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Cytotoxic and Apoptotic Effects of 17α-Ethynylestradiol and Diethylstilbestrol on CHO-K1 Cells**

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Summary

There is considerable concern about the substances present in the environment and their potential to interfere with the endocrine system of vertebrates. Among these, the so-called endocrine-disrupting compounds, which can modulate or disrupt developmental and reproductive processes, substances with estrogenic activity have attracted most attention. Concerns about the presence of these compounds in the environment have led to the development of screening and testing assays that are able to detect such substances and evaluate their potential to induce adverse effects. *In vitro* systems such as mammalian and fish cell lines have become of growing importance in toxicity testing of such compounds. The cytotoxic and apoptotic effects induced by 17α-ethynylestradiol and diethylstilbestrol were studied on CHO-K1 cell line. Trypan blue exclusion method was used to determine the cell viability. Cytotoxicity of 17α-ethynylestradiol (0.34–34 μM) and diethylstilbestrol (0.37–37 μM) was found to be concentration-dependent with IC_{50} values of 12.8 and 10.4 μM after 72 h of exposure, respectively. In treated CHO-K1 culture cell death was assessed by determining morphological changes by haematoxilyn and eosin staining, nuclear morphology by fluorescein diacetate/propidium iodide staining and fluorescence microscopy, DNA fragmentation by TUNEL method and translocation of phosphatidyl serine by flow cytometry. The obtained results showed that 17α-ethynylestradiol induced apoptosis, while diethylstilbestrol induced necrosis in the treated CHO-K1 cells.

Key words: apoptosis, CHO-K1 cells, cytotoxicity, diethylstilbestrol, 17α-ethynylestradiol, necrosis

Introduction

The presence of chemicals that influence the endocrine system of humans and animals has become a major problem worldwide (*1*). The most potent active endocrine disrupting compounds (EDCs) present in the environment belong to the steroids, formed naturally by humans and animals or produced synthetically. The number of EDCs detected in the environment continuously increases and the need for assessing the potential risk of such compounds has become of outmost importance. Many compounds from industry (plastics, detergents, pesticides, cosmetics, pharmaceuticals, *etc.*) to which humans are unintentionally exposed due to aquatic pollution (*2*) also

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have impact on the endocrine system. The chemical structures of these compounds show a high diversity so it is difficult to predict their estrogenic activity and potency to cause adverse effects on exposed organisms. Therefore, the use of cell lines as an *in vitro* model for the evaluation of toxicity has important contribution in elucidating physiological and molecular mechanisms induced by xenobiotics (*3*). 17α-Ethynylestradiol (EE2) and diethylstilbestrol (DES) are synthetic estrogens chosen as representatives of EDCs in this work, since their residues enter the environment mostly through the effluents of sewage treatment works. Although they are present in the environment in low concentrations (in ng/L), the increased concern about the exposure of different organisms is present in public and scientific community (*4,5*). Synthetic estrogen EE2 is widely used as a major component of contraceptive pills as well as in other medical indications (*e.g.* for the treatment of menopausal disorders). DES was used extensively as a therapeutic drug for pregnant women in the 1960s, but it was recognized as a transplacental teratogen. Prenatally DES-exposed women and men have adverse symptoms such as: increased tendency for spontaneous abortion, cell adenocarcinoma, low sperm count and testicular cancers (*6*). DES is currently used for treatment of prostate cancer (*7*) and breast cancer (*8*), as well as in veterinary clinics for urinary incontinence.

The aim of the present study is to determine whether the cytotoxic effect of synthetic estrogens EE2 and DES on CHO-K1 cell line is due to apoptosis or necrosis. Morphological changes in dying cells were examined after haematoxylin and eosin, and fluorescein diacetate/propidium iodide staining. Induced cell death in the treated CHO-K1 cells was assessed by TUNEL method using fluorescence microscopy and by flow cytometry.

Materials and Methods

Cell line and culture conditions

CHO-K1 cells, derived from the ovary of an adult Chinese hamster, were of the American Type Culture Collection (CCL-61) origin (Manassas, VA, USA). CHO-K1 cells are used in the biotechnological production of recombinant proteins as well as for toxicity testing (*9, 10*). Cells were cultured in Dulbecco's modified Eagle's medium DMEM/F12 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % FBS at 37 °C in a humidified atmosphere containing 5 % CO_2. Antibiotics were not used during the experiments.

Cell viability assay

For experimental purposes, CHO-K1 cells collected in the exponential growth phase were seeded in 24-well plates at the initial concentration of $5 \cdot 10^4$ cells/mL and allowed to attach for 24 h before EE2 or DES treatment. Stock solutions of EE2 (3.4 mM) and DES (3.7 mM) were prepared by dissolving EE2 or DES (both from Sigma-Aldrich) in absolute ethanol. The final EE2 or DES concentration was obtained by appropriate dilution of the stock solution in the culture medium. Cells were exposed to 0.34–34 μM EE2 or 0.37–37 μM DES during 72 h. The final ethanol concentrations in the medium of the treated or control cells were 0.1 % for each sample. Cytotoxicity of EE2 or DES was examined by the measurement of cell viability using Trypan blue (TB) exclusion method (*11*). After EE2 or DES treatment, the cells were trypsinized and the cell suspension (20 μL) was mixed with 0.4 % Trypan blue dye solution (20 μL). Cells were counted on Fuchs-Rosenthal haemocytometer after 72 h of exposure. All experiments were performed in at least triplicate and within single experiment each EE2 and DES concentration was tested in triplicate.

Haematoxylin and eosin staining

Approximately 10^5 cells/mL were seeded in 6-well plates, allowed to attach for 24 h and then exposed to 17 μM of EE2 or 18.5 μM of DES during 72 h. The cells were washed with phosphate buffered saline (PBS) and then fixed in 70 % methanol at –20 °C for 10 min. Cells were stained with haematoxylin and eosin according to the Kiernan method (*12*) and morphological changes were observed immediately by Olympus BX51 microscope (Olympus, Tokyo, Japan).

Fluorescein diacetate and propidium iodide staining

Approximately 10^5 cells/mL were seeded in 6-well plates, allowed to attach for 24 h and then exposed to 17 μM of EE2 or 18.5 μM of DES during 48 h. The cells were washed with PBS, trypsinized, centrifuged and resuspended in 0.2 mL of PBS. Cell staining with fluorescein diacetate (FDA) and propidium iodide (PI) was performed according to the method described by Jones and Senft (*13*) and immediately examined using the fluorescent microscope Olympus BX51 with integrated camera.

TUNEL assay

Cells were seeded on culture slides with chambers (BD Labware, Falcon, NY, USA), allowed to attach for 24 h and then exposed to 17 μM of EE2 or 18.5 μM of DES. After 48 h of treatment, TUNEL method was performed using *In situ* Cell Detection Kit, Fluorescein (Roche Applied Science, Mannheim, Germany). Cells were fixed onto glass slides with 4 % paraformaldehyde in PBS and the assay was carried out according to the manufacturer's instructions. After co-staining with DAPI (Sigma-Aldrich), the cells considered apoptotic, or TUNEL-positive, were green, because of the catalytic incorporation of FITC-labelled dUTP by the terminal deoxynucleotidyl transferase (TdT) at 3'-OH end of fragmented DNA. Cells were examined immediately by fluorescence microscopy using Olympus Provis AX70 with integrated Nikon DXM 1200 camera (Nikon, Tokyo, Japan).

Flow cytometry assay

Cytotoxic effect induced by EE2 or DES treatment was analyzed by flow cytometry (*14*). Distinction of apoptotic cells from the live ones was determined by fluorescein isothiocyanate (FITC) conjugation of Annexin V (Annexin V-FITC), which binds to phosphatidyl serine (PS), a hallmark of early apoptosis events. Propidium iodide (PI) was used to discriminate late apoptotic/necrotic cells from the live ones. Both attached and detached CHO-K1 cells were collected after 48 h of treatment with EE2 (3.4–85 μM) or DES (3.7–92.5 μM), centrifuged, washed

twice with DMEM/F12 culture medium and resuspended in cold DMEM/F12 with 10 % of FBS to a final concentration of 10^6 cells/mL. Samples were incubated for 10 min at room temperature with Annexin V-FITC and PI, and analyzed by Cytomics FC500 MPL flow cytometer (Beckman Coulter, Miami, FL, USA) to detect viable (Annexin V-FITC-negative and PI-negative), apoptotic (Annexin V-FITC-positive and PI-negative) and necrotic cells (Annexin V-FITC-negative and PI-positive). A minimum of 10 000 cells were analyzed per sample.

Statistical analysis

The obtained data were expressed as the mean value± standard deviation (S.D.) of three independent experiments performed in triplicate. One-way analysis of variance (ANOVA), followed by Dunnett's significant difference test, was used to determine statistically significant ($p<0.05$) differences from the untreated controls. The IC_{50} value, defined as the concentration that reduces the survival of treated cells to 50 % of control cells, was calculated from the concentration-effect curves using the equation of related polynomial trend lines for TB assay.

Results and Discussion

Concerns about the presence of EDCs in the environment have led to the development of screening assays that are able to detect substances which interfere with the endocrine system of vertebrates. Among these so-called EDCs, substances with estrogenic activity have attracted most attention. To evaluate their potential to induce adverse effects in humans and animals, *in vitro* models (mostly mammalian and fish cell lines) have become important in toxicity testing of such compounds.

To determine the viability of CHO-K1 cells after exposure to EE2 and DES, cell number was assessed using Trypan blue exclusion method. The effects of different EE2 concentrations on CHO-K1 cells, expressed as a percentage of control, are summarized and shown in Fig. 1a. A statistically significant ($p<0.05$) stimulatory response was observed when CHO-K1 cells were exposed to lower doses of EE2 (0.34 and 1.7 μM). The observed stimulatory effect of EE2 in CHO-K1 culture can be explained by chemical hormesis, a reproducible and relatively common biological phenomenon characterised by a low dose stimulation and high-dose inhibition (*15*). Stimulatory effect of EE2 at low concentrations (0.01 and 1 nM) was demonstrated in primary cultures of fish testes, while higher concentrations of EE2 (100 nM) showed inhibitory effect on cell proliferation (*16*). Also, the observed increase in cell proliferation can be considered as proof of EE2 estrogenicity in CHO-K1 cells since Hertz (*17*) defined estrogen as a substance that can elicit mitotic stimulation of female genital tract tissue or induce proliferation of estrogen-responsive target cells *in vitro*. At the subsequent concentrations of EE2 (17 and 34 μM), a significant decrease ($p<0.05$) in the CHO-K1 cell viability was observed. The cell viability of the exposed cells dropped to (38±5) and (12±4) % compared to control cells, showing concentration dependency. When CHO-K1 cells were exposed to DES (0.37–37 μM), significant cytotoxic effect ($p<0.05$) was observed at all tested concentrations in a concentration-dependent manner (Fig. 1b). Antiproliferative effect of DES at concentrations of 1, 5 and 10 μg/mL was detected in the prostatic carcinoma cell line LNCaP and MRC-5 cells (*18*). Also, DES influenced cell viability of human lymphocytes at 5, 10, 15 and 20 μM, showing dose and time dependency (*19*). The concentration-dependent decrease in cell viability of CHO-K1 cells after exposure to EE2 and DES observed in this study was consistent with our previous study when MTT and Neutral red assays were performed (*20*). In order to compare cytotoxicity of EE2 and DES, IC_{50} values were calculated and were 12.8 μM for EE2 and 10.4 μM for DES, showing higher toxicity of DES in CHO-K1 culture.

Morphological changes induced by EE2 and DES were observed after 72 h of treatment by haematoxylin and eosin staining. Control CHO-K1 cells (Fig. 2a) were well attached and retained characteristic epithelial morphology. Due to shrinkage, treated cells lost adhesion contacts that led to cell monolayer disruption. In both treated samples (Figs. 2b–c) intensive nuclear staining was visible, probably due to chromatin condensation (pyknosis), which indicates apoptosis. In DES-treated CHO-K1 cells cytoplasm vacuolization was also observed (Fig. 2c). Accumulation of vesicular structures in the cytosol of the damaged cells may indicate formation of autophagosome. Although autophagy is considered as cell's major adaptive (survival) strategy in response to various triggers or toxic injury (*21*) and thus may precede apop-

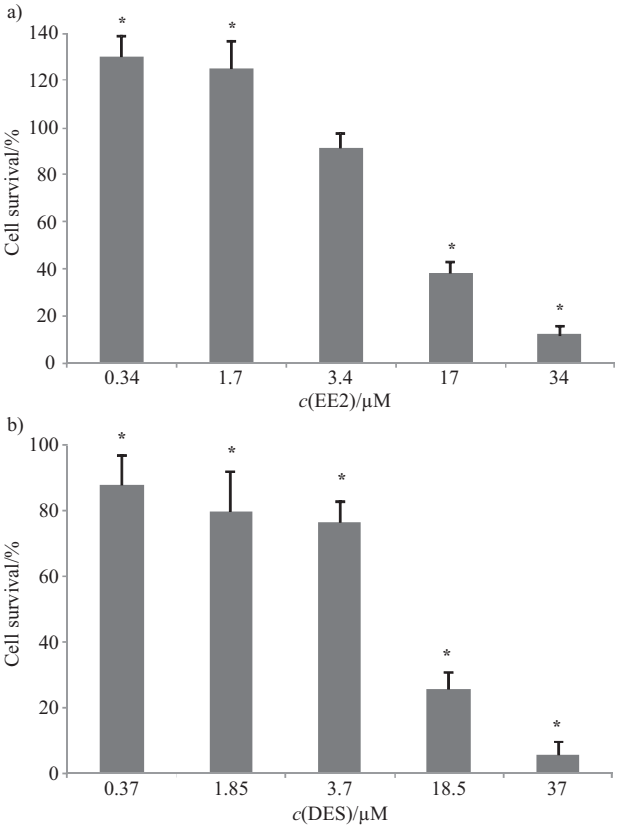

Fig. 1. Effects of: a) EE2 (0.34–34 μM) and b) DES (0.37–37 μM) on cell survival in CHO-K1 culture after 72 h of exposure using Trypan blue assay. Data expressed as percentage of unexposed control cells±S.D. of three replicates for each concentration; *significant difference from control ($p<0.05$)

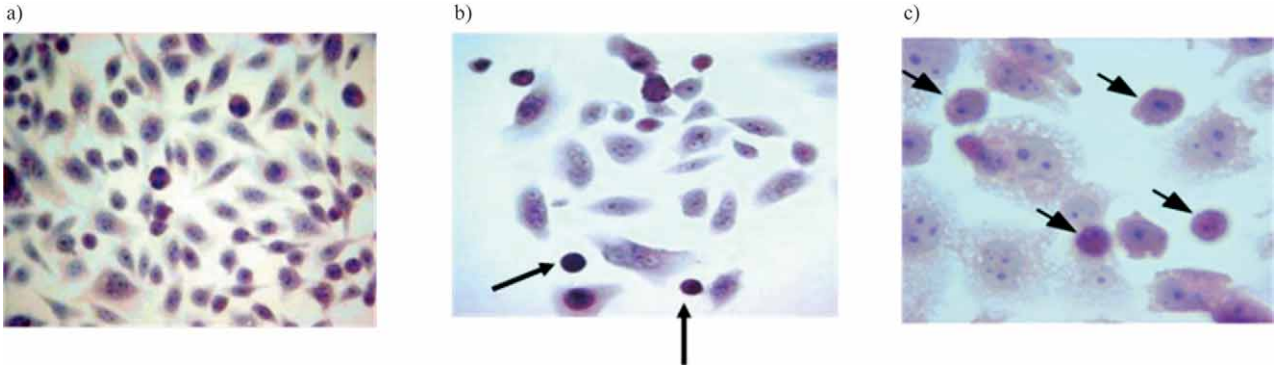

Fig. 2. Photomicrographs of CHO-K1 cells stained with haematoxylin and eosin: a) control cells, and cells treated for 72 h with b) 17 μM EE2 and c) 18.5 μM DES. Apoptotic cells and bodies are intensively stained (arrows). In DES-treated cells, cytoplasmic vacuolization can be observed. Magnification: a) and b) 600× and c) 1000×

tosis (22), it also has to be considered as a hallmark of programmed cell death type II (*i.e.* autophagic cell death). To further investigate whether the observed cytotoxicity is a result of apoptosis or necrosis, nuclear morphology of EE2 and DES-treated CHO-K1 cells was evaluated using FDA and PI staining (Figs. 3a–c). In control sample (Fig. 3a), most of the cells are viable, as evidenced by FDA hydrolysis and the subsequent green fluorescence of the hydrolytic product. As shown in Fig. 3b, red fluorescence of PI increases in the cells exposed to 17 μM of EE2, but not significantly. In contrast, most DES-treated cells (Fig. 3c) are PI-positive, indicating cell death by necrosis. One of the late apoptosis hallmarks is DNA fragmentation that occurs through endonuclease activity (23), and therefore TUNEL method was applied for *in situ* visualisation of DNA fragmentation at a single-cell level. Figs. 4a–c show fluorescence of DAPI in blue representing live cells, while green fluorescence of incorporated FITC-dUTP presents TUNEL-positive, *i.e.* apoptotic cells. Increased number of apoptotic cells is seen in both, EE2 and DES-treated CHO-K1 cells (Figs. 4e–f). The biochemical characteristic of early phase of apoptosis is translocation of PS from the inner side of the plasma membrane to the outer layer. Since Annexin V has a high affinity for PS, it is commonly used as an apoptosis marker. In combination with PI, this allows quantitative discrimination of live, apoptotic and necrotic cells by flow cytometry. CHO-K1 cells were evaluated after 48 h of EE2 (3.4–85 μM) or DES (3.7–92.5 μM) treatment, in respect to the control cells. In CHO-K1 cells treated with EE2, an increase of apoptotic/necrotic cells is not dose-dependent (Fig. 5a). The highest rate of apoptotic cells (23.9 %) was detected in sample treated with 34 μM EE2 (Fig. 5b). Interestingly, samples treated with higher concentrations of EE2 (51 and 85 μM) had more viable and less apoptotic cells than those treated with 34 μM EE2. Although higher concentrations of EE2 have shown lower cell viability assessed by Trypan blue exclusion (data not shown), this effect cannot be explained by cell death as there was no evidence of increased rate of apoptosis or necrosis in these samples. Inhibitory effect provoked by 51 and 85 μM of EE2 might be a consequence of G0/G1 cell-cycle block, but this requires further investigation.

The effect of DES (3.7–92.5 μM) on CHO-K1 cells was also determined by flow cytometry (Fig. 6). Percentage of apoptotic, necrotic and live cells in every sample after 48 h of treatment is shown in Fig. 6a, while a representative 'dot-plot' chart (Fig. 6b) shows CHO-K1 cells treated with 92.5 μM of DES, where the highest rate of necrosis (95.3 %) was detected. The observed DES-induced effects are dose-dependent, since an increase in DES concentrations lowered cell viability and increased the number of necrotic cells. Therefore, we can assume that DES cytotoxicity is related to necrosis induction. Roan *et al.* (*10*) reported DES-induced apoptosis in CHO-K1 cells at 100

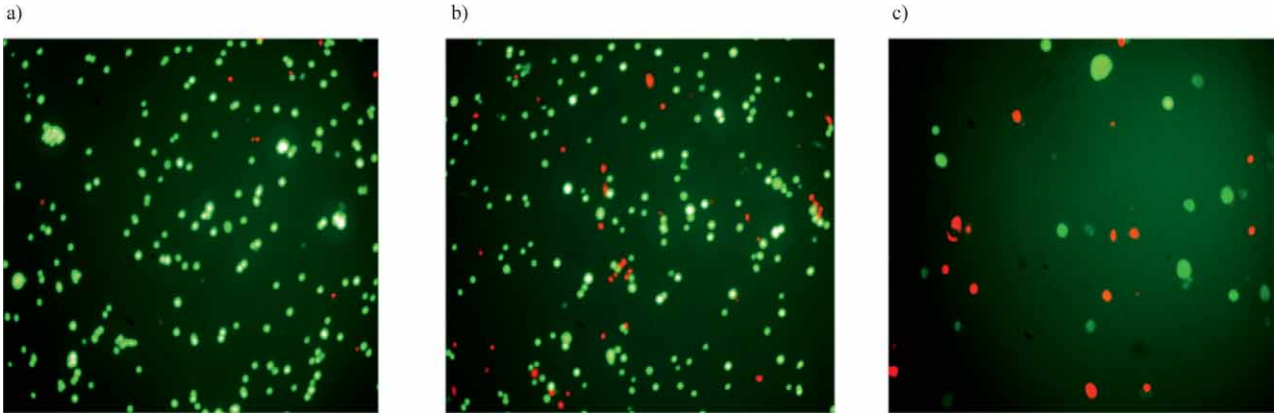

Fig. 3. Photomicrographs of CHO-K1 cells stained with FDA and PI: a) control cells, and cells treated for 48 h with b) 17 μM EE2 and c) 18.5 μM DES. Viable cells shown in green (a), while apoptotic/necrotic cells (b, c) were observed in treated samples. Magnification: 400×

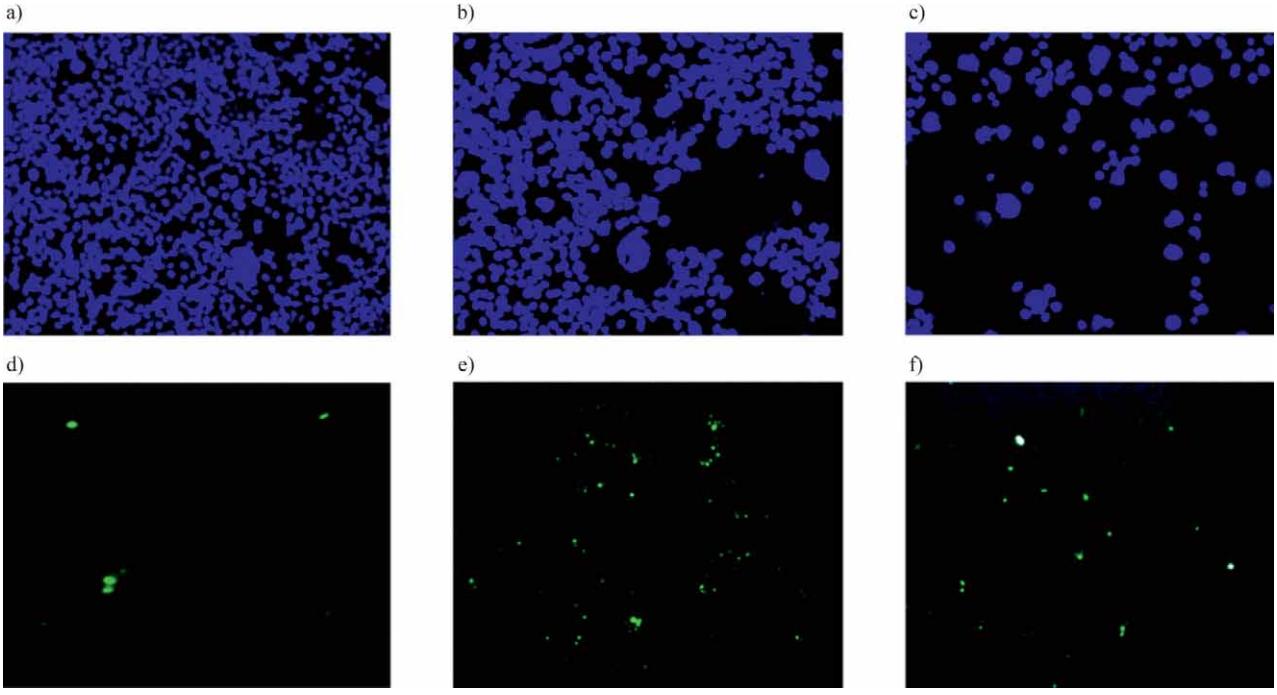

Fig. 4. Photomicrographs of CHO-K1 cells analyzed by TUNEL method and co-stained with DAPI. Upper row (a, b, c) depicts cell populations stained with DAPI (blue), while green fluorescence of incorporated dUTP-FITC presents TUNEL positive *i.e.* apoptotic cells (d, e, f). Control CHO-K1 cells (a, d) and cells treated for 48 h with 17 μM EE2 (b, e) or 18.5 μM DES (c, f). Magnification 200×

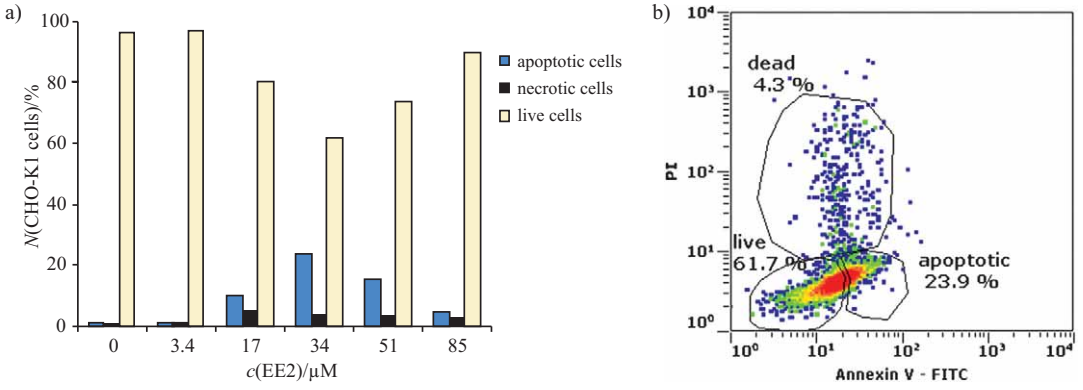

Fig. 5. Flow cytometric analysis of CHO-K1 cells treated with: a) EE2 (3.4–85 μM) is shown as percentage of apoptotic, necrotic and live cells in each sample, while b) 'dot-plot' chart represents CHO-K1 cells treated with 34 μM EE2 for 48 h

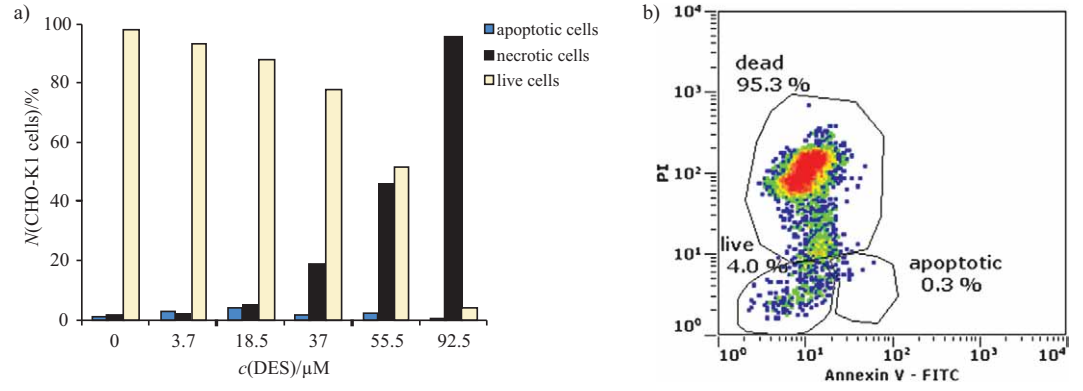

Fig. 6. Flow cytometric analysis of CHO-K1 cells treated with: a) DES (3.7–92.5 μM) is shown as percentage of apoptotic, necrotic and live cells in each sample, while b) 'dot-plot' chart represents CHO-K1 cells treated with 92.5 μM DES for 48 h

μM during overnight treatment, and Konac *et al.* (*19*) reported apoptosis induction in human lymphocytes *in vitro* after 24 h of exposure to 5, 10 and 15 μM DES, while 5 μM DES during 48 and 72 h exposure increased the rate of necrotic cells. It has to be kept in mind that there is a considerable interplay between different types of cell death and a high degree of flexibility in the cell's response to changes of (micro)environmental conditions or presence of death stimuli. Thus the cell's response may shift gradually from the elimination of damaged proteins by autophagy and survival to autophagic or apoptotic cell death, the failure of which eventually may result in necrosis. Taken all together, we can conclude that toxic effects of DES, as well as type of cell death induced, considerably differentiate depending on the used cell line, concentration and time of exposure.

Conclusions

EE2 showed dual effects on cell viability of CHO-K1 cells, as determined by Trypan blue exclusion method, depending on the applied concentration. Lower concentrations of EE2 (0.34 and 1.7 μM) had stimulatory effects on cell proliferation, while higher concentrations (17 and 34 μM) had a cytotoxic effect. All tested concentrations of DES (0.37–37 μM) showed cytotoxic effects in a concentration-dependent manner. Morphological changes, nuclear morphology, DNA fragmentation, translocation of PS and cell viability were examined in EE2 and DES-treated CHO-K1 cells. Taken together, the obtained results suggest that reduced cell viability observed in EE2 (17 and 34 μM) and DES (0.37–37 μM) treated CHO-K1 cells can be related to apoptosis and necrosis induction, respectively.

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References

1. T. Colborn, F.S. vom Saal, A.M. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, *Environ. Health Perspect. 101* (1993) 378–384.
2. S. Tayama, Y. Nakagawa, K. Tayama, Genotoxic effects of environmental estrogen-like compounds in CHO-K1 cells, *Mutat. Res. 649* (2008) 114–125.
3. J. Kniewald, I. Kmetič, V. Gaurina-Srček, Z. Kniewald, Alternative models for toxicity testing of xenobiotics, *Arh. Hig. Rada Toksikol. 56* (2005) 195–204.
4. K.L. Thorpe, R.I. Cummings, T.H. Hutchinson, M. Scholze, G. Brighty, J.P. Sumpter, C.R. Tyler, Relative potencies and combination effects of steroidal estrogens in fish, *Environ. Sci. Technol. 37* (2003) 1142–1149.
5. S. Schrager, B.E. Potter, Diethylstilbestrol exposure, *Am. Fam. Physician, 69* (2004) 2395–2400.
6. M. Marselos, L. Tomatis, Diethylstilbestrol: I, pharmacology, toxicology and carcinogenicity in humans, *Eur. J. Cancer, 28* (1992) 1182–1189.
7. D.S. Scherr, W.R. Pitts Jr, E.D. Vaughan, Diethylstilbestrol revisited: Androgen deprivation, osteoporosis and prostate cancer, *J. Urology, 167* (2002) 535–538.
8. P.E. Lønning, P.D. Taylor, G. Anker, J. Iddon, L. Wie, L.M. Jørgensen *et al.*, High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy, *Breast Cancer Res. Treat. 67* (2001) 111–116.
9. I. Kmetič, V. Gaurina Srček, I. Slivac, B. Šimić, Z. Kniewald, J. Kniewald, Atrazine exposure decreases cell proliferation in *Chinese hamster ovary* (CHO-K1) cell line, *Bull. Environ. Contam. Toxicol. 81* (2008) 205–209.
10. C.J. Roan, C.C. Huang, H.H. Cheng, J.M. Chien, C.T. Chou, K.L. Lin *et al.*, Diethylstilbestrol-induced estrogen receptor-dependent $[Ca^{2+}]_i$ rises and apoptosis in Chinese hamster ovary (CHO) cells, *J. Recept. Signal. Transduct. Res. 28* (2008) 307–322.
11. R.I. Freshney: *Animal Cell Culture: A Practical Approach*, D. Rickwood, B.D. Hames (Eds.), IRL Press, Oxford, UK (1992).
12. J.A. Kiernan: *Histological and Histochemical Methods*: *Theory and Practice*, Scion Publishing Ltd, Bloxham, UK (2008) pp. 96–97.
13. K.H. Jones, J.A. Senft, An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide, *J. Histochem. Cytochem. 33* (1985) 77–79.
14. Z. Darzynkiewicz, G. Juan, X. Li, W. Gorczyca, T. Murakami, F. Traganos, Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis), *Cytometry, 27* (1997) 1–20.
15. E.J. Calabrese, L.A. Baldwin, Hormesis: The dose-response revolution, *Annu. Rev. Pharmacol. Toxicol. 43* (2003) 175–197.
16. M. Song, H.O. Gutzeit, Effect of 17α-ethynylestradiol on germ cell proliferation in organ and primary culture of medaka (*Oryzias latipes*) testis, *Dev. Growth Differ. 45* (2003) 327–337.
17. R. Hertz: The Estrogen Problem – Retrospect and Prospect. In: *Estrogens in the Environment II: Influences on Development*, J.A. McLachlan (Ed.), Elsevier Science Publishing Inc, New York, NY, USA (1985) pp. 1–11.
18. P. Schulz, H.W. Bauer, W.P. Brade, A. Keller, F. Fittler, Evaluation of cytotoxic activity of diethylstilbestrol and its mono- and diphosphate towards prostatic carcinoma cells, *Cancer Res. 48* (1988) 2867–2870.
19. E. Konac, A. Ekmekci, V. Barkar, A. Yilmaz, D. Erbas, Effects of diethylstilbestrol in human lymphocytes *in vitro*: A dose and time-dependent study on genotoxic, cytotoxic and apoptotic effects, *Mol. Cell. Biochem. 276* (2005) 45–53.
20. K. Radošević, T. Tonković, I. Slivac, Z. Kniewald, V. Gaurina Srček, Comparison of cytotoxicity induced by 17α-ethynylestradiol and diethylstilbestrol in fish CCO and mammalian CHO-K1 cell lines, *Bull. Environ. Contam. Toxicol. 86* (2011) 252–257.
21. W. Bursch, A. Karwan, M. Mayer, J. Dornetshuber, U. Fröhwein, R. Schulte-Hermann *et al.*, Cell death and autophagy: Cytokines, drugs, and nutritional factors, *Toxicology, 254* (2008) 147–157.
22. R. Kim, M. Emi, K. Tanabe, S. Murakami, Y. Uchida, K. Arihiro, Regulation and interplay of apoptotic and non-apoptotic cell death, *J. Pathol. 208* (2006) 319–326.
23. L. Gollapudi, M.M. Oblinger, Stable transfection of PC12 cells with estrogen receptor (ER*alpha*): Protective effects of estrogen on cell survival after serum deprivation, *J. Neurosci. Res. 56* (1999) 99–108.