of GR-regulated genes, whereas at high dose it disrupts GR gene transcption interfering with hormone receptor binding (Ahir et al. 2013; Sun et al. 2016). Other EDCs are able to interact with GR system. For example, it has been demonstrated that polychlorinated biphenyls (PCBs) downregulate brain GR expression in fish (Aluru et al. 2004; Nesan and Kurrasch 2016). Other compounds have seen to act on HPA axis. Among EDCs, NP was shown to strongly stimulate the whole HPA axis inducing a time-dependent stimulation of CRF, ACTH and corticosterone release in reptile bioindicator *Podarcis sicula* lizards (De Falco et al. 2014). Moreover, NP was able to induce histological changes of adrenal glands with the presence of totally degranulated chromaffin cells (De Falco et al. 2014). Another class of potent environmental pollutant are dioxins that comprises 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This compound has shown to reduce CRH mRNA in hypothalamus of monkeys (Shridhar et al. 2001; Nesan and Kurrasch 2016).

Conclusion

EDCs are compounds of different chemical nature and are widely disseminated in the environment in which we live. This causes the exposure of human and animal populations to occur at any time of life and at different doses. One characteristic of EDCs is their ability to act at very low concentrations, interacting with hormone systems and altering the homeostasis of different organs and systems. It is also evident that a precise and unambiguous classification is practically impossible since many of them

have biphasic behaviors and are capable of activating different molecular pathways depending on the cellular system considered. This particular feature focuses on the behavior of EDCs that we are constantly exposed to in which individual compounds may be present, each with different activity, which in combination can trigger synergies that lead to complete and profound endocrine destruction and all systems connected to it. For this reason, the understanding of the molecular mechanisms that EDCs can activate is of paramount importance to orient themselves in the endocrine interference they have determined and to try to stem the large amount of related pathologies.

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Nonylphenol acts on prostate cells via estrogen molecular pathways

Abstract

A large body of evidence supports the idea that estrogens play a prominent role in the patho-physiology of the prostate. Epidemiological data, as well as results deriving from *in vitro* and *in vivo* studies suggest a relation between xenoestrogens exposure and prostate diseases. Thus, in this work we studied the effects of nonylphenol (NP), a well known xenoestrogen on human adenocarcinoma prostate cells (LNCaP), investigating on cellular proliferation, cell cycle, cellular localization and expression of estrogen receptors and expression of genes involved in prostate diseases. Moreover, we performed the same experiments with 17β- estradiol (E2), the most estrogen circulating in humans. We demonstrated the ability of low concentration of NP ($1x10^{-10}$ M) to induce proliferation of LNCaP, S-phase progression, and cytoplasm-nucleus translocation of ERα with its major expression. Moreover, we observed an up-regulation of key target genes involved in cell cycle and inflammation process. These data suggest the harmful effects of xenoestrogens on prostate cells and highlight some aspects of molecular pathways involved in prostate responses to xenoestrogens.

Keywords: EDCs, estrogens, xenoestrogens, prostate cells, nonylphenol

1. INTRODUCTION

Prostate development is influenced by the levels of circulating androgens and estrogens (Prins and Korach, 2008). In recent years, there are several data, both from *in vitro* and *in vivo* studies which demonstrate the pivotal role of estrogens in prostate physiology, exerting both protective and deleterious effects (Ho et al., 2011). When estrogen levels increase and conversely androgen levels decrease, prostate cells reprogram their cell cycle, resulting in an increased proliferation, which in turn lead to an aberrant growth of the gland As final results, the prostate undergoeses in pathological states, such as benign prostatic hyperplasia and prostate cancer. The increase in the number of cases of prostate diseases, especially in industrialized countries suggest a strictly association with the exposure to environmental pollutants (Sweeney et al., 2015). The endocrine disrupting chemicals (EDCs) are ubiquitous chemicals, found in many parts of the world, that act as endogenous hormones, mimicking and evoking their same molecular pathways (Giulivo et al., 2016). Humans are exposed to EDCs through skin absorption, inhalation and ingestion of contaminated food and water (Nappi et al., 2016). Several different studies have already reported the harmful effects of EDCs on reproductive and non-reproductive systems (De Falco et al., 2015; Nappi et al., 2016; Nesan and Kurrasch, 2016; Roseboom, 2012). EDCs can act on different molecular targets and EDCs with estrogens mimicking actions are called xenoestrogens. The most studied xenoestrogens are Bisphenol A (BPA) and alkylphenols (AP), which include Octylphenol (OP) and Nonylphenol (NP). These compounds are widely used in plastics formulation as non ionic surfactants, in agricultural products, in personal care products and they have been found as contaminants in rivers, lakes, seas, groundwater and sediments (Asimakopolous et al., 2012; Careghini et al., 2015). Xenoestrogens have been reported to bind both estrogen receptor α (ERα) and β (ERβ) and to induce proliferation of many cell lines (Forte et al., 2016; In et al., 2015). In particular, NP is able to stimulate breast cancer cells proliferation and to cause reproductive abnormalities (Bechi et al., 2010; Choi et al., 2012; In et al., 2015). Regarding the prostate, epidemiological studies suggest the relation between EDCs and prostate diseases and the expression of estrogen receptors (ERs) in prostate has been demonstrated.

Moreover, *in vitro* studies as well as animal data suggest a positive association between prostate diseases and EDCs exposure (Alvanja et al., 2003). An increased risk of prostate cancer was found in agricultures exposed to pesticides belong to the class of EDCs (Alvanja et al., 2003; Koutros et al., 2013). In rats, neonatal treatment with BPA was reported to induce prostatic intraepithelial neoplasia (Ho et al., 2006; Prins et al., 2011) and to increase cell proliferation in the primary prostatic ducts of mice (Timms et al., 2005). Similarly, neonatal and developmental exposure to estradiol and BPA was shown to induce prostate carcinogenesis in rats and to target human progenitor prostate stem cells (Ho et al., 2006; Prins et al., 2011; Prins et al., 2014). Recently, Tarapore et al. (2014) found in prostate cancer patients significant urinary levels of BPA compared to non prostate cancer patients. In the same study, they demonstrated that BPA is able to induce centrosome abnomarmalities and neoplastic transformation in several prostate cell lines. Moreover, BPA was found to induce prostate cells migration in LNCaP cells (Derouiche et al., 2013). We recently demonstrated tha NP induces cell proliferation of human non tumorigenic prostate cells, affecting estrogen related gene expression and ERα cellular localization (Forte et al., 2016). Given this background, in this work we evaluated the effects of NP, comparing to the endogenous hormone 17β- estradiol (E2) on human adenocarcinoma prostate cells (LNCaP). These cells represent a useful *in vitro* model of prostate, since they are hormone responsive and express all the prostate specific markers (Horoszewicz et al., 1983). Furthermore, they are often used for the study of the actions of esogenous and endogenous compounds on prostate. We analyzed the effects on proliferation, cell cycle, migration ability, localization of ERs and genetic expression of key target genes involved in the normal growth and pathological states of prostate. The aim of this work is to best characterize the involvement of estrogen and xenoestrogen in prostate phato-physiology, also giving data for the risk management of human exposure to EDCs.

2. MATERIALS AND METHODS

2.1 Cell culture

LNCaP cells (CRL-1740™ American Type Culture Collection, Manassan, VA) were grown in RPMI 1640 (Gibco, Invitrogen), supplemented with 10% FBS, 2mM glutamine, 1X non essential amminoacid, 1X penicillin/streptomycin, 10µg/mL gentamycin (Euroclone) at 37°C, 5% CO_2 in an humidified incubator. When 70% confluent, cells were enzymatically detached with trypsin-edta (Euroclone) and seeded in a new cell culture flask. Medium was changed twice a week. Cells were used from passage 9 to 20.

2.2 Chemicals

Nonylphenol (NP) and 17β- estradiol (E2) were purchased from Sigma Aldrich and dissolved in DMSO. Then, NP and E2 were diluted in RPMI 1640 red- phenol free at the concentrations used for the experiments. Control cells were treated with vehicle (DMSO 0,01%).

2.3 Treatment

LNCaP were treated with NP and E2 from $1x10^{-6}$ M to $1x10^{-12}$ M for 48h in order to perform the MTT assay. MTT assay allowed us to set compounds concentration to use for the subsequent experiments. Immunofluorescence was

performed after 2h and 6h of exposure with NP $1x10^{-10}$ M and E2 $1x\ 10^{-9}$ M. FACS, western blot and RT-qPCR were carried out after 48h of exposure to NP $1x10^{-10}$ M and E2 $1x\ 10^{-9}$ M.

2.4 MTT assay

NP and E2 effects on cell viability were evaluated through MTT assay. LNCaP were seeded at a density of $1x10^4$/ well in 96 multiwell and starvated (FBS 1%) for 24h and exposed for 48h to NP and E2 ($1x10^{-6}$ M to $1x10^{-12}$ M) .Briefly, 10µL of MTT were added to each well. After 4h 37°C 5% CO_2 of incubation, a solution of isopropanol and DMSO (1:1) was added in order to dissolve the crystal of formazan produced in each well. Then, the solution was read at 570 nm using a microplate reader. The value of absorbance is proportional with the number of living cells. Each MTT assay was performed in triplicate.

2.5 FACS analysis

FACS analysis was used to evaluate the distribution of LNCaP cells in cell cycle phases, analyzing the content of DNA after 48h of treatment with NP $1x10^{-10}$ M and E2 $1x10^{-9}$ M. In order to synchronize cells in G0/G1 phase, after 1% FBS starvation, LNCaP were washed with PBS, centrifugate at 800 g for 5min and fixed in 70% ethanol. Then, pellet was resuspended in 100 µg/ml RNAse for 30 min at 37°C. After, 20 µg/ml propidium iodide was added to each sample at 4°C for 30 min in the dark. FACScan™ flow cytometry system (Becton Dickinson, San Jose, CA) was used for analyze cells distribution. For each sample, $5x10^4$ events were analyzed and percentage distribution in each phase of cell cycle was calculated. Each experiment was performed in triplicate.

2.6 Immunofluorescence

LNCaP cells were cultured in 4-well chamber slides (Sarstedt, Nürnbrecht, Germany) at a density of $5x10^4$ cells overnight. After 24 h 1% FBS starvation, cells were treated with NP $1x10^{-10}$ M and E2 $1x10^{-9}$ M for 2h and 6h. Then, cells were fixed with ice cold methanol for 10 min, washed with PBS, permeabilized with 0,4% Triton in PBS for 10min and blocked with 5% Normal goat serum (NGS) for 30min. Then, cells were incubated overnight at 4°C with anti-human ERα antibody (Santa Cruz SC-544) or anti-human ERβ antibody (Santa Cruz SC-8974), diluted 1:100 in 1% NGS. For detection of ERα and ERβ, Alexa Fluor 488 (diluted 1:200 in 1% NGS) was used for 1h at 37°C in the dark. Cell nuclei were stained for 3min with 1µg/ml Höechst and the images were taken on an Axioskop (Carl Zeiss) epifluorescence microscope using a 40x objective. Axiocam MRc5 and the acquisition software Axiovision 4.7 (Carl Zeiss) were used to capture the images in different channels (Alexa Fluor 488, Höechst 33258). Three experiments were performed for each experimental conditions and the fields were randomly chosen.

2.7 Protein extraction and western blot analysis

For protein extraction LNCaP cells were seeded in 10 cm cell dishes. After 48h of treatment with NP $1x10^{-10}$ M and E2 $1x10^{-9}$M, the dishes with confluent control and treated cells were putted on ice for 10 min and washed with ice cold PBS. Then, PBS-EDTA was added, cells were scraped, collected and centrifugated for 5 min at 3000 rpm at 4°C. Pellets obtained were resuspended for 30 min with RIPA lysis buffer containing protease and phosphatase inhibitors cocktail (Santa Cruz). Homogenates were centrifugated at 12,000 g for 20 min and total protein amounts were defined using BCA protein assay reagent kit (PIERCE). For each sample, 50 µg of proteins were boiled for 5 min in SDS buffer [50 mMTris-HCl (pH 6.8), 2 g 100 mL^{-1}SDS, 10% (v/v) glycerol, 0.1 g 100 mL^{-1}Bromophenolblue], separated on 10%

SDS-PAGE and transferred to a PVDF membrane for blotting (Trans-Blot® Semi-Dry Transfer Cell, Biorad). The membranes were incubated for 1 h with blocking buffer (TBS, 0.05% Tween-20 and 5% milk) at room temperature and after blocking were incubated overnight at 4° C with primary antibodies diluted in TBS-T containing 2% milk. Primary antibodies used were: rabbit polyclonal anti-human ERα (1:200, Santa Cruz – sc544) , rabbit polyclonal anti-human ERβ (1:200, Santa Cruz sc-8974) and rabbit polyclonal anti-human β-actina (1:200, Santa Cruz sc-7210). The day after, the membranes were washed four times for 10 minutes in TBS, 0.05% Tween-20 before a 1h incubation with secondary antibody. Secondary antibody used was goat anti-rabbit IgG (HRP) (1:3000; Abcam ab-6721) and it was diluited in TBS-T containing 2% milk. After incubation, the membranes were washed again four times for 10 minutes and specific protein bands were detected with chemiluminescence using the C-DiGit Chemiluminescent Western Blot Scanner (LI-COR).Western blots were analyzed using Image Studio Software to determine optical density (OD) of the bands. The OD reading was normalized to β-actin to account for variations in loading. Western blots were performed as previously reported in Di Lorenzo et al. (2017) and all experiments were performed in triplicates.

2.8 RNA extraction and RT-qPCR

Expression levels mRNA of estrogen target, proliferation, and inflammation genes were analyzed using real-time PCR. Total RNA from control and treated LNCaP with NP $1x10^{-10}$ M and E2 $1x10^{-9}$ M for 48h, was extracted using Trizol (Life Technologies). After purification of genomic DNA with TURBO DNA-free™ Kit (Ambion, Life Technologies), the total amount of RNA was quantified with a NanoDrop spectrophotometer. cDNAs were synthesized from 1 µg RNA using the High Capacity cDNA Reverse Transcriptase (Life Technologies) and quantitative PCR was performed by using the 7500 Real-Time PCR System and SYBR® Select Master Mix 2X assay (Applied Biosystem). All primers used were designed according to the sequences published on GenBank using Primer Express software version 3.0. The amount of target cDNA was calculated by comparative threshold (Ct) method and expressed by means of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using hypoxanthine phosphoribosyltransferase 1 (HPTR1) as housekeeping gene.

Primers used:

Cyclin D1: FOR 5'-CGTGGCCTCTAAGATGAAGGA-3'; REV 5'-CGGTGTAGATGCACAAGCTTCTC-3';
Ki67: FOR 5'-CCCGTGGGAGACGTGGTA-3'; REV 5'-TTCCCGTGACGCTTCCA-3';
p53: FOR5'- TCTGTCCCTTCCCAGAAAACC-3'; REV5'-CAAGAAGCCCAGAAACGGAAA 3';
c-myc: FOR 5'-AGGGTCAAGTTGGACAGTGTCA-3': REV 5'- TGGTGCATTTTCGGTTGTTG-3';
IL-8: FOR 5'-CTGGCCGTGGCTCTCTTG-3'; REV 5'-CTTGGCAAAACTGCACCTTCA-3';
IL-1β: FOR 5'-ACGATGCACCTGTACGATCACT-3'; REV 5'-CACCAAGCTTTTTTGCTGTGAGT-3';
HPTR1: FOR5'-GACTTTGCTTTCCTTGGTCAGGCA-3'; REV 5'- ACAATCCGCCCAAAGGGAACTGA-3'.

2.9 Statistical analysis

Statistic analysis was performed by Graph Pad Prism 5 software. Data are expressed as mean values ±SEM for the indicated number of independent determinations. The statistical significance was calculated by the Student's t-test for FACS analysis. For the MTT assay, migration assay, western blot and qPCR analyses the one way ANOVA with Bonferroni's multiple comparison test was performed and differences were considered statistically significant when the P values was at least <0.05. All of the experiments were performed in triplicate and repeated at least three times.

3. RESULTS

3.1 Mtt assay

We assessed the effects of NP and E2 on LNCaP proliferation after 48 h of exposure. NP had a significant effect on LNCaP cell proliferation at 10^{-10} M (**Fig. 1a**). E2 stimulated LNCaP proliferation at 10^{-9} M (**Fig. 1b**). No effects were observed neither at the highest concentrations (from 10^{-6} M to 10^{-8} M) used neither at the lowest (from 10^{-10} M to 10^{-12} M).

3.2 FACS analysis

Cell cycle cells distribution was performed with FACS analysis using propidium iodide staining after 48h of treatment. As shown in **Fig. 2** both NP $1x10^{-10}$ M (**Fig. 2c**) and E2 $1x10^{-9}$ M (**Fig. 2b**) increased cell distribution in S phase and decreased G0/G1 phase compared to control cells. Comparing both treatments (**Fig. 2d),** we didn't observe differences in cell distribution between E2 and NP treated cells.

3.3 Immunofluorescence

ERα and ERβ cellular localizazion were studied with fluorescence microscopy after two different time of treatment: 2h and 6h. In LNCaP, ERα appears to be prevalently localized in the cytoplasm, with no evident fluorescent signal in cell nuclei (**Fig. 3**). E2 induced a nuclear localization of ERα at both time considered, without any localization in the cytoplasm. In contrast, NP act on ERα translocation after 6h of treatment, with a cytoplasmic localization after 2h. ERβ is prevalently located in the cytoplasm of LNCaP (**Fig. 4**); not E2 neither NP acted on ERβ translocation which remained cytoplasmatic at both times of treatment.

3.4 Western blot analysis

Western blot analysis was performed in order to evaluate estrogen receptors expression. Results showed ERα (66 KDa), ERβ (56 KDa) both in control and treated LNCaP cells **(Fig. 5a).** The densitometric analysis revealed higher levels of ERα both with NP and E2 treatment **(Fig. 5b).** NP treatment did not interfere with ERβ protein expression (**Fig. 5c**), instead E2 significantly enhanced its expression **(Fig. 5c).**

3.5 RT-qPCR

We studied the expression of genes involved in cell cycle regulation as well as -in inflammation (**Fig. 6 a-f**) signaling pathways after the treatment with NP $1x10^{-10}$ M and E2 $1x10^{-9}$ M. NP increased mRNA levels of Ki67 (**a**) and CICD1 (**b**) while decreased p53 gene expression (**c**). E2 LNCaP treated cells shown an up-regulation of CICD1 (**b**) and c-Myc (**d**), and a down-regulation of p53 (**c**). Regarding inflammation genes, only NP showed a significant effect, increasing mRNA levels of IL8 (**e**) and IL1β (**f**).

4. DISCUSSION

In recent years, emerging evidence suggest a relation between prostate diseases and human exposure to environmental pollutants, such as EDCs (Soto and Sonnenschein, 2010). Notably, cases of prostate cancer and prostate linked pathologies are increased in parallel to industrialization and Europe and US present the highest incidence. However, most of the data supporting this hypothesis prevalently derived from epidemiological studies. One of the explanations of the association between EDCs and prostate disease is the estrogen like action of a class of EDCs, called xenoestrogens. In this regard, it is well known the role of estrogens as the main responsible for the normal and aberrant growth of the prostate (Hu et al., 2011; Leung et al., 2010; Nelles et al., 2011). Thus, we decide to investigate the molecular pathways involved in the action of nonylphenol (NP) on LNCaP prostate cells. In parallel, we performed the same experiments with the estrogen 17β- estradiol (E2). The first data obtained regard the effects on the cellular proliferation. After 48h of exposure, NP and E2 at $1x10^{-10}$ M and $1x10^{-9}$ M, respectively, enhanced the cellular proliferation and at the same concentration they also induced the cell progression in S phase of cell cycle. Then, to highlight a possible estrogen receptors involvement, we studied their cellular localization and expression. Interestingly, both NP and E2 act only on ERα cytoplasm- nucleus translocation, although with different times, 2h for E2 and 6h for NP. Differently from E2, NP induced a strong increase only in ERα expression and it did not interfere with ERβ expression Some data reported the role of ERα in the proliferation and carcinogenesis of prostate cells, while ERβ seems to play a protective role for the gland, inducing apoptosis (Hartman et al., 2012). Thus, our data suggest the estrogen like action of NP, probably mediated via ERα. Finally, through qPCR analysis, we studied changing in gene expression of genes involved in proliferation and inflammation pathways. To note that most of these genes are well known estrogen downstream targets, as well as marker of prostate pathologies. Not all the genes investigated showed significant change after the treatment. The most effects on gene were the up- regulation of Ki67, CICD1, IL-8 and IL1β. All of the four genes resulted up-regulated in prostate diseases (Dey et al., 2013; Sfanos and De Marzo 2012). In particular, IL-8 is considered as prognostic markers of prostate diseases (Araki et al., 2007). In our case, our results suggest that the up-regulation of Ki67, CICD1, IL-8 and IL1β after NP treatment, turn into increase cell proliferation and S phase cell distribution. Interestingly, gene expression after E2 treatment not completely overlap with NP treatment: c-Myc and Ki67 resulted up- regulated only after E2 and c-Myc treatment, respectively. Moreover, E2 didn't affect gene expression of IL8 and IL1β, compared to LNCaP NP treated cells. Probably, NP, plus than E2, evokes an inflammation process in LNCaP cells. In addition, ERα is not the only pathway that mediate the effect of NP. In conclusion, we can summarize our results in some points: 1) estrogens and xenoestrogens stimulate proliferation, phase S progression of LNCaP, that may be a risk factors for prostate cancer 2) ERα switch in the nucleus after NP and E2 exposure, increasing gene expression of target genes 3) NP probably act with a mechanism not dependent from estrogen signaling pathways.

Figure captions

Fig. 1 MTT assay of 48 h exposure to 17β- estradiol (E2) and nonylphenol (NP). NP stimulates LNCaP proliferation with a significant effect at $1x10^{-10}$ M (**a**). E2 increases LNCaP proliferation at $1x10^{-9}$ M (**b**). Control cells were treated with vehicle (0,01% DMSO). Data represent the mean ± SE of three independent experiments (**P<0,01)

Fig. 2 Cell cycle analysis of 48 h exposure to 17β- estradiol (E2) and nonylphenol (NP). Rappresentative histograms (**a**, **b**, **c**) and relative data summary of the cell cycle distribution (**d**). $1x10^{-9}$ M E2 (**b**) and $1x10^{-10}$ M NP (**c**) increase cells in phase S, consequently decreasing G0/G1 cell accumulation. Control cells were treated with vehicle (0,01% DMSO). Data represent the mean ± SE of three independent experiments (**P<0,01)

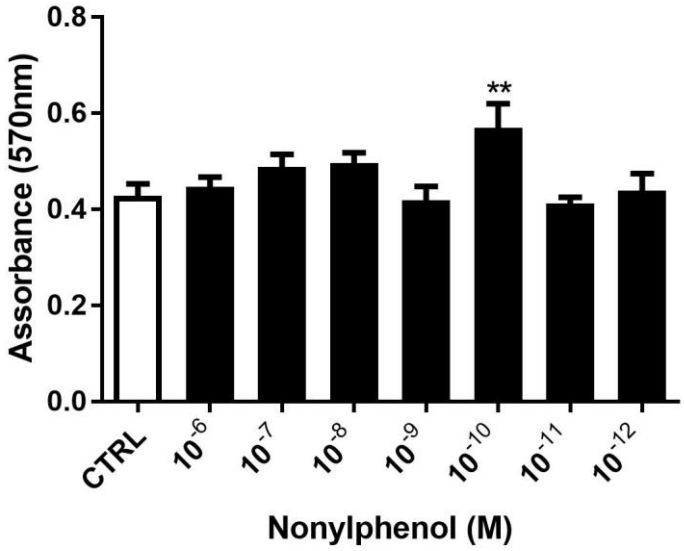
Fig. 3 ERα localization in LNCaP cells treated with 17β- estradiol (E2) and nonylphenol (NP). ERα appears to be localized in the cytoplasm in control cells. E2 induced ERα translocation at both time considered (2h, 6h). NP induced a switch cytoplasm- nucleus after 6h. LNCaP were plated in chamber slide under hormone deprived conditions. ERα (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10μm

Fig. 4 ERβ localization in LNCaP cells treated with 17β- estradiol (E2) and nonylphenol (NP). Treatment didn't affect cellular localization of ERβ, that was localized in the cytoplasm. LNCaP were plated in chamber slide under hormone deprived conditions. ERβ (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence. Scale bar 10μm

Fig.5 Western blot analysis. The graphs represented the optical density (O.D.) ratio of ERα (b), ERβ (c), normalized on β actin. Look at the text for more details. (* $p < 0,005$; **** $p < 0,01$; ***** $p < 0,001$).**

Fig. 6 qPCR analysis of genes involved in cell cycle regulation and inflammation processes (a-f). To note the different actions on gene expression of nonylphenol (NP) and 17β- estradiol (E2). Look the test for details. (*P<0,05; **P<0,01; ***P<0,001)

FIG. 1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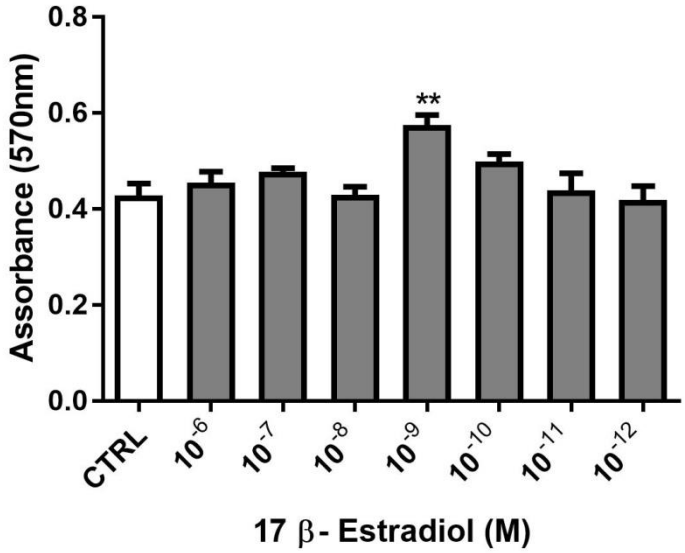
Nonylphenol (M)

a)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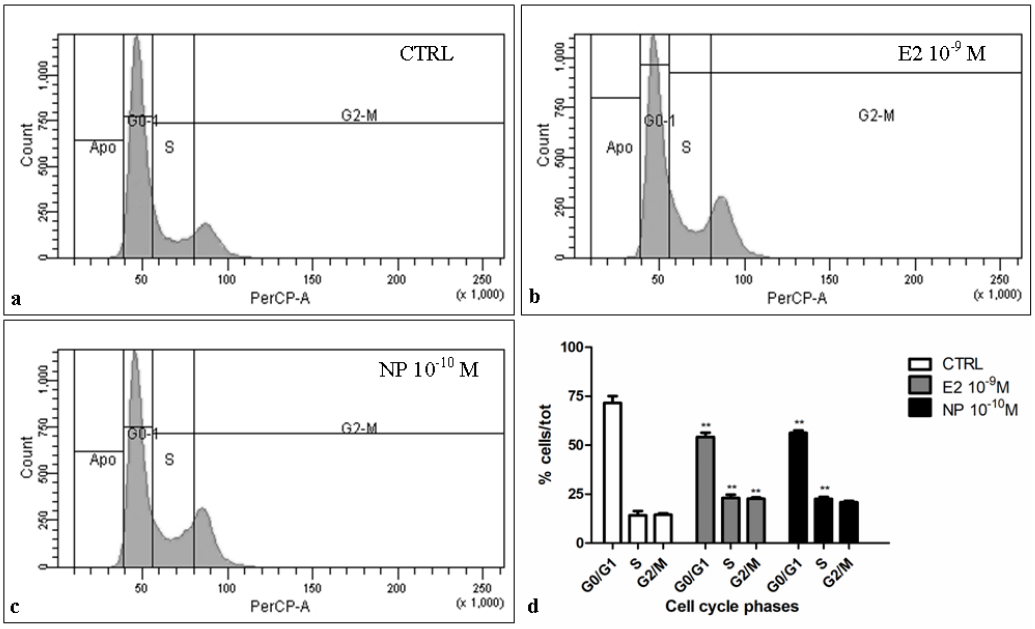
17 β- Estradiol (M)

b)

FIG.2: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E2 10-9 M
NP 10-10 M
Apo
G0-1
S
G2-M
Count
PerCP-A
(x 1,000)
a
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c
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% cells/tot
CTRL
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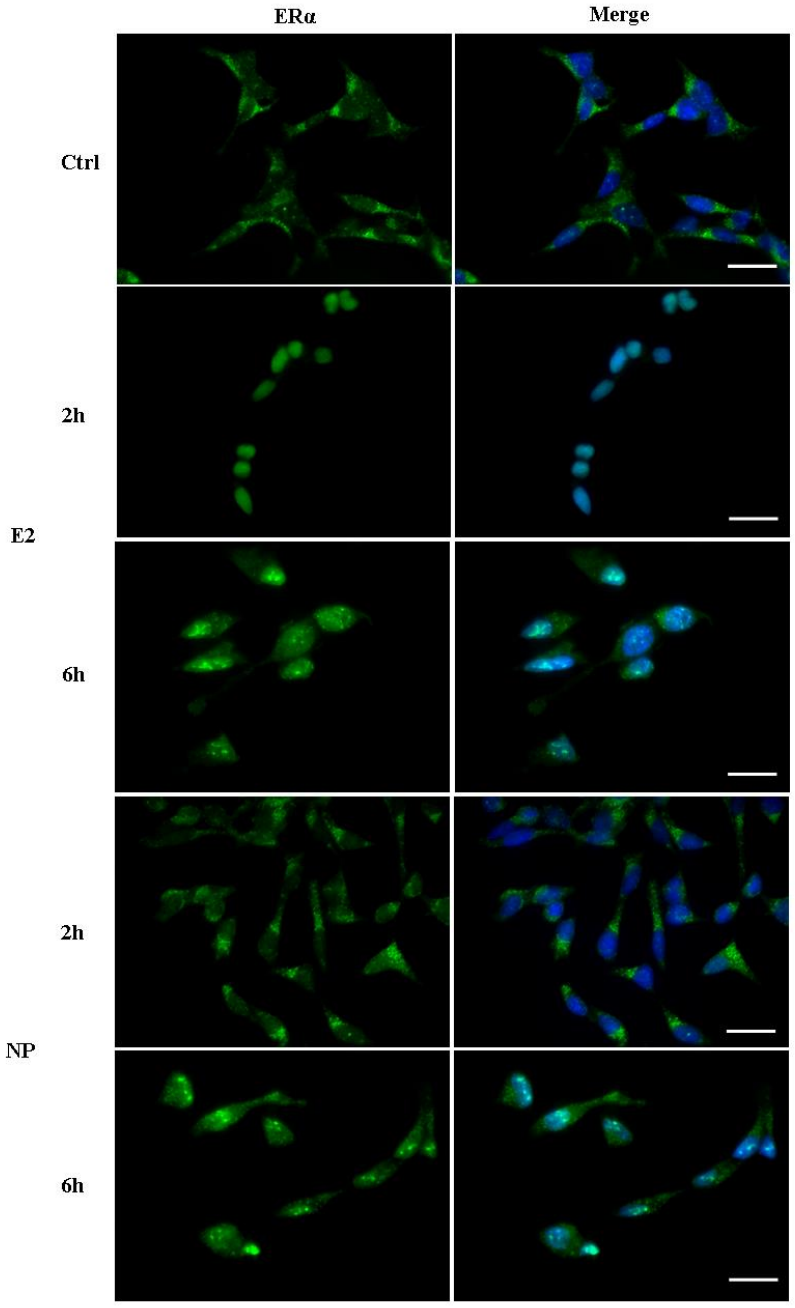
NP 10-10M
G0/G1
S
G2/M
Cell cycle phases

Fig.3:



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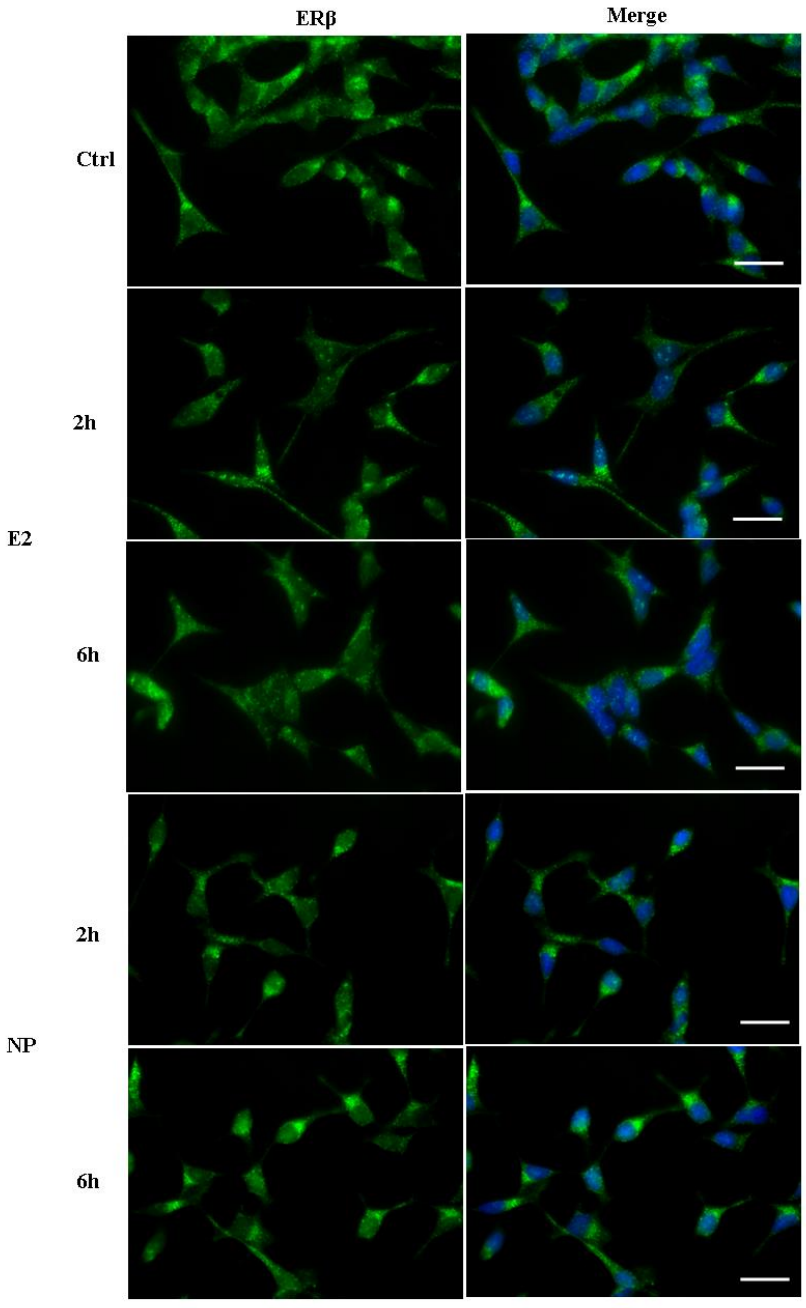

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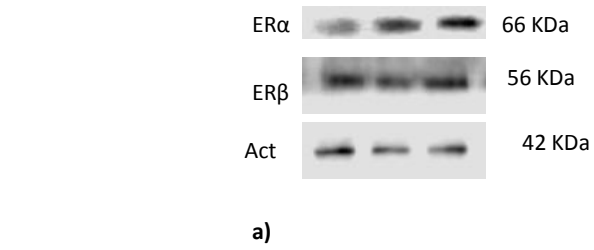

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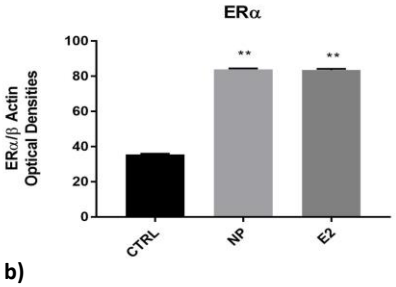

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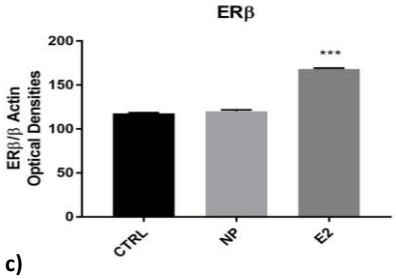
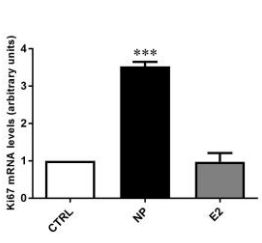


c)

FIG.6:



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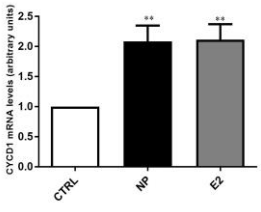

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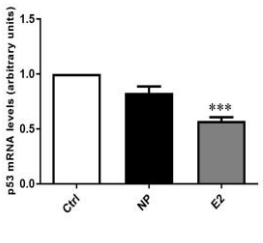

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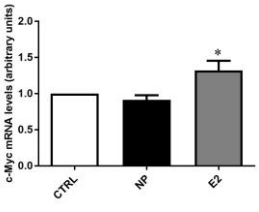
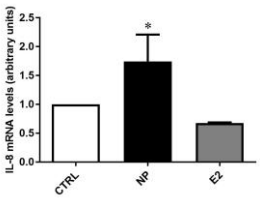
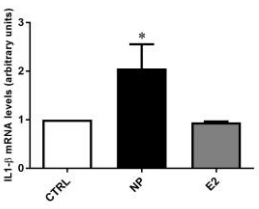


d)



e)



f)

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5. Conclusions

There is growing evidence to suspect that lifestyle factors and environmental exposure to endocrine disrupting chemicals (EDCs) can potentially contribute to the trends in the occurrence of reproductive health problems.

Endocrine disrupting chemicals comprise a class of different chemical origin compounds used in several different applications such as pesticides, herbicides, food packaging or in the formulation of personal care products. Their extensively diffusion in sediment, soil, water and atmosphere make them routinely available for human exposure which can occur at any time of life and at different doses.

Data showed reinforce the link between EDCs exposure and male reproductive tract disorders.

In particular, data confirm the ability of EDCs to act at very low dose following the typical non monotonic dose-response.

The effects of EDCs on the male reproductive system are notable attributed to the interactions of these chemicals with the normal production of steroid hormone but it is really interesting to note that the interaction with estrogen receptors may be an additional mode of action of EDCs on male reproductive system.

Moreover, it is very important to highlight the ability of the different substances tested to interfere with the same hormonal pathway inducing different effects on the same target.

Furthermore, results from *in vivo* study suggest that fetal life need special attention because exposure during this window of susceptibility may induce adverse effects later in life.

Additional studies are mandatory to determine the relevance of some hot points in the link between EDCs and infertility, including critical windows of susceptibility for various target tissues, dose-response curves, and potential synergistic effects of mixtures of EDCs which human are daily no-stop exposed.

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