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Comparison of Cytotoxicity Induced by 17α-Ethynylestradiol and Diethylstilbestrol in Fish CCO and Mammalian CHO-K1 Cell Lines

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Abstract The effects of synthetic estrogens 17α-ethynylestradiol (EE2) and diethylstilbestrol (DES) were compared on cell proliferation and morphology in Channel Catfish Ovary (CCO) and Chinese Hamster Ovary (CHO-K1) cells. EE2 exposure (0.1 and 0.5 μg/mL) induced stimulatory effect on CCO and CHO-K1 cell proliferation, while higher concentrations (1–10 μg/mL) showed cytotoxic effects. Increase in DES concentrations mainly resulted in dose-depended increase in cytotoxicity in both cell lines. Morphological changes induced by EE2 and DES exposure (5 μg/mL) showed disrupted cell monolayer and increased number and size of lysosomes. Comparison of IC_{50} values showed almost equal sensitivity towards cytotoxicity of tested compounds in CCO and CHO-K1 cells.

Keywords CCO cells · CHO-K1 cells · Cytotoxicity · Synthetic estrogens

In recent years, there have been increasing concerns regarding environmental endocrine disrupting chemicals which influence the endocrine system, and cause adverse effects in fish, wildlife and humans (Colborn et al. 1993). Contaminants present in the aquatic environment such as pesticides (Donohoe and Curtis 1996), degradation products of non-ionic surfactants (Jobling et al. 1996), natural and synthetic steroids (Mills and Chichester 2005), and wood-derived phytoestrogens (Mellanen et al. 1996) have shown reproductive abnormalities in fish populations. Ekwall (1995) proposed that the lethal dose of a chemical in an environmental sample is the dose that actually kills an organism through toxicity at the cellular level. His "basal cytotoxicity concept" is a basis for relating in vitro cytotoxicity data to in vivo acute lethality. The rising interest in analysis of toxic effects of various pharmaceutical and chemical agents accumulated in the natural aquatic environment requires an increase in the number of fish cell lines available for systematic and routine toxicology investigations, prior to use of in vivo methods (Tan et al. 2008), since cytotoxicity studies can provide information about toxic potency of xenobiotics usually more rapidly and at a lower cost than in vivo studies.

A change in cell viability as a general response to a toxicant assesses fundamental cellular activities which may be expressed by many cell lines and in similar ways when toxicity is measured by various viability criteria (Babich and Borenfreund 1991). Two assays that are commonly used for the assessment of general cytotoxicity are the tetrazolium salt reduction (MTT) and neutral red (NR) assays (Fent 2001; Castaño and Gómez-Lechón 2005). The MTT assay is based on the measurement of mitochondrial metabolic activity by detection of the reduction of the soluble yellow MTT tetrazolium salt to purple MTT formazan by action of mitochondrial succinate-dehydrogenase (Mosmann 1983). The NR assay is based on the accumulation of neutral red dye in lysosomes of viable cells (Borenfreund and Purner 1985).

In this work, we used the fish ovary cell line (i.e., CCO), derived from the ovaries of juvenile channel catfish. It has been used for small-scale processes for channel catfish virus vaccine production (Bowser and Plumb 1980) and recently, for research in aquatic toxicology (Tan et al.

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2008). The mammalian cell line (i.e., CHO-K1) is derived from the ovaries of the Chinese hamster. It has been used in the biotechnological production of recombinant proteins as well as for toxicity testing (Kmetič et al. 2008; Roan et al. 2008). EE2 and DES were chosen as model estrogens, since their residues have entered the environment through the effluents of sewage treatment works (Ternes et al. 1999; Yang et al. 2008), causing concern about the environmental exposure of different organisms (Thorpe et al. 2003; Schrager and Potter 2004).

The aim of this study was to test and compare the effects of two selected synthetic estrogens, 17α-ethyniylestradiol (EE2) and diethylstilbestrol (DES), on cellular toxicity in ovarian cells from a fish and a mammal.

Materials and Methods

CCO cells, derived from the ovaries of Channel Catfish (*Ictalurus punctatus*) were of American Type Cultures Collections (ATCC: CRL-2772) origin. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% inactivated foetal bovine serum (FBS) (GIBCO, Paisley, Scotland, UK) at 30°C in a humidified atmosphere containing 5% CO_2. CHO-K1 cells, derived from the ovaries of the Chinese hamster were of ATCC (CCL-61) origin. Cells were cultured in DMEM/F12 medium (Sigma, 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 with either of the cell lines.

For experimental purposes, CCO and CHO-K1 cells collected in the exponential growth phase were seeded in 24-well plates at the initial concentration of 5×10^4cells/mL and allowed for 24 h to attach before treatment with EE2 or DES. Stock solutions (1 mg/mL) were prepared by dissolving EE2 or DES (both from Sigma, St. Louis, MO, USA) 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 0.1; 0.5; 1, 5 and 10 μg/mL EE2 (0.34–34 μM) or 0.1; 0.5; 1, 5 and 10 μg/mL DES (0.37–37 μM) for 72 h. The final ethanol concentrations in the medium of treated or control cells were 0.1% for each sample.

Cytotoxicity of EE2 or DES was examined by the measurement of cell viability using the MTT and NR assays. The MTT assay was performed according to the method described by Mosmann (1983). After treatment with EE2 or DES, cells were washed with phosphate-buffered saline (PBS). 100 μL of MTT (Sigma–Aldrich Chemie, Steinheim, Germany) dissolved in PBS buffer (5 mg/mL) was added to each well with 1 mL of fresh medium and cells were incubated for 4 h. At the end of the incubation period, the media was discarded and 1 mL of DMSO was added to each well. The plates were mechanically shaken until purple formazan crystals were dissolved and absorbance was measured at 570 nm using a spectrophotometer (Helios, Thermo Electro Corporation). Uptake of the NR dye (Merck, Darmstadt, Germany) by the lysosomes was measured as described by Borenfreund and Purner (1985). After treatment with EE2 or DES, the medium was removed and cells were washed with PBS. NR solution (50 μg/mL) was added in each well and cells were incubated for 3 h. The cells were de-stained by adding 1 mL of de-staining solution (1% glacial acetic acid; 50% ethanol, 49% distilled water), followed by shaking for 20 min. The absorbance was measured at 540 nm using a spectrophotometer.

Data were expressed as the mean ± standard deviation (SD) 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 untreated controls. The IC_{50} value, defined as the concentration affecting 50% of cells compared to untreated control cells, was calculated from the concentration-effect curves using equations of related polynomial trend lines for the MTT and NR assays.

Light microscopy was used to investigate the morphological changes during the treatment. CCO and CHO-K1 cells (1×10^5 cells/mL) were seeded in the wells and exposed to EE2 or DES (5 μg/mL) for 72 h. The cells were washed with PBS and the adherent cells were stained with crystal-violet (Sigma–Aldrich, Chemie, GmbH, Germany) and NR dye. Morphological images of cells were taken using an inverted microscope (Carl Zeiss, Germany).

Results and Discussion

The effects of different EE2 concentrations to CCO and CHO-K1 cells as determined by the MTT and NR assay are summarized and shown in Fig. 1a–b. The results of the MTT assay demonstrated a dose-dependent significant cytotoxic effect ($p < 0.05$) at 1, 5 and 10 μg/mL EE2 in both cell types (Fig. 1a). The same concentrations of EE2 as determined by the NR assay showed dose-dependent cytotoxicity in the CCO cell line (Fig. 1b), and significant cytotoxic effects ($p < 0.05$) in the CHO-K1 cells, but not in a concentration-dependent manner. Cell viability of CHO-K1 cells after exposure to 10 μg/mL of EE2 was still 70% when compared to control cells. High lysosomal activity was shown, preventing the calculation of an IC_{50} value (Table 1). A notable observation was that EE2 exposure (0.1 and 0.5 μg/mL) in CCO and CHO-K1 cells stimulated cell proliferation as determined by the MTT and

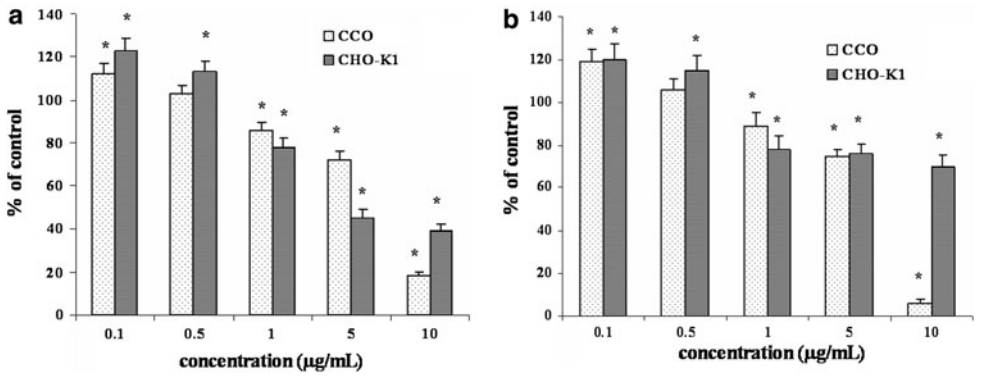

Fig. 1 Effects of different EE2 concentrations (0.1–10 µg/mL or 0.34–34 µM) in CCO and CHO-K1 cultures determined after 72 h exposure using the MTT (**a**) and NR assays (**b**) *denotes significant difference from control (*$p < 0.05$)

NR assays. This effect can be explained by chemical hormesis, a reproducible and relatively common biological phenomenon characterised by low dose stimulation and high-dose inhibition (Calabrese and Baldwin 2003). Kocan et al. (1985) described "stimulation" in fish cells at low concentrations of a toxic substance, with marked toxicity demonstrated at higher critical dose levels. Our results are in agreement with this finding, as increased MTT and NR uptake occurred at low concentrations in both cell lines, while cytotoxic effects were observed at higher concentrations of EE2. Stimulation of cell proliferation, as determined by the NR assay, was also detected in RTG-2 fish cells after exposure to zinc salts (Ní Shúilleabháin et al. 2004) and in PHLC-1 fish cells exposed to some pharmaceuticals as detected by the MTT assay (Caminada et al. 2006).

Table 1 IC_{50} values (µg/mL) for CCO and CHO-K1 cells following exposure to 17α-ethynylestradiol and diethylstilbestrol for 72 h as derived by the MTT and NR assays

Cell line	Assay	EE2 (µg/mL)	DES (µg/mL)
CCO	MTT	7.2	4.5
	NR	6.8	6.5
CHO-K1	MTT	6.2	4.2
	NR	–	6.0

An increase in cell proliferation in both CCO and CHO-K1 cells can be considered as evidence of estrogenicity for tested compounds. Hertz (1985) defined estrogen as a substance that can elicit mitotic stimulation of the tissue of a female genital tract or induce proliferation of estrogen-responsive target cells in vitro.

The effects of different DES concentrations examined in CCO and CHO-K1 cells by the MTT and NR assays are shown in Fig. 2a–b. Obtained results of the MTT assay showed that DES only in concentrations of 5 and 10 µg/mL induced significant cytotoxic effect ($p < 0.05$) in CCO cells (Fig. 2a), while in CHO-K1 cells all tested concentrations of DES induced significant cytotoxic effect ($p < 0.05$). The results of the NR assay showed significant stimulatory effect ($p < 0.05$) in CCO and CHO-K1 cells when 0.1 and 0.5 µg/mL of DES was examined (Fig. 2b). This effect was detected only by the NR assay, indicating high lysosomal activity in both cell lines in the presence of low DES concentrations. Significant cytotoxic effects

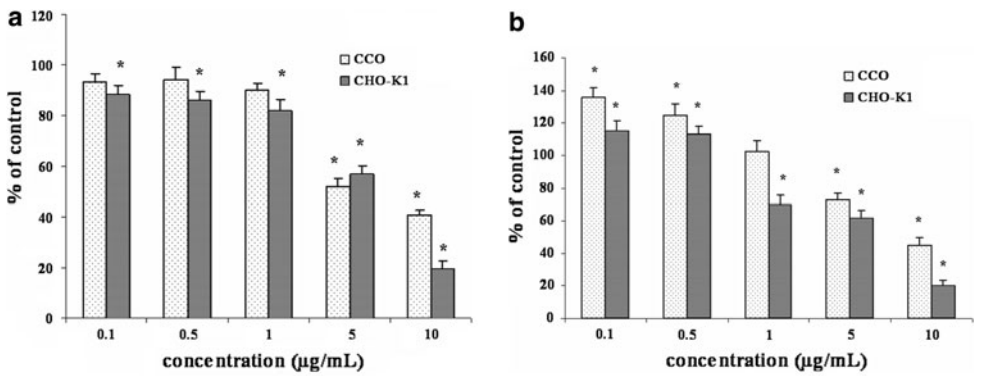

Fig. 2 Effects of different DES concentrations (0.1–10 µg/mL or 0.37–37 µM) in CCO and CHO-K1 cultures determined after 72 h exposure using the MTT (**a**) and NR (**b**) assays *denotes significant difference from control (*$p < 0.05$)

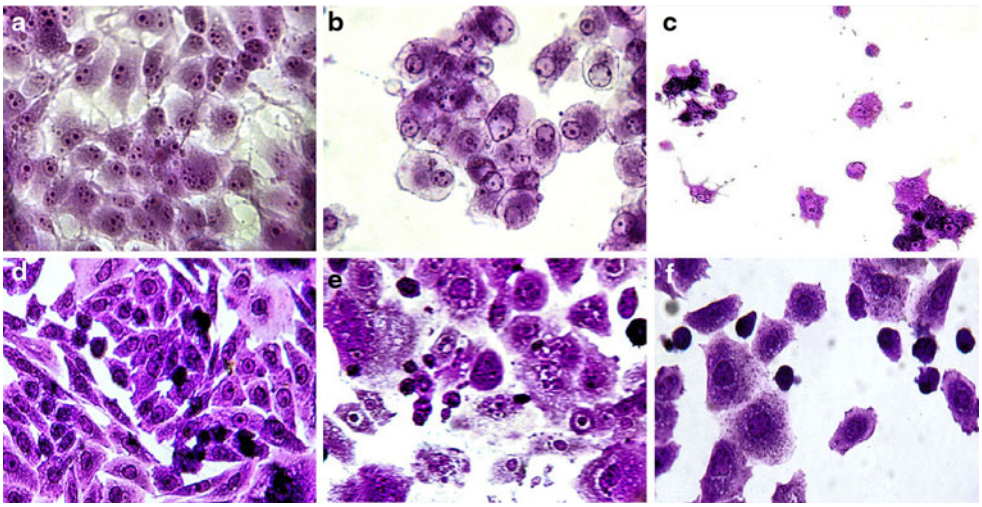

Fig. 3 Morphology of CCO (**a**, **b**, **c**) and CHO-K1 (**d**, **e**, **f**) cells stained with crystal-violet. In comparison to control cells (**a**), 72 h exposure of CCO cells to 5 μg/mL EE2 (**b**) or 5 μg/mL DES (**c**) produced loss of cell monolayer integrity, general damage and rounding effects. In comparison to control cells (**d**) 72 h exposure of CHO-K1 cells to 5 μg/mL EE2 (**e**) or 5 μg/mL DES (**f**) produced decrease in cell number and disruption of cell monolayer. Vacuolization of cytoplasm and some apoptotic cells were also visualized. Magnification ×400

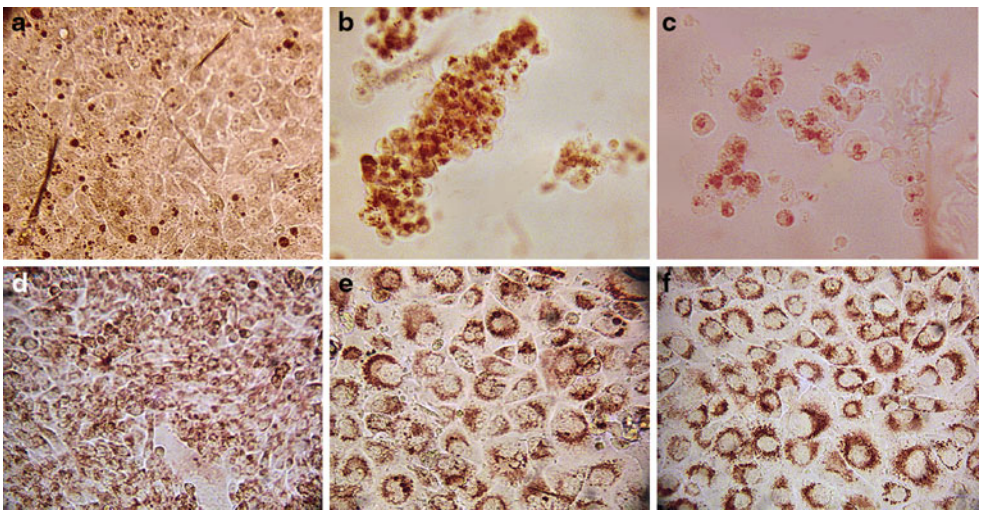

Fig. 4 Morphology of CCO (**a**, **b**, **c**) and CHO-K1 (**d**, **e**, **f**) cells stained with NR. Increased number and size of lysosomes after 72 h of exposure to 5 μg/mL EE2 (**b**, **e**) or 5 μg/mL DES (**c**, **f**) was observed due to stimulated NR uptake. Magnification ×400

($p < 0.05$) were detected in CHO-K1 cells by the NR assay when concentrations of 1, 5 and 10 μg/mL of DES were tested. In vitro studies have shown that DES induces cytotoxic effects in various cells, such as human lymphocytes (Konac et al. 2005). Also, Roan et al. (2008) observed biphasic (i.e., enhancing or inhibiting) effects of DES on cell viability depending on concentration, which is in agreement with our observed results.

Morphological changes induced by EE2 and DES exposure in CCO and CHO-K1 cells were examined by light microscopy after staining with crystal-violet (Fig. 3a–f) and NR (Fig. 4a–f). Crystal-violet staining showed typical fibroblast morphology for control CCO cells (Fig. 3a) while exposure to 5 μg/mL of EE2 (Fig. 3b) and DES (Fig. 3c) for 72 h resulted in lost monolayer integrity with adjacent cells no longer in contact. Rounding and reduction in number of cells was also observed. Control CHO-K1 cells were well attached and showed typical epithelial morphology (Fig. 3d). Exposure to 5 μg/mL of EE2 (Fig. 3e) and DES (Fig. 3f) for 72 h produced severe injuries, like decreased cell number and disruption of the cell monolayer. Vacuolization of cytoplasm was also observed in treated CHO-K1 cells. More intensively stained cells in Fig. 3e and f are probably apoptotic cells since the entire

nucleus is seen as a single ball (pyknosis) due to condensation of chromatin. This should be confirmed by other methods. When CCO and CHO-K1 cells were exposed to EE2 and DES (5 μg/mL) for 72 h and stained with NR (Fig. 4a–f), the stimulation of the uptake of NR is visible, showing an increase in the number and size of lysosomes, especially in the CHO-K1 cell line. This is in agreement with our toxicity findings obtained in the NR assay.

In order to compare cytotoxicity induced by EE2 and DES in CCO and CHO-K1 cells, IC_{50} values for the MTT and NR assay were calculated, and summarized in Table 1. Both assays resulted in similar IC_{50} values when CCO and CHO-K1 cells were exposed to EE2, while slightly higher sensitivity of the MTT assay was found when cells were treated with DES. These differences between cytotoxicity assays indicate intracellular effects on specific organelles and may suggest that DES has effect on the mitochondria. The comparison of IC_{50} values didn't show noticeable differences between CCO and CHO-K1 cells exposed to EE2 and DES, indicating similar sensitivity of fish and mammalian cell lines towards the cytotoxicity of tested synthetic estrogens. Castaño and Gómez-Lechón (2005) from a literature survey estimated a good correlation of IC_{50} values for fish and mammalian cells, allowing them to conclude that both fish and mammalian cells can predict basal toxicity equally well, which is in agreement with our obtained results.

In conclusion, CCO and CHO-K1 cells were found to be suitable in cytotoxicity testing of 17α-ethynylestradiol and diethylstilbestrol using the MTT and NR assays. The comparison of cytotoxic effects, according to IC_{50} values, showed that CCO and CHO-K1 cell lines are almost equally sensitive and both cell lines may be useful in the prediction of the cytotoxicity of synthetic estrogens and other environmental disrupting chemicals. To minimize the use of in vivo acute fish bioassays, we suggest that the CCO cell line may be a good biological system for monitoring environmental disrupting chemicals in the aquatic environment.

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