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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

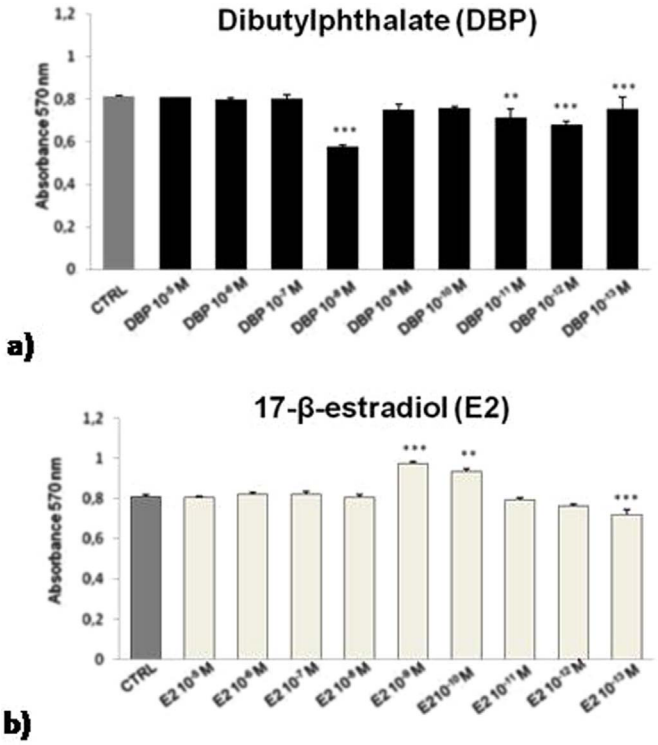

Fig. 1. MTT assay after 24 h of exposure to dibutylphthalate (DBP) and 17β-estradiol (E2) from 10^{-5} M to 10^{-13} M. In graphs are reported the absorbencies measured at 570 nm which correlates with number of living cells. (**a**) DBP induce a decrease of cell viability at 10^{-8} M; (**b**) E2 stimulates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

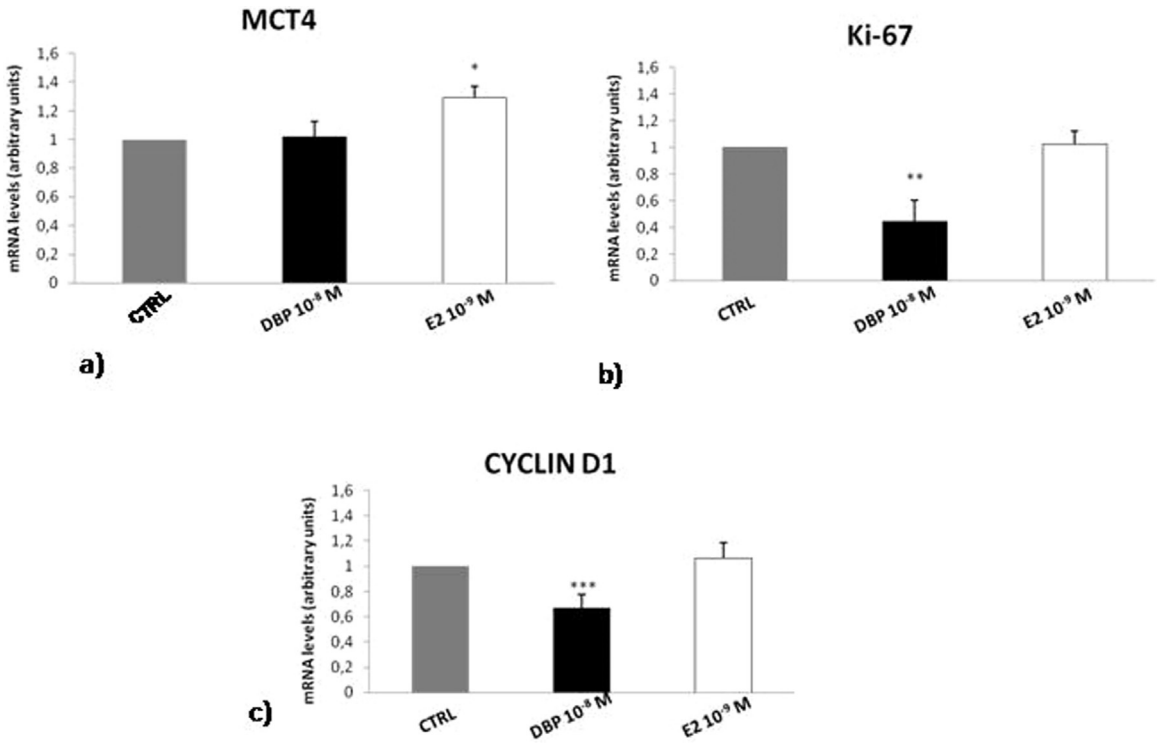

Fig. 2. qPCR analysis after 24 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β- estradiol (E2) 10^{-9} M. RT-qPCR was performed to evaluate expression of genes involved in cell cycle regulation such as MCT4, Ki67 and Cyclin D1. To note the different actions on gene expression of DBP and E2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

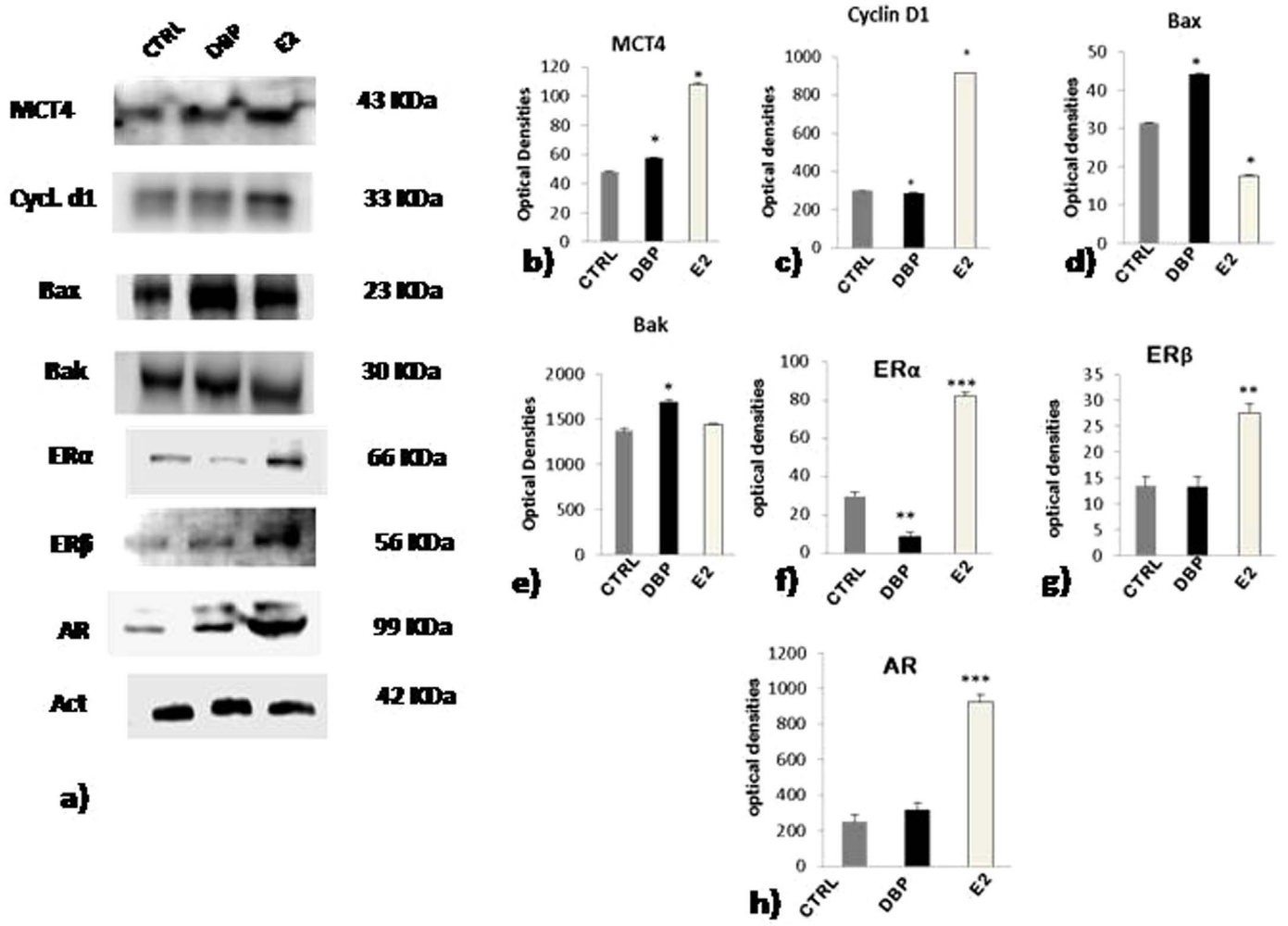

Fig. 3. Western blot analysis after 24 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β- estradiol (E2) 10^{-9} M. The graphs represented the optical density (O.D.) ratio of MCT4 (b), cyclin D1 (c), Bax (d), Bak (e), ERα (f), ERβ (g), AR (h) normalized on β actin. Look at the text for more details.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

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

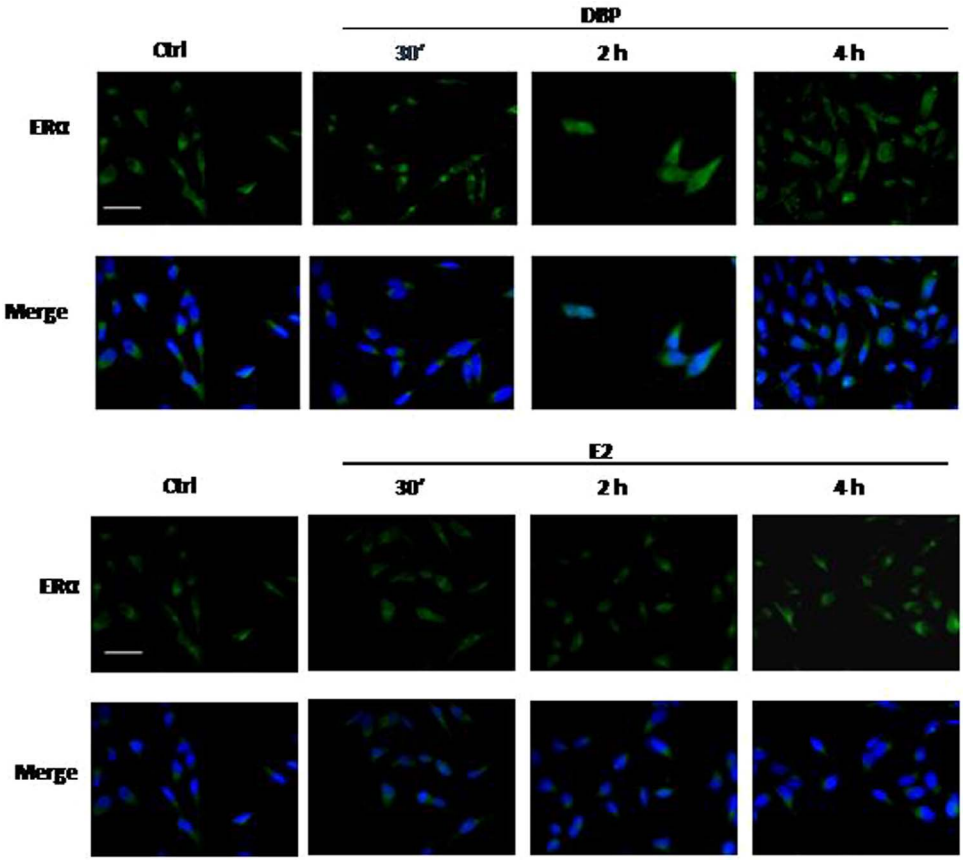

Fig. 4. ERα localization after 30′, 2 h and 4 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β-estradiol (E2) 10^{-9} M. ERα appears to be localized in the cytoplasm in control cells. DBP induced a cytoplasm-nuclear translocation after 4 h of exposure. E2 switch cytoplasm-nucleus after 2 h and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

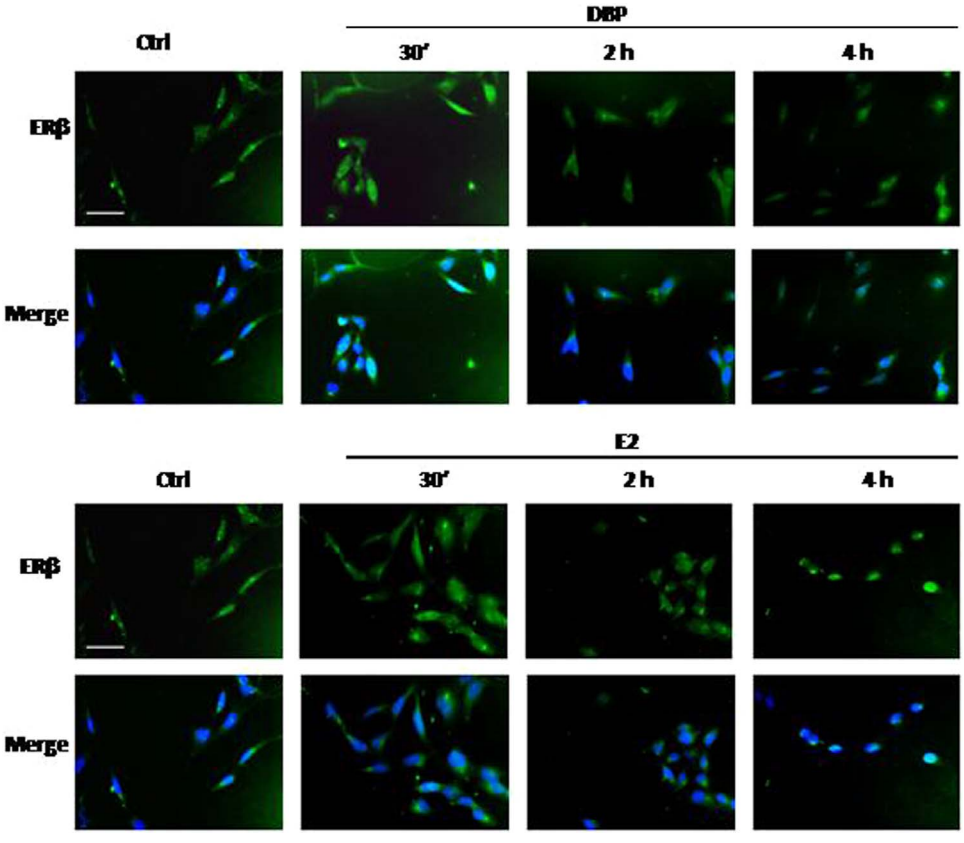

Fig. 5. ERβ localization after 30′, 2 h and 4 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β-estradiol (E2) 10^{-9} M. ERβ appears to be localized in the cytoplasm in control cells. DBP did not affects ERβ localization. E2 induced a cytoplasm-nucleus translocation only after 4 h. (Alexa Fluor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

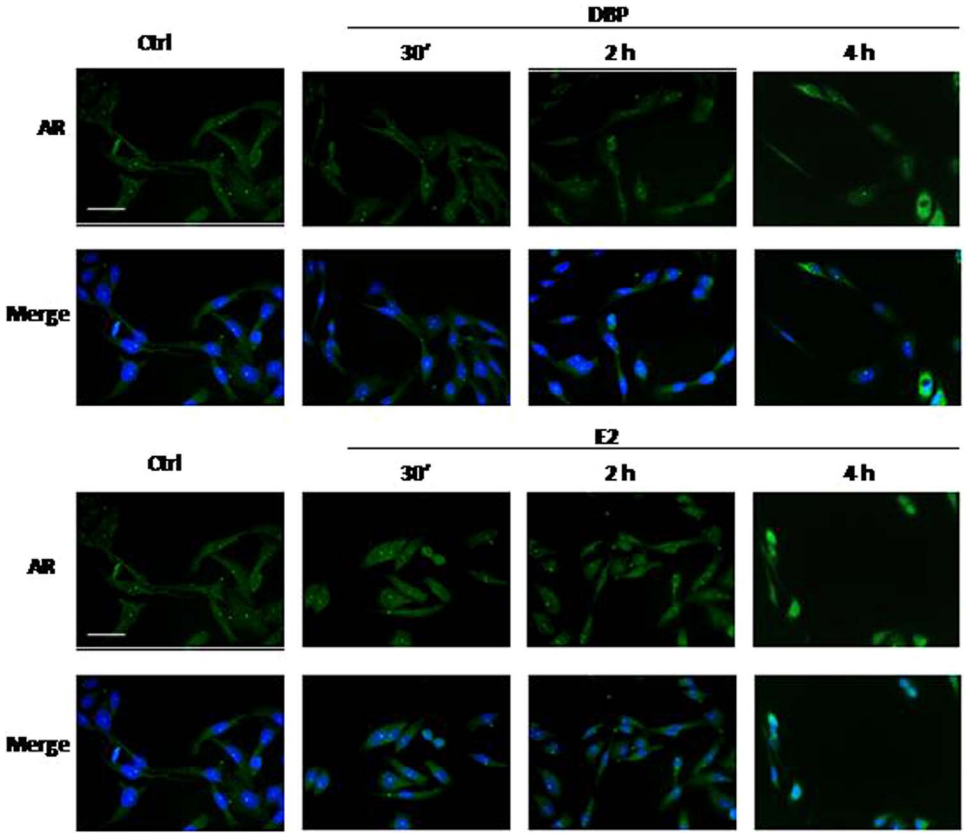

Fig. 6. AR localization after 30′, 2 h and 4 h of exposure to dibutylphthalate (DBP) 10^{-8} M and 17β-estradiol (E2) 10^{-9} M. DBP did not affects AR localization. E2 induced AR cytoplasm-nucleus translocation only after 4 h. (Alexa Fluor 488) and nuclear staining (Höechst) were analyzed by immunofluorescence.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