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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