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Effects of Xenoestrogenic Environmental Pollutants on the Proliferation of a Human Breast Cancer Cell Line (MCF-7)

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Abstract. A human breast cancer cell line (MCF-7) was used to develop an *in vitro* screening assay for the detection of xenoestrogenic environmental pollutants. MCF-7 cells were cultured in DMEM containing 5% fetal bovine serum (FBS). An estrogenic response was defined as an increase in the frequency of proliferating MCF-7 cells, and was measured using a thymidine analog, bromodeoxyuridine, and flow cytometry. Di-2-ethylhexyl phthalate (DEHP) and 4-n-nonylphenol (4-n-NP) were used as model chemicals. The proliferation rate of S-phase cells after 24 h of exposure to various concentrations of 17β-estradiol and to model compounds was compared with a positive and a negative control, containing 1 nM 17β-estradiol and 0.1% ethanol, respectively. DEHP and 4-n-NP increased the frequency of proliferating MCF-7 cells in a dose-dependent manner. The lowest concentration that significantly increased the proliferation of MCF-7 cells was 10 μM for DEHP and 1 μM for 4-n-NP.

The results showed that the assay is accurate and quick to perform. It may prove a valuable tool for screening potential estrogen-mimicking environmental pollutants.

During recent years it has been proposed that certain chemicals or their metabolites *i.e.* 1,1,1-trichloro-2-2-bis(4-chlorophenyl) ethane (DDT), polychlorinated biphenyls (PCBs), alkylphenols and phthalate esters exhibit characteristics similar to 17β-estradiol (E2), the female sex hormone (Ahlborg *et al.* 1995; Soto *et al.* 1995). Estrogens are important in both males and females in various aspects of growth, development, and morphological differentiation, as well as in the development and regulation of sexual and reproductive behavior (Eckert *et al.* 1988). Prenatal exposure to excessive amounts of estrogen can cause reproductive defects in human as well as animal fetuses (Hileman 1994). In 1978, Johnstone *et al.* showed that orally administered E2 induced sex reversal in juvenile male atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhyncus mykiss*).

Nonsteroidal substances with widely different chemical structure exhibit the same characteristics as E2 (Soto *et al.* 1991). It is therefore difficult to predict if a substance will have estrogenic properties by only examining its structure. Domestic and industrial effluents, together with leachates from waste disposal sites, contain a range of chemicals of which many may be estrogenic (Jobling *et al.* 1995). Effluents from sewage treatment works, for example, contain alkylphenols (White *et al.* 1994) and phtalate esters are released into drinking water from plastic piping and packaging material (Thuren 1988).

Alkylphenols were introduced in the 1940s and are used in paints, herbicides, pesticides, and as nonionic surfactants and plastic additives (Soto *et al.* 1991; White *et al.* 1994). For example, Soto *et al.* (1991) showed that nonylphenol (NP) is released from plastic (polystyrene) centrifuge tubes. It has been estimated that 60% of the alkylphenolic compounds produced end up in the aquatic environment, mostly via sewage effluents. The metabolites formed, *e.g.* NP, tend to accumulate in river sediments and sewage sludge. It has been shown that NP is capable of stimulating vitellogenin gene expression in trout hepatocytes, gene transcription in transfected cells, and the growth of breast cancer cell lines (*i.e.* MCF-7) (White *et al.* 1994).

Phthalate esters are a group of compounds that are all diesters of phthalic acid. They are used primarily as plasticizers in polyvinylchloride (PVC) plastics and to a lesser degree in paints, lacquers, pesticides, cosmetics, ammunition, and lubricants. Di-2-ethylhexyl phthalate (DEHP) is a colorless liquid added to plastics to make them flexible. PVC especially can contain as much as 40% DEHP. PVC is used in many common items such as toys, shower curtains, vinyl upholstery, adhesives, coatings, and as components of paper and paperboard. It is also used in the production of disposable medical gloves and in plastic tubing in hemodialysis equipment (US Department of Health and Human Services 1991). Since phthalates are not chemically bound to the plastic material, only dissolved in it, DEHP may be leached from the plastic (Thuren 1988). The reported human daily intake of DEHP varies between 0.3–2 mg/day depending on food intake (US Department of Health and Human Services 1991; Wams 1987). Some risk groups, such as people undergoing blood transfusions, can receive doses of up to 300 mg DEHP (Wams 1987). Also, people receiving hemodialysis on a regular basis (*i.e.* three times a week) receive an average dose of 40 mg/day (Wams 1987).

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The first identifications of estrogenic substances relied on rodent bioassays that use stimulation of uterine growth as an indication of an estrogenic response (Clark *et al.* 1980). This type of bioassay, although accurate, is very time consuming and thus impractical to test large numbers of xenobiotics. Since then, bioassays using breast cancer cells have been developed (Darbre *et al.* 1983; Dembinski and Green 1982; Mellanen *et al.* 1996; Soto *et al.* 1992; White *et al.* 1994) to predict the effects of estrogens and estrogen mimics.

Dolbeare *et al.* (1983) was first to develop a method for the simultaneous flow cytometric measurement of cellular DNA-content and thymidine analog, bromodeoxyuridine (BrdU), incorporation into cellular DNA.

The human breast cancer cell line (MCF-7) is known to respond to estrogen stimuli by increasing DNA-synthesis, *i.e.* the cells in S-phase, *in vitro.* Therefore, this cell line is often used to study cell proliferation by estrogens and estrogen-mimicking substances (Mellanen *et al.* 1996; Soto *et al.* 1992; White *et al.* 1994).

The aim of this study was to develop an easy and effective method for the screening of xenoestrogenic environmental pollutants. The test is based on an *in vitro* bioassay combined with flow cytometric measurements of cell proliferation.

Materials and Methods

Charcoal-Dextran Stripping of FBS

Two slightly different methods were used for the stripping of fetal bovine serum (FBS).

Method 1. 433 mg charcoal (Sigma) and 7.2 mg dextran T-70 (Pharmacia) was added to 50 ml ice-cold NaCl (0.9%). The solution was shaken on ice for 15 min and then centrifuged (1,700 *g* for 10 min, 4°C). The supernatant was replaced by another 50 ml ice-cold 0.9% NaCl and centrifuged again. The supernatant was replaced by 10 ml ice-cold 0.9% NaCl. 2.5 ml of the dextran-charcoal solution was added to 50 ml FBS and shaken for 1 h at 4°C after which the supernatant was centrifuged and sterile filtered.

Method 2. 125 mg charcoal (Sigma) and 1.25 mg Dextran T-70 (Pharmacia) in 50 ml 0.25 M sucrose/1.5 mM $MgCl_2$/10 mM Hepes (pH 7.4) was incubated overnight at 4°C. The solution was centrifuged for 10 min at 500 *g* to pellet the charcoal. The supernatant was replaced with equal amounts of FBS. The tube was throughly shaken and incubated at 4°C for 24 h. It was then centrifuged (10 min 1,700 *g*) and the supernatant was sterile filtered.

Vials used to keep and prepare cd-FBS were made of glass or polypropylene because it has been shown that polystyrene-plastics leach NP, an estrogenic xenobiotic (Soto *et al.* 1991).

Estrogen Analysis

The estrogen contents of FBS and cd-FBS, stripped using two different methods, were analyzed and compared using a Diagnostic Products Cooperation (DPC) kit with minor alterations (Duchens *et al.* 1994).

Cell Line and Cell Culture Conditions

MCF-7 cells, originally from the American Type Culture Collection (ATCC), were donated by S. Kärenlampi, University of Kuopio, Kuopio, Finland.

MCF-7 cells were grown as monolayer cultures in phenol red–free Dulbeccos Modified Eagles Medium (DMEM, Sigma) supplemented with 5% fetal bovine serum (FBS, Gibco), 2 nM L-gluthamine (Ajinomoto), insulin (10 ng/ml, Sigma), penicillin (6 mg/ml, Sigma), and streptomycin (5 mg/ml, Sigma) in a humified incubator at 37°C and 5% CO_2/95% air.

Pilot Studies

In order to evaluate the optimal conditions for the cultivation of MCF-7 cells, a preliminary study was conducted in which a range of parameters were investigated. These included suitable concentration of cd-FBS in the culture medium, effect of insulin withdrawal on cell growth, suitable initial plating density, and differences in growth stimulation attributed to addition of E2 to cells cultivated in medium containing FBS or cd-FBS. An appropriate solvent for estrogen-mimicking chemicals was also chosen.

Experimental Procedure

The cells were subcultured in petri dishes, 5 cm in diameter, at an initial density of 150,000 cells per plate in 5 ml of DMEM with supplements as described above. After 24 h the medium was changed to an experimental one containing 5% dc-FBS + various concentrations of the chemicals tested. After another 24 h, 50 µl of 10^{-3}M BrdU (Sigma) was added to each petri dish. One hour later the cells were resuspended into solution with the use of trypsine enzyme in PBS (without Ca- or Mg- ions) and stored in 2 ml of ethanol (70%) in test tubes overnight.

For experiments aimed at determination of the doubling time of the cell population, the BrdU containing medium was after 1 h replaced by medium without BrdU and cell cultivation continued for various periods of time according to Begg *et al.* (1985). The cells were then resuspended into solution as above.

Staining procedure: The following day, the cells were centrifuged at 400 *g* for 10 min and 1 ml of pepsin/2 M HCl (0.2 mg/ml) was added. The test tubes were left in the dark at room temperature for 1 h. Three milliliters of washing solution (PBS without Ca- and Mg- ions with 0.5% Tween 20, Merck) was added and the cells were resuspended into solution. The cells were centrifuged again and the supernatant was exchanged for 2 ml of washing solution, after which the cells were centrifuged. The supernatant was discarded and 100 µl of the primary antibody M744 (1:12.5 in washing solution, Dako) was added. The cells were left in the dark for 30 min at room temperature, after which 2 ml of washing solution was added and the test tubes centrifuged. The supernatant was discarded and 100 µl of the secondary antibody F313 (1:50 in washing solution, Dako) was added. The cells were left in the dark for 30 min at room temperature, after which 2 ml of washing-solution was added and the test tubes centrifuged. The supernatant was discarded and 200 µl propidium iodide solution (10 µg/ml in PBS) added.

The flow cytometric analysis was conducted with a FACStar^{PLUS} flow cytometer (Becton Dickinson, Sunnyvale, CA) equipped with an argon laser (Spectra-Physics 168B, Spectra-Physics, Mountain View, CA) tuned to 488 nm and operating at 200 mW. FITC (BrdU-antibody)-fluorescence pulse height (FL1-H) was collected through a 530/30-nm bandpass filter and PI (DNA-content)-fluorescence pulse height, -width, and -area (FL3-H, FL3-W, and FL3-A, respectively) through a 660/20-nm bandpass filter.

PI-fluorescence was collected using linear amplification, while the FITC-fluorescence was collected using logarithmic amplification. A four-parameter (FL1-H, FL3-H, FL3-W, and FL3-A) listmode file was collected from each sample comprising up to 30,000 events. Data were collected on a FACStation running CELLQUEST software version 1.2.2. An analysis gate consisting of FL3-A and FL3-W was set to

exclude debris and aggregates. The singlets were divided into proliferating and nonproliferating cells (Figure 1).

For determination of cell population doubling time, T_{pot} was calculated according to Begg *et al.* (1985).

Exposure of Potentially Xenoestrogenic Substances to MCF-7 Cells

MCF-7 cells were exposed to various concentrations of 4-n-nonylphenol (purity 99.9%, Larodan AB, Malmö, Sweden), di-2-ethylhexyl phthalate (purity 99.6%, Neste-Oxo, Stenungsund, Sweden), or dimethylsulfoxide for 24 h, after which they were prepared for flow cytometric analysis. The cell proliferation rate was measured and related to a positive control, *i.e.* cells exposed to 1 nM E2, and to a negative control, containing 0.1% ethanol. E2, DEHP, and 4-n-NP were dissolved in 99.5% ethanol to a thousand times the final concentration (0.1% in medium). The concentrations measured ranged between: 4-n-NP: 0.01–100 μM; DEHP: 0.1–500 μM; DMSO: 0.1–1.0% μM. Data were calculated according to Equation 1 below

$$[(S - E)/(E - R)] + 1.00 \times 100\% \quad (1)$$

where S = proliferation of sample, R = proliferation of negative control, E = proliferation of positive control.

Statistical Analysis

Statistical analysis of the data obtained by flow cytometric analysis was performed using the Mann-Whitney *U* test. This was done in order to test for the presence of significant differences in the frequency of proliferating cells exposed to different concentrations of potential estrogen mimics compared with a negative control.

Results and Discussion

Exposure to 10 and 100 μM di-2-ethylhexyl phtalate (DEHP) increased the proliferation rate of the cells to 45%, and at 500 μM to 65%, of that of the positive control. At 0.1 and 1 μM DEHP any observed increase in cell proliferation was not significant (Figure 2). At 1 and 10 μM, 4-n-nonylphenol increased the proliferation rate of the cells to 21 and 45%, respectively, of that of 17β-estradiol (Figure 3). At higher concentrations (50 μM), NP had an inhibitory effect on cell proliferation and at even higher levels (100 μM) it was lethal to the cells (data not shown). At 0.01 and 0.1 μM NP any observed changes in cell proliferation were not significant. DMSO increased cell proliferation rates to 72, 69, 65, and 49%, at 0.1, 0.2, 0.4, and 0.6%, respectively, of that of the positive control (Figure 4). At higher concentrations (0.8 and 1.0%) DMSO decreased cell proliferation (data not shown).

The medium used in the present study contained 5% fetal bovine serum. Serum contains various hormones and growth factors that affect cell proliferation. The E2 content of the FBS used in this study was found to exceed 400 pM. When the FBS was stripped using a charcoal-dextran solution (method 2) to remove steroids, less than 1 pM E2 remained in the serum. When the FBS was stripped using method 1, 15 pM E2 remained in the serum. Henceforth, only method 2 was used.

Pilot studies indicated that a suitable concentration of cd-FBS in the culture medium was 5%. At lower concentrations and without the addition of insulin, cell growth was decreased. The initial plating density of the cells was important; the proliferation rate of the cells depended on their density when seeded, however, there also had to be enough cells to be able to use the flow cytometer. Cells cultured in medium containing 5% FBS did not respond to further addition of 1 nM E2; these cells had already reached their peak proliferative rate. Cells cultivated in medium containing 5% cd-FBS responded to an addition of E2. At 1 nM E2, the cells had reached a maximum proliferation rate and further additions of E2 had no enhancing effect. We chose 1 nM E2 as a positive control. At 0.1, 10, and 50 nM E2 the frequency of proliferating cells was between 80–90% of that of 1 nM E2, whereas 0.01 nM E2 only had a proliferative effect of 6% of that of the positive control. Studies using MCF-7 cells for the detection of xenoestrogenic chemicals have shown that E2 stimulates MCF-7 cell growth at a concentration of 0.1–1 nM (White *et al.* 1994), or at 0.03 nM E2 (Soto *et al.* 1991). In both these studies the amount of cells or nuclei were counted after 5 or 6 days, respectively, of exposure. In the present study, the lowest value to induce an increase in the frequency of proliferating S-phase cells was 0.01 nM E2.

In a study by Soto *et al.* (1995) NP was shown to be estrogenic at a concentration of 1 μM, and a full agonist to E2. In hepatic rainbow trout cells NP stimulated vitellogenin synthesis and secretion at concentrations of 1 μM and above; it was also shown that NP displaced E2 from its receptor (White *et al.* 1994). Also, Danzo (1997) showed that NP reduced the binding of 17β-estradiol to the estrogen receptor by 75%. In the present study, the lowest concentration to induce a significant increase in the frequency of proliferating MCF-7 cells was 1 μM 4-n-NP.

Soto *et al.* (1994) states that a final solvent concentration in culture medium of 0.1% dimethylsulfoxide or ethanol did not affect cell yields. We have found the DMSO used in the present study to be an unsuitable solvent due to its stimulating effect on MCF-7 cell proliferation at low concentrations. We used DMSO as a solvent in several experiments with 4-n-NP but the results obtained were highly irregular and the use of DMSO was discontinued. Ethanol had a slight inhibitory effect on MCF-7 cell proliferation, however at a solvent concentration of 0.1% its effects were negligible.

The proliferation rate of the negative control used at each set of experiments (0.1% ethanol) varied considerably (ranging from 10–35%) between experiments, even though the procedure was carried out identically each time. Due to this, the data collected at each experiment was related to a negative and a positive control (1 nM E2) from the same experiment. The MCF-7 ATCC cell line has been shown by Villalobos et al. (1995) to be sensitive to both E2 and NP. They also showed that the percentage of S-phase cells was 20 and 32% depending on treatment (cells cultured in medium containing 10% charcoal-dextran–treated fetal bovine serum and cells cultured in medium containing 10% fetal bovine serum, respectively). These figures are consistent with our results, although as stated before, the proliferation rates vary somewhat between experiments. This variation might be due to the ''age'' of the cells, *i.e.* the number of passages, or to the density of cells before subculturing them to a new experiment. Another possibility is the fact that we collected the cells after only 24 h in the experimental medium and they might still have been in an initial lag phase or in some cases just passed over to an exponential phase.

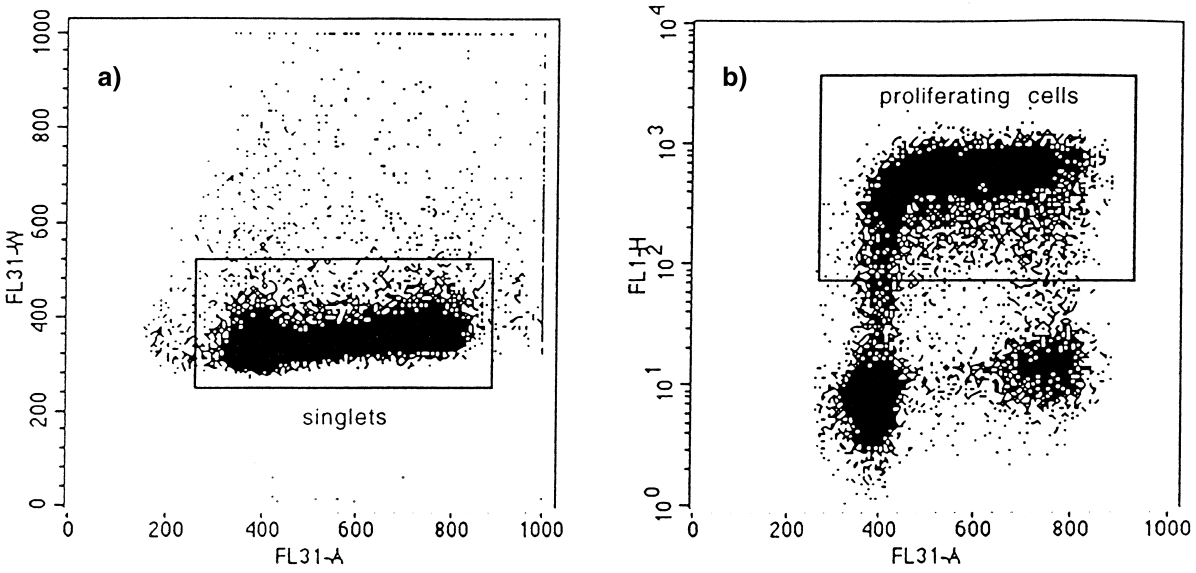

Fig. 1. Flow cytometric analysis of MCF-7 cell proliferation. (a) shows the area/width distribution used to exclude debris and aggregates with the analysis gate for singlets indicated. In (b), the analysis region used for evaluation of frequencies of proliferating bromodeoxyuridine-incorporating cells is indicated

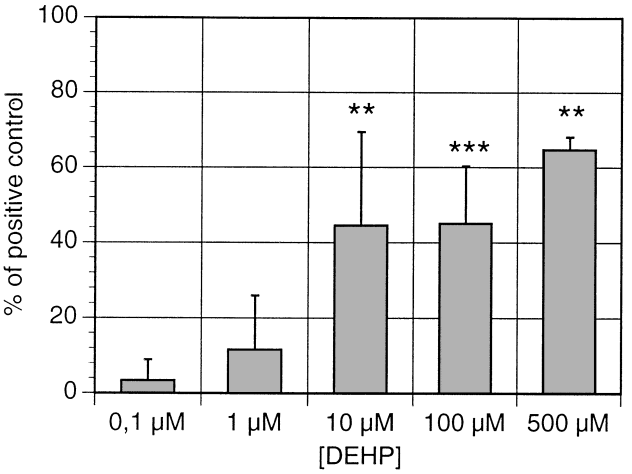

Fig. 2. Effect of di-2-ethylhexyl phthalate on the proliferation of MCF-7 cells relative to a positive control, given as percentage of positive control proliferation (positive control = 1 nM 17β-estradiol). Data were calculated according to Equation 1. MCF-7 cells were cultured for 24 h at an initial density of $1.5 \cdot 10^5$ cells in 5 ml medium supplemented with 5% charcoal-dextran stripped fetal bovine serum and various concentrations of di-2-ethylhexyl phthalate. The proliferation of the negative control (vehicle) has been set to 0%, and that of the positive control to 100%. **P $<$ 0.01 and ***P $<$ 0.001 denote a significant increase in the frequency of proliferating S-phase cells compared with the negative control. Values are given as mean ± 1 SD. Each value is the mean of 4–7 individual measurements

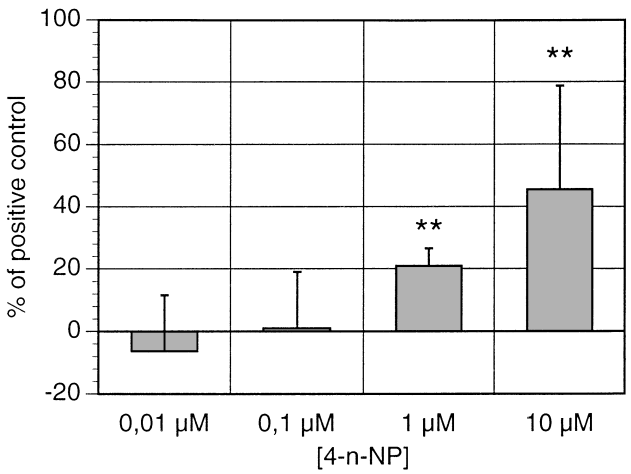

Fig. 3. Effect of 4-n-nonylphenol on the proliferation of MCF-7 cells relative to a positive control, given as percentage of positive control proliferation (positive control = 1 nM 17β-estradiol). Data were calculated according to Equation 1. MCF-7 cells were cultured for 24 h at an initial density of $1.5 \cdot 10^5$ cells in 5 ml medium supplemented with 5% charcoal-dextran stripped fetal bovine serum and various concentrations of 4-n-nonylphenol. The proliferation of the negative control (vehicle) has been set to 0%, and that of the positive control to 100%. **P $<$ 0.01 denotes a significant increase in the frequency of proliferating S-phase cells compared with the negative control. Values are given as mean ± 1 SD. Each value is the mean of 4–6 individual measurements

Villalobos *et al.* (1995) has compared the growth pattern of different MCF-7 cell stocks. It seems that cell numbers increased exponentially starting 2 days after plating. The MCF-7 ATCC cell stock differed in that the increase in cell numbers was not as prominent. We found the doubling time of the cell population to be 42 ± 3.9 h for cells cultured in DMEM containing 5% charcoal-dextran–treated FBS, and 32 ± 2.8 h for cells cultured in DMEM containing 5% FBS. According to the results published by Villalobos *et al.* (1995), MCF-7 ATCC cells had a doubling time of 49 ± 2.7 h and 59 ± 3.1 h, when cultured in phenol red–free DME with 10% fetal bovine serum and phenol red–free DME with 10% charcoal-dextran–treated fetal bovine serum, respectively.

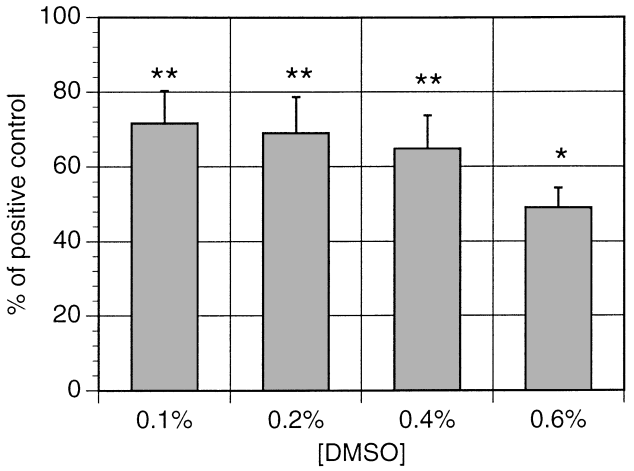

Fig. 4. Effect of dimethylsulfoxide on the proliferation of MCF-7 cells relative to a positive control, given as percentage of positive control proliferation (positive control = 1 nM 17β-estradiol). Data were calculated according to equation 1. MCF-7 cells were cultured for 24 h at an initial density of $1.5 \cdot 10^5$ cells in 5 ml medium supplemented with 5% charcoal-dextran stripped fetal bovine serum and various concentrations of dimethylsulfoxide. The proliferation of the negative control (vehicle) has been set to 0%, and that of the positive control to 100%. *P $<$ 0.05 and **P $<$ 0.01 denote a significant increase in the frequency of proliferating S-phase cells compared with the negative control. Values are given as mean $\pm$ 1 SD. Each value is the mean of 5–6 individual measurements

In short, 17β-estradiol induced an increase in the frequency of proliferating MCF-7 cells. Di-2-ethylhexyl phtalate significantly increased the proliferation of MCF-7 cells at concentrations greater than or equal to 10 μM in a similar manner to 17β-estradiol. 4-n-nonylphenol significantly increased the proliferation of MCF-7 cells at a concentration of 1–10 μM in a similar manner to 17β-estradiol. Two different solvents were evaluated; dimethylsulfoxide at low concentrations (0.1–0.6%) increased and at high concentrations (0.8–1.0%) decreased the frequency of proliferating S-phase cells, and was therefore not used in subsequent experiments.

The assay proved to be accurate, quick, and easy to perform. Small variations in the proliferation rate of MCF-7 cells could be detected using flow cytometric analysis, and the data was very reliable as up to 30,000 cells were collected for each measurement. The exposure time for xenoestrogenic substances was short, 24 h, and the data were quickly evaluated using flow cytometric analysis.

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