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Cytotoxic effects of di (2-ethylhexyl) phthalate on cultured mammalian cells

A. Peropadre[1], P. Fernández Freire[1], O. Herrero[2], J. M. Pérez Martín[1] and M. J. Hazen[1,*]

[1]Grupo de Toxicología celular, Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain, [2]Grupo de Biología y Toxicología Ambiental, Facultad de Ciencias, Universidad Nacional de Educación a Distancia, 28040 Madrid, Spain

ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is a high production volume chemical included on the first candidate list of "substances of very high concern" brought out by the European Chemicals Agency. Despite the available information, the basal cytotoxicity of this compound in mammalian cells is poorly understood. In this work, Vero cells derived from the kidney of green monkey were used to determine the cytotoxic effects of DEHP, using four biochemical endpoints: neutral red uptake, tetrazolium (MTT) reduction, LDH activity and cell protein content. Membrane-based assays were the most sensitive for detecting cytotoxicity in our experimental system and revealed that DEHP targets lysosomal membranes. An interesting finding of this study relate to moderate permeabilization of lysosomes during DEHP-mediated cytotoxicity in Vero cells, which did not trigger cell death and was completely reversible after drug withdrawal. Our results not only provide useful information on the primary subcellular targets and mechanisms underlying the toxicity of DEHP in mammalian cells, but also support the importance of considering multi-parametric methods to define the toxicological profile of chemical compounds.

*Corresponding author: M. J. Hazen,
Departamento de Biología (Lab. A-110),
Facultad de Ciencias, Universidad Autónoma de Madrid,
c/ Darwin 2, 28049 Madrid, Spain.
mariajose.hazen@uam.es

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INTRODUCTION

Phthalates are a group of chemicals that have become ubiquitous industrial pollutants in the environment during the past several decades [1, 2]. Of particular concern is di (2-ethylhexyl) phthalate (DEHP), the most widely used plasticizer for polyvinyl chloride (PVC) products, which have a broad range of applications such as in building materials, food packaging and medical devices [3]. DEHP readily leaches from plastic surfaces, since it is not chemically bound to the PVC polymer, resulting in widespread nonoccupational exposure through multiple routes [4-6].

Although human epidemiological data are limited and inconclusive to date, a causal relationship has been suggested between DEHP exposure and reproductive or birth defects [7-9], pulmonary troubles [10], obesity-related outcomes [11] and thyroid dysfunction [12]. On the other hand, large studies involving hundreds of laboratory animals have definitively revealed that DEHP induces a range of harmful effects on the liver, lungs and kidneys [13-15], and causes toxicity in developing embryos [16] and reproductive system [17, 18]. Consequently, DEHP is listed as a category 2 reproductive toxicant under EU Directive 2001/59/EC [19] on classification and labeling of dangerous substances. In long-term experiments,

the compound increases the incidence of hepatocellular tumors in rats and mice, due to peroxisome proliferation [20, 21]. Although there is little epidemiological evidence, DEHP has been reclassified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (Group 2B) [22].

It is well recognized, even if extrapolations are often questionable, that research using whole animals has been essential in determining adverse human health effects. Nevertheless, toxicology testing has broadened its scope considerably and reliable *in vitro* methods are nowadays available, that may be incorporated into the risk assessment process [23-25]. In this respect, singular attention has been paid to cell-based assays since, according to the basal cytotoxicity concept proposed by Ekwall 1995 [26], most chemicals cause toxicity by interference with fundamental cellular pathways.

Cell cultures of mammalian origin likely represent the most suitable models for extrapolating data obtained in experimental conditions for human risk assessment [27] because they provide more accurate data than those obtained from acute toxicity studies in rodents [28]. Nonetheless, there are few reports in the literature concerning the toxicity of DEHP at the cellular level. Recent studies, conducted in diverse mammalian cell lines, showed that the compound exhibits anti-apoptotic properties [29], interferes with fatty acid homeostasis [30], decreases cell growth [31], alters gene expression profiles [32] and impairs insulin binding and glucose oxidation [33].

The present work was designed to analyze the cytotoxic potential of DEHP in Vero cells, a monkey kidney-derived cell line that has proved to be a useful tool for studying mechanisms underlying toxic responses induced by chemical agents currently found in the environment [34-37]. In order to distinguish between general cytotoxicity and effects on specific organelles as well as to increase the reliability of the results obtained, four basal cytotoxicity assays were performed at different incubation times. Our findings, although simplified with respect to the *in vivo* situations, characterize a novel cytotoxic activity of DEHP in mammalian cells and provide additional data that may contribute to a mechanistic justification for continued evaluation of this important toxicant.

MATERIALS AND METHODS

Chemicals

Di (2-ethylhexyl) phthalate (CAS No 117-81-7), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl-tetrazolium bromide), bovine serum albumin, β-Nicotinamide adenine dinucleotide (NADH) and sodium pyruvate were purchased from Sigma Chemical Co. (USA). Neutral red was obtained from Merck (Germany), Coomassie® Brilliant Blue G-250 reagent from Bio-Rad (USA) and all other chemicals were of analytical grade. Reagents for cell cultures were products of Lonza (Switzerland).

Cell culture and DEHP treatments

Vero cells (monkey kidney) were grown at 37 °C in 25 cm^2 flasks (Falcon, Becton Dickinson, USA) under a 5% CO_2 humidified atmosphere, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Exponentially growing cells were seeded at a density of 10^5 cells/ml into 24-well plates for quantitative evaluation or into 12-well plates, containing a sterile glass coverslip in each well, for morphological studies. After an overnight incubation, cells were treated for 24-48 h with a new medium containing serial dilutions of DEHP and subsequently washed with PBS and processed according to the different experimental procedures.

Stock solutions of 10^{-2} M and 10^{-3} M di (2-ethylhexyl) phthalate were prepared in ethanol and maintained in darkness at room temperature. The working solutions, ranging from 1-100 μM (0.39-39.05 μg/ml), were prepared before use in DMEM supplemented with 1% FCS and sterilized by filtration through a 0.22 μm Millipore® filter. Ethanol concentrations in medium did not exceed 1% including for the control groups.

Cytotoxicity evaluation

Four spectrophotometric methods were applied to determine the cytotoxic activity of DEHP. The absorbance values at appropriate wavelengths were

recorded using a Spectrafluor microplate reader (Tecan, Austria).

Cell proliferation and/or detachment was estimated by quantifying total protein content (TPC) according to the method of Bradford [38], using Coomassie® Brilliant Blue G-250 reagent and bovine serum albumin as standard. MTT assay, that involves reduction of the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl-tetrazolium bromide) by viable cells to purple formazan, was performed according to the method of Mosmann [39]. Briefly, after DEHP treatments, cells were incubated for 2 h with MTT in DMEM at a final concentration of 0.5 mg/ml. The medium was then replaced with dimethyl sulfoxide (DMSO) for formazan solubilization.

Neutral red uptake (NRU) into the lysosomes of viable cells was evaluated as described by Borenfreund and Puerner [40]. In brief, after DEHP exposure, culture medium was replaced with new medium containing neutral red (50 μg/ml). Following an incubation period of 3 h, the medium was removed and intracellular neutral red was extracted by addition of 50% aqueous ethanol containing 1% acetic acid. The activity of intracellular lactate dehydrogenase (LDH; EC1.1.1.27), as a measure of cell membrane integrity, was determined by the UV method [41], following the oxidation of NADH in the presence of sodium pyruvate as substrate.

Microscopy

Differential interference contrast (DIC) microscopy was used to observe the distribution and integrity of lysosomes loaded with neutral red. Microscopic observations were carried out using an Olympus BX-61 epifluorescence microscope (Tokyo, Japan), equipped with an HBO 100W mercury lamp. The images were acquired with a CCD camera Olympus DP-70 and processed using the software Olympus DP controller 1.1.1.65 and Adobe Photoshop 9.0 (Adobe Systems Inc.). All comparative images (treated vs. untreated samples) were obtained under identical microscope and camera settings.

Data analysis

Statistical analysis was carried out using the GraphPad Prism 5.0 software. The results were analyzed by Student's t test for comparing paired samples and analysis of variance (ANOVA) with Bonferroni as *post hoc* test for multiple samples. Each data point represents the arithmetic mean ± standard deviation of at least three independent experiments. EC50 values (50% effective concentration) were obtained using non-linear regression.

RESULTS

Cytotoxic activity of DEHP

The dose-response curves for the different cytotoxicity assays evaluated in Vero cells are depicted in Figure 1. Among the biomarkers analyzed, the neutral red uptake and MTT metabolization were the most sensitive at 24 h, showing statistically significant differences between exposed and unexposed cells from concentrations ≥ 10 μM. When the treatment was extended up to 48 h, the dose-response curves followed a similar profile but revealed a moderate time-dependent increase in DEHP toxicity. All the assays, with the exception of LDH leakage test, showed differences between treated and control cells at concentrations ≥ 5 μM. Median effective concentrations (EC50) were obtained only with the membrane-based methods; NRU assay was particularly sensitive with EC50 values about 6-fold lower than those estimated when LDH leakage test was employed (Table 1).

Some conclusions started to emerge when comparing results from the different measured endpoints. First, Bradford and MTT methods were found to give equivalent results, since statistical analysis revealed no significant differences between both assays in any experimental condition (Student´t test, $p \leq 0.01$). Second, although the plasma membrane integrity was compromised in the presence of DEHP, according to LDH assay, enzyme leakage was revealed at doses higher than those needed for significant reduction of neutral red uptake (ANOVA, Bonferroni $p \leq 0.01$). Consequently, the dye incorporation into DEHP-treated cells was followed spectrophotometrically to examine in depth the lysosomal membrane integrity. Neutral red accumulation increased gradually over time, reaching a maximum at 3 hours in control cultures and cells treated with

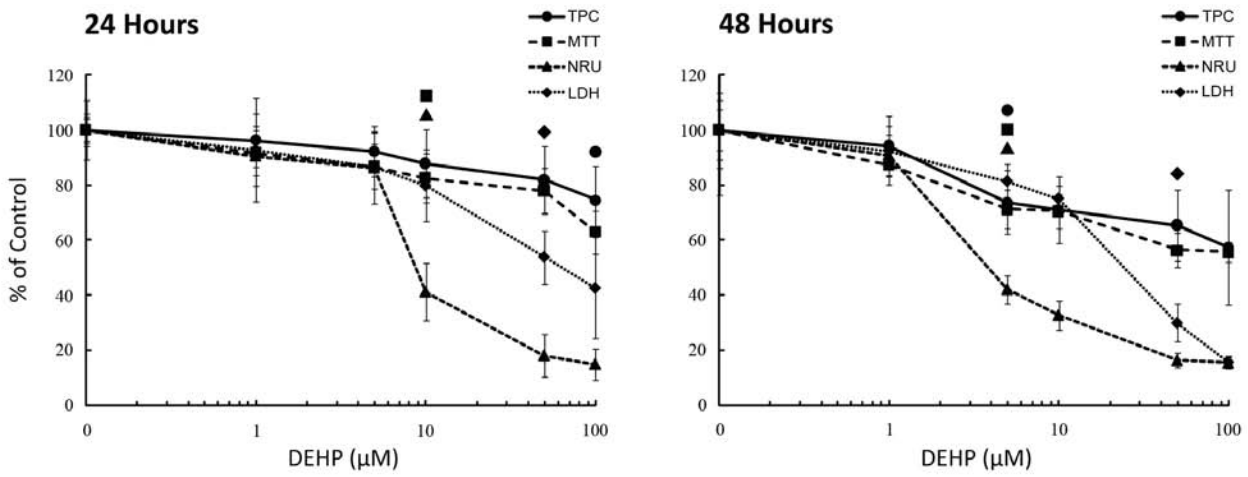

Figure 1. Dose-response curves obtained in the four cytotoxicity assays after treatment of Vero cells with increasing DEHP concentrations for 24 and 48 h. Data are expressed as percentage of that found in respective control cultures. Statistically different values in treated cells compared to control cells were obtained from concentrations marked with their respective symbol ($p \leq 0.01$).

Table 1. EC50 values of DEHP at different incubation times determined by the four cytotoxicity assays.

	EC50 (μM)			
	TPC	**MTT**	**NRU**	**LDH**
24 h	> 100.00	> 100.00	9.57	64.16
48 h	> 100.00	> 100.00	4.93	23.16

concentrations ≤ 5 μM. However in cells exposed to higher DEHP concentrations, neutral red uptake tended to decline as incubation progressed (Figure 2). Therefore, the dose-and time-dependent diminution of neutral red into DEHP-treated cells suggests dye efflux due to lysosomal membrane destabilization.

In order to investigate the possible reversibility of lysosomal damage, Vero cells treated for 24 h with the compound were allowed to recover for another 24 h in fresh culture medium and neutral red uptake was reassessed. To make sure that our results were accurate and not due to differences in cell number, dye absorbance values in each well were normalized to total protein content. Data are expressed as percentage of recovery in Figure 3a, according to the following formula: [(NR absorbance/μg protein)$_{24+24h}$/(NR absorbance/μg protein)$_{24h}$] x 100. There was no evidence of lysosomal damage upon removal of DEHP, but a dose-related increase in the uptake of neutral red that even exceeded the values in the corresponding controls. This result involves the complete reversion of the DEHP effects on lysosomal membranes, and was confirmed when Vero cells loaded with neutral red were visualized by DIC microscopy (Figure 3b).

DISCUSSION

The new EU regulation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) that came into force on June 2007 (EC 1907/2006) [42], includes specific requirements to promote alternative methods based on the 3R principle (Replacement, Reduction and Refinement of laboratory animal use). These requirements have been recently reinforced by the new European law regulating the Legislation for the protection of animals used for scientific purposes (EC 2010/63/UE) [43]. Thus, application of new approaches to improve testing strategies is currently a major challenge for the scientific community, industry and regulatory agencies.

In the present study, the Vero cell line was used as an *in vitro* model to evaluate the cytotoxic effects of DEHP, a high production volume chemical included on the first candidate list of "substances of very high concern" brought out by the ECHA (European Chemicals Agency) under the REACH legislation [44]. Cultured cells are a valuable tool

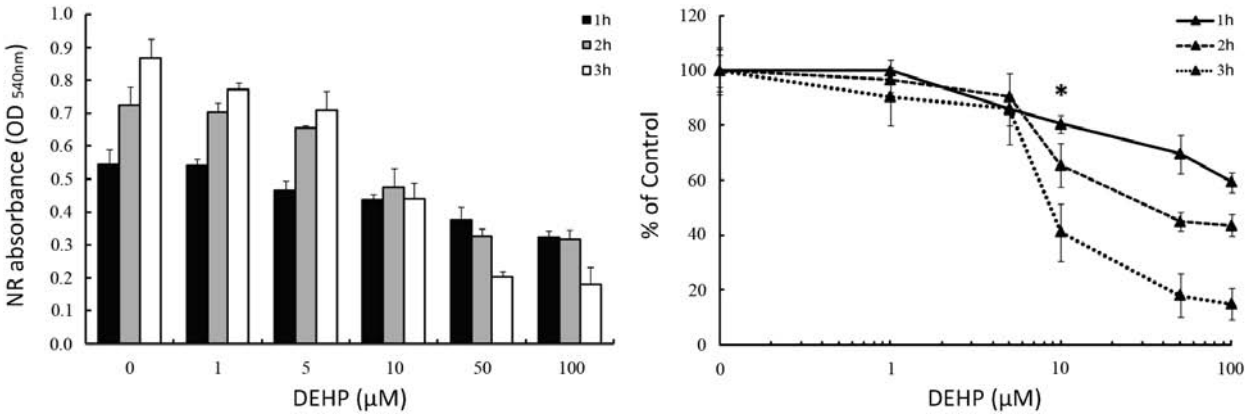

Figure 2. Kinetics of neutral red uptake in Vero cells exposed to DEHP for 24 h. The left panel depicts the time course of absorbance changes associated with dye uptake for DEHP-treated cells. Data in the right panel are expressed as percentage of the respective control cultures. Statistically different values in treated cells compared to control cells were obtained from concentration 10 μM, marked with an asterisk ($p \leq 0.05$).

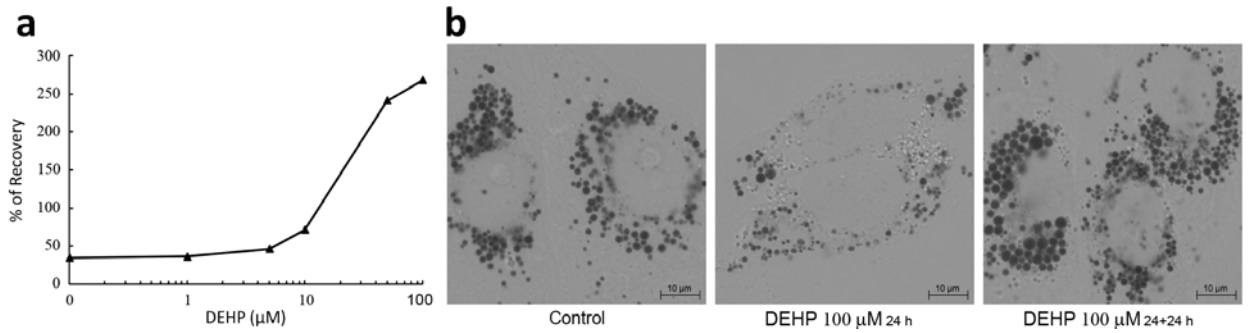

Figure 3. Recovery of lysosomal stability assessed with the neutral red assay in pretreated Vero cells, after 24 h of DEHP withdrawal. (a) Dye absorbance values normalized to total protein content and expressed as percentage of that found in respective control cultures. (b) Representative images of neutral-red-loaded cells after different treatment conditions.

for mechanistic analysis of toxic compounds and may constitute an appropriate alternative for early toxicity assessment [27]. However, accuracy and reliability of the data generated in cell cultures depend to a great extent on the experimental design and procedures chosen for the study [45]. Our experimental protocol was based on the overall scheme of *in vitro* basal cytotoxicity testing, proposed by the Interagency Coordinating Committee on the Validation of Alternative Toxicological Methods [46]. Accordingly, DEHP-induced toxicity in Vero cells was estimated using four well-accepted endpoints for cell proliferation and viability, complemented by microscopic observations.

The quantitative determination of the total protein content, a toxicity indicator of cell proliferation and/or detachment, showed no significant changes in cell density 24 h after treatment with DEHP at concentrations < 100 μM. However, Vero cells were already affected by the compound since all the other assays revealed a dose-dependent reduction in the fraction of viable cells. A prolonged incubation for 48 h led to a moderate increase in the cytotoxic activity of DEHP and allowed in most cases the detection of adverse effects at lower concentrations.

Different results were reached depending on the endpoint used to analyze cell viability that are explained by the characteristics of each assay. MTT metabolization, mainly attributed to mitochondrial enzymes, was significantly reduced by DEHP exposure at concentrations ≥ 10 μM and

≥ 5 μM after 24 and 48 h, respectively. This effect was to some extent expected because mitochondrial dysfunction caused by DEHP has previously been reported both *in vitro* [47, 48] and *in vivo* [49]. Nonetheless, the performance of the MTT assay was compared with that of the Bradford method because a decline in MTT reduction rates might reflect cytotoxicity or diminished cell proliferation [39]. Despite equivalence between both assays over the entire concentration range studied, MTT test appeared to be more sensitive at short exposures suggesting an early mitochondrial dysfunction in DEHP-treated cells. Since mitochondria modulate specific signaling pathways that affect cell cycle progression [50], further investigation will be necessary to analyze whether decreased metabolic activity of Vero cells would be responsible for the delay or slight growth inhibition observed after DEHP exposure.

The assays measuring changes in membrane integrity were the most accurate in revealing DEHP cytotoxicity in our experimental system. Under identical treatment conditions, lysosomal membrane permeabilization detected in terms of neutral red release, was more prominent than plasma membrane damage quantified by leakage of cytosolic LDH. Therefore, we considered that lysosomal compartment is a key subcellular target of the compound, in agreement with previous studies that reported lysosomal stress after DEHP exposure in rabbit alveolar macrophages [51], rodent hepatocytes [52, 53] and digestive cells of mussels *Mytilus galloprovincialis* [54].

Loss of lysosomal integrity and the consequent release of hydrolases into the cytosol is a rather common event in chemical-induced cell injury that represents an important step in ultimate apoptotic or necrotic cell death [55, 56]. On the other hand, restricted lysosomal leakage causes degenerative alterations which can be efficiently repaired by autophagy [55, 57]. Under our experimental conditions, the decline in lysosomal function involved no loss of cell integrity and was completely reversible after DEHP withdrawal. Moreover, both the biochemical and microscopic results showed that treated Vero cells following a recovery period of 24 h, accumulated neutral red to a greater extent than the respective control cells. It should be noted that increased uptake of vital dyes by lysosomes is consistent with volume expansion of the acidic compartments during the course of adaptation to cellular stress [58, 59]. We can therefore assume that Vero cells were able to recover from a partial destabilization of lysosomal membranes triggering an adaptive physiological response in order to limit cellular injury caused by DEHP. This can be considered as one important finding of our study since, although *in vitro* results are very difficult to compare with those obtained in animal studies, most of the adverse effects of DEHP observed *in vivo* are reversible or do not progress after cessation of treatments [3].

CONCLUSION

Taken together, our results contribute to elucidate the primary subcellular targets and mechanistic aspects of di (2-ethylhexyl) phthalate toxicity in cultured mammalian cells. The present experiments indicated that DEHP treatments essentially involve initial lysosomal destabilization and late plasma membrane damage, resulting in sublethal cytotoxic effects. Further studies are underway to extend these initial findings, as well as to verify if the toxic activity of DEHP observed in this work was cell-type-specific or consistently found in cell lines of human origin.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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