Di(2-ethylhexyl) phthalate inhibits glutathione regeneration and dehydrogenases of the pentose phosphate pathway on human colon carcinoma cells

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Abstract

Phthalates, particularly di(2-ethylhexyl) phthalate (DEHP), are compounds widely used as plasticizers and have become serious global contaminants. Because of the bioaccumulation of such substances, the food chain is at risk. The food contamination by some phthalates has been linked to different side effects in experimental animals. That is why we have chosen the intestinal system's cells which represent the primary targets of these compounds to test their toxic effects. Human colon carcinoma cells (HCT 116) were chosen to elucidate whether DEHP triggers oxidative stress and apoptosis. Our results indicated that DEHP is cytotoxic; it induces the overexpression of Hsp70 protein and causes oxidative damage through the generation of free radicals leading to lipid peroxidation induction and the increase of superoxide dismutase (SOD) and catalase (CAT) activities. In addition, cell treatment with DEHP resulted in a glutathione (GSH) content decrease and a decrease in the glutathione reductase (GR) activity. As new evidence provided in this study, we demonstrated that the DEHP affected the two enzymes' activities of the oxidative phase of the pentose phosphate pathway: Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). This leads to a decrease in the level of NADPH used by the GR to maintain the regeneration of the reduced GSH. We also demonstrated that such effects can be responsible for DEHP-induced apoptosis.

Keywords Di(2-ethylhexyl) phthalate · Oxidative stress · Glutathione metabolism · Pentose phosphate pathway · Apoptosis

Introduction

Phthalates belong to the products called “emerging” contaminants (ECs). They are a group of chemicals used to soften and increase the flexibility of plastic (Melo-Guimaraes et al. 2013). Di(2-ethylhexyl) phthalate (DEHP) is the most common plasticizer, generally used in the industry for the poly-

meric product production such as polyvinyl chloride (PVC) (Kroschwitz and Howe-Grant 1999; Nalli et al. 2006; Yuwatini et al. 2013) and non-polymeric products, such as cosmetics, perfumes, medical devices, and food packaging (Huang et al. 2008). The exposure of the general population to this phthalate is via dermal and especially via oral routes. In fact, DEHP was detected in drinking water regularly ingested daily (Zhu and Qiu 2011; Liu et al. 2013). In addition, food and packaging are a source of human exposure. The human body can be exposed to DEHP from both aqueous and soil sources (Kueseng et al. 2007). Nakamiya et al. (2005) reported that the human body introduces around 25 mg of DEHP every day only from food. Moreover, Sharman et al. (1994) estimated that DEHP at concentrations of 12 mg/L and 2000 mg/L was detected, respectively, in European milk and cheese. Besides, it was reported that about 1–10 mg/kg/d of DEHP was daily ingested by the infant from breast milk and

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cow's milk (Kamrin 2009). Similarly, Huang et al. (2008) showed that DEHP was also found in *Oreochromis niloticus niloticus* and the fishes *Liza subviridis* and reached the concentrations of 129.5 mg/kg d.w. and 253.9 mg/kg d.w., respectively.

DEHP absorbed in the body is rapidly hydrolyzed by unspecific lipases to MEHP and 2-ethylhexanol (Albro 1986). MEHP is further metabolized to cytochrome P450 (CYP)-mediated oxidative and dealkylated metabolites in the human liver and at a lower rate in the human intestine. These metabolites are mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH MEHP), mono(2-ethyl-5-oxohexyl) phthalate (5-Oxo MEHP), phthalic acid (PA), mono(2-ethyl-5-carboxypentyl) phthalate (5-carboxy MEPP), and mono(2-carboxymethylhexyl)phthalate (2-carboxy MMHP). Only MEHP, 5-OH MEHP, and 5-Oxo MEHP were glucuronidated in human liver microsomes in vitro (Choi et al. 2012; Joo et al. 2011). DEHP can also be metabolized by enzymes produced by gastrointestinal microflora or gut contents. Rowland et al. (1977) demonstrated that rat gut contents from the stomach, small intestine, and cecum have been shown to metabolize 1.0, 22.1, and 6.9%, respectively, of the DEHP dose following 16 h of incubation. However, the metabolism proportion occurring in human feces was lower (0.6%). Rowland (1974) concluded that the metabolism of DEHP in rats is partially related to bacteria since treatment with antibiotics during incubation reduced the overall metabolism of DEHP. Since DEHP is absorbed and metabolized in the intestines, it has been necessary to test its toxic effects on its first exposure route, the intestinal tract.

Many investigations demonstrated that long-term exposure to DEHP can cause toxic effects on animals as well as in humans even at low concentrations (Quan et al. 2005; Takeshita et al. 2006; Yuan et al. 2010). The gastrointestinal tract (GI) represents the first barrier to food contaminations such as phthalates. Information on the oral toxicity of DEHP in humans is limited to gastrointestinal symptoms (mild abdominal pain and diarrhea) in two individuals who ingested a single large dose of the compound. Studies in rats, mice, and other rodent species show that DEHP has a low order of acute oral toxicity, with some data indicating that young animals are more susceptible than adults. However, the most adverse effects that have been reported after DEHP exposure are with other tissue forms. In fact, DEHP was known initially as an endocrine disruptor causing disturbance of androgen-dependent development, alterations in the quality of the sperm, increase in spontaneous abortions, and precocious puberty in adolescent female rats (Maffini et al. 2006; De Coster and van Larebeke 2012; Liu et al. 2018). Besides, several studies, using in vivo models, have shown that DEHP exposure causes immune system alteration, neurotoxicity, nephrotoxicity, and liver damage (Carlisle et al. 2009; Yuan et al. 2017). Muscogiuri and Colao (2017) linked cardiovascular diseases to obesity caused by the exposure to this phthalate.

DEHP exposures to primary cells of the GI tract are not already demonstrated by other experiments. Therefore, the present study deals with the in vitro investigation of the toxic effect of DEHP toward cells derived from the GI tract like HCT 116. These cells have been widely used in toxicological studies (Chae et al. 2008; Yang et al. 2008; Chengzhi 2009; El-Khatib et al. 2010; Ben Salem et al. 2016). Our study aimed to elucidate the mechanism of action of DEHP-induced toxicity in HCT 116.

Material and methods

Chemicals

DEHP, low-melting-point agarose (LMPA), and normal-melting-point agarose (NMPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cell culture medium (RPMI 1640), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin-EDTA, penicillin/streptomycin mixture, and L-glutamine (200 mM) were obtained from GIBCO-BCL (UK). 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) was supplied by Molecular Probes (Cergy-Pontoise, France). All other chemicals used were of analytical grade.

Methods

Cell culture

Human colon carcinoma cells (HCT 116) cell line was obtained from the American Type Culture Collection (ATCC® CCL-247™) and was cultured in RPMI 1640 medium at 37 °C with 5% CO₂. The medium was supplemented with 10% FBS, 1% of mixture penicillin (100 IU/ml) and streptomycin (100 µg/ml), and 1% L-glutamine (200 mM). DEHP was dissolved in pure ethanol. Desired concentrations were obtained by diluting DEHP in cell culture media so that the percentage of ethanol did not exceed 0.5%. To obtain the studied concentrations in the cell culture media, the DEHP treatment volume was negligible and represented about 0.5% of the total medium volume. In these conditions, untreated cells and cells receiving this low vehicle volume responded in the same manner.

Cell toxicity assay (MTT assay)

The MTT assay (tetrazolium salt reduction assay) is a test evaluating cell viability and gives an idea on cellular mitochondria activity (Mosman 1983). DEHP at different concentrations (25–240 µM) was tested on HCT 116 cells seeded in 96-well plates (25 × 10³ cells/well) after 24 h incubation at 37 °C. Untreated cells were taken as a negative control.

Cells were then incubated with the MTT solution for 3 h. At the end of the incubation, the absorbance of crystals of formed formazan was read at 570 nm using a microplate reader spectrophotometer (BioTek Elx800). The results were expressed as a percentage conversion of MTT in the treated cells compared to the untreated cells. All assays have been repeated 3 times.

Reactive oxygen species determination and oxidative stress status

To assess the oxidation in biological systems, DCFH-DA fluorometric assay is commonly used (Cathcart et al. 1983; Gomes et al. 2005; Chen and Wong 2009). It is based on the measurement of the DCF fluorescence at 522 nm. The DCF fluorescent probe is the final result of the initial non-fluorescent DCFH-DA probe transformation into non-fluorescent DCFH thanks to intracellular esterases and then to DCF in the presence of ROS (Le Bel et al. 1992). Cells seeded on 24-well culture plates (Polylabo, France) at 10^5 cells/well were treated with DEHP (40, 65, and 130 μ M corresponding to IC₅₀/4, IC₅₀/2, and IC₅₀, respectively) for 24 h at 37 °C. After incubation, cells were stained with 20 μ M DCFH-DA. A positive control treatment for 24 h with H₂O₂ (20 μ M) was also used. ROS production was measured after 30 min of incubation at 37 °C by fluorometric detection of DCF oxidation on a fluorimeter (BioTek FLx800) with an excitation wavelength of 485 nm and an emission wavelength of 522 nm. The DCF fluorescence intensity is relative to the intracellular ROS content. Results are expressed as the ratio of intensity of fluorescence in treated cells to that of the corresponding fluorescence in the control.

Measurement of the lipid peroxidation

Malondialdehyde (MDA), as an index of lipo-peroxidation, was quantified spectrophotometrically (Ohkawa et al. 1979). For this purpose, 7.5×10^5 cells seeded on 6-well plates were treated with DEHP, for 24 h at 37 °C. After the elimination of treatment, cold PBS was used to wash cells and then ice-cold 1.15% KCl to lyse the cell suspension by homogenization. Mixtures prepared with 0.1 ml of cell lysates, 1.5 ml of 20% acetic acid (pH 3.5), 0.2 ml of 8.1% SDS, and 1.5 ml of 0.8% thiobarbituric acid were heated to 95 °C for 120 min, cooled to room temperature, and shaken vigorously after adding 5 ml of n-butanol and pyridine (15:1, v:v). Finally, the absorbance at 546 nm of the supernatant was measured after centrifugation at 4000 rpm for 10 min. According to a standard curve, the MDA concentration was determined, and value results were expressed as nmoles of malondialdehyde/mg of proteins.

Measurement of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured according to the method of Marklund and Marklund (1974). When the superoxide radical ($O_2^{\cdot-}$) is present, the pyrogallol is autoxidized and then can be quantified spectrophotometrically at 440 nm. The SOD by catalyzing the dismutation of $O_2^{\cdot-}$ will inhibit the autoxidation of pyrogallol. Thus, the activity of this enzyme was estimated by the amount of enzyme necessary to inhibit 50% of the autoxidation of the pyrogallol and was expressed as enzyme units/mg of proteins.

Measurement of catalase (CAT) activity

The activity of the catalase (CAT) was established by a spectrophotometric method in HCT 116 cells after 24 h of DEHP treatment. It consists to follow the H₂O₂ decomposition over time at 240 nm (Acuna et al. 2009). One unit of CAT activity was referred to the μ moles of H₂O₂ consumed/minute/mg of protein.

Determination of the GSH/GSSG ratio

The enzymatic method described by Rahman et al. (2007) was used to quantify the rate of GSH and GSSG in HCT 116 cell lysates after 24 h of DEHP treatment. We used perchloric acid to deproteinize cell lysates followed by centrifugation at 10000 g for 10 min at 4 °C. Total glutathione and GSSG were analyzed in the supernatants. GSH was evaluated by measuring the reduction of DTNB by GSH at 412 nm, while GSSG was determined after GSH scavenging by 2-vinylpyridine which forms a pyridinium salt when complexed with thiol groups. The GSH/GSSG ratio was calculated and used as an oxidative stress marker.

Measurement of glutathione reductase activity

Glutathione reductase activity was determined by the method described by Carlberg and Mannervik (1975). It is determined spectrophotometrically at 340 nm by measuring the NADPH disappearance. Briefly, the assay mixture consisted of phosphate buffer (0.1 M, pH 7.6), HCT 116 cell homogenate after 24 h of DEHP treatment, EDTA (0.5 mM), and NADPH (0.1 mM). The enzymatic reaction was initiated by addition of GSSG (1 mM). GR activity was calculated as nmol NADPH oxidized/min/mg of protein using molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Activity determination of dehydrogenase enzymes from the pentose phosphate pathway.

Glucose-6-phosphate dehydrogenase (G6PD) activity was assayed in HCT 116 cells after 24 h of DEHP treatment by the method described by Tian et al. (1999). Is a spectrophotometric measurement at 341 nm based on the conversion of

NADP⁺ to NADPH. The conversion of NADP⁺ to NADPH is done by G6PD or the second enzyme 6-phosphogluconate dehydrogenase (6PGD). Firstly, glucose 6-phosphate is converted by the G6PD to 6-phosphogluconolactone, which is rapidly hydrolyzed by the 6PGD to 6-phosphogluconate. Then, to determine G6PD and 6PGD activities, we must calculate the PGD activity separated as well as the total enzyme activity (G6PD and 6PGD). We obtain the G6PD activity by subtracting the 6PGD activity from the total dehydrogenase activity. The total dehydrogenase activity was obtained by adding both dehydrogenase enzymes' substrates, while the 6PGD activity was determined by adding only the substrate of this enzyme. The enzyme activity was calculated as nmol NADP reduced/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Mitochondrial membrane potential ($\Delta\Psi$ m) assay

The mitochondrial membrane potential determination is based on the capacity of retention of the cationic fluorescent dye, rhodamine-123, by the cell membrane. HCT 116 cells (2.5×10^5 cells/well in 96-well plates) were treated with DEHP (32.5, 65, and 130 μM corresponding to IC₅₀/4, IC₅₀/2, and IC₅₀, respectively) for 24 h. Next, cells were incubated with rhodamine-123 for 15 min (37 °C, 5% CO₂). The rhodamine-123 uptake was estimated by fluorometric detection. The results were expressed as the percentage of rhodamine fluorescence absorbed by the treated cells from the fluorescence measured in the untreated cells.

Protein extraction and Western blotting

After 24 h of DEHP treatment, HCT 116 cell proteins were obtained after cell lysis with the lysis buffer containing HEPES 0.5 M 0.5% Nonidet-P40, 1 mM PMSF, 1 mg/ml aprotinin, and 2 mg/ml leupeptin, pH 7.4. Equal amounts of proteins were transferred onto membrane of nitrocellulose after SDS-PAGE separation. Membranes were incubated overnight at 4 °C with appropriate primary antibodies: anti-Hsp70 (SC-10789, Santa Cruz Biotech. Inc), anti-Bax (SC-7480, Santa Cruz Biotech. Inc), and anti-Bcl2 polyclonal (SC-7382, Santa Cruz Biotech. Inc). The same membrane was incubated with anti- β -actin (ab8227, Abcam, Cambridge, UK) for 1 h with the secondary antibody. Membranes were then incubated with the secondary polyclonal antibody conjugated with horseradish peroxidase for 1 h at room temperature. Proteins were detected after immunoreactive protein staining by adding the chromogenic substrates BCIP/NBT. ImageJ software was used to calculate the relative density (RD), and the intensity of each protein was normalized to β -actin. The data is then as a ratio between the intensity of the protein tested in the treated cells and that in the untreated cells.

Alkaline single-cell gel electrophoresis assay (comet assay)

We used ordinary microscope slides covered with agarose to spread nuclei from HCT 116 cells treated with DEHP for 24 h. Then, an electrophoresis of 25 V was then applied under alkaline conditions in the dark for 30 min. After staining with ethidium bromide, about 100 nuclei per slide were visualized and counted. The result was obtained after a score calculation based on the DNA damage degree which is relative to the intensity of the fluorescence in the tail (Collins et al. 1996).

Statistical analysis

Each experiment was done three times separately, and data were expressed as the mean $\pm$ SD of the means. To determine differences between groups and their respective controls, we used one-way ANOVA followed by Tukey-Kramer post hoc test. Differences were considered significant at $P < 0.05$.

Results

DEHP induces cell death in HCT 116 cells

To evaluate the influence of DEHP on intestinal cells (HCT 116), the MTT assay was performed. The data obtained indicated that this compound induces a decrease in HCT 116 cells' metabolic ability to reduce MTT in a dose-dependent manner, which reveals its cytotoxic effect (Fig. 1). The half maximal inhibitory concentration (IC₅₀) value determined after 24 h of cell treatment was about 130 μM .

DEHP induces Hsp70 expression in HCT 116 cells

The expression level of Hsp70 was evaluated. This protein is expressed to protect cells against stressful conditions. HCT 116 cells were treated with concentrations of 32.5 μM , 65 μM , and 130 μM corresponding to IC₅₀/4, IC₅₀/2, and IC₅₀, respectively. As shown in Fig. 2a, we found that the exposure to DEHP for 24 h increases the expression of Hsp70 in HCT 116 cells compared to the control group, as evidenced by the immunoblotting assay followed by scanning densitometry illustrated in Fig. 2a, which was further confirmed by results of.

DEHP induces ROS production in HCT 116 cells

To check the oxidative stress status in HCT 116 cells in response to DEHP for 24 h, we measured the production of fluorescent DCF (the result of DCFH oxidation mostly by H₂O₂). Our results demonstrated that DEHP treatment at increasing concentrations (32.5 μM , 65 μM , and 130 μM)

Fig. 1 Cytotoxic effect of DEHP on HCT 116 cells. Cells were treated with DEHP at the indicated concentrations for 24 h. Cell viability was determined using the MTT assay and expressed as percentages of viability. Data are expressed as the mean $\pm$ SD of three independent experiments

DEHP induces oxidative stress

Fig. 2 DEHP induces oxidative stress in HCT 116 cells after 24 h of exposure. **(a)** DEHP induces Hsp70 protein overexpression. The Hsp70 protein level was analyzed by Western blot. β -Actin was used as a loading control. *RD* relative density as described in the Material and Methods **(b)** DEHP induces ROS generation. Intracellular ROS generation was measured by analyzing DCF fluorescence, and H₂O₂ (20 μM) was considered as a positive control. **(c)** DEHP induces lipid

peroxidation. The peroxidation of lipids was recorded by measuring the accumulation of MDA. Cells were incubated for 24 h with DEHP (32.5, 65, and 130 μM). Data are expressed as the mean $\pm$ SD of three independent experiments. * P < 0.05 and *** P < 0.001 values are significantly different from control; ^{xxx} P < 0.01 and ^{xxxx} P < 0.001 values are significantly different from cells treated with H₂O₂ (as positive control)

induces a significant ROS generation compared to the control group (Fig. 2b).

DEHP induces lipid peroxidation in HCT 116 cells

As an index of lipid peroxidation, the intracellular concentration of MDA was determined in HCT 116 cells. After 24 h of incubation with DEHP, the MDA level increased from the basal level of 0.19 ± 0.02 μmol MDA/mg of protein to 0.25 ± 0.01 μmol of MDA/mg of protein, 0.46 ± 0.01 μmol of MDA/mg of protein, and 0.58 ± 0.03 μmol of MDA/mg of protein in HCT 116 cells treated with DEHP 32.5, 65, and 130 μM , respectively (Fig. 2c). Additionally, treated cells with H_2O_2 (20 μM), used as positive control, increased the MDA level to 0.97 ± 0.01 μmol of MDA/mg of protein.

DEHP alters antioxidant enzymes' activities in HCT 116 cells

The cellular protection against oxidative stress can be detected by a change in the antioxidant enzymes' activities. SOD catalyzes the dismutation of the highly reactive superoxide anion

to H_2O_2 . Figure 3a illustrates a significant increase in the SOD activity after cell treatment with DEHP for 24 h. Indeed, the SOD activity measured increased from basal value of 2.65 ± 0.26 U/mg of protein in control group to 3.31 ± 0.90 U SOD/mg of protein, 6.04 ± 0.63 U SOD/mg of protein, and 9.46 ± 0.92 U SOD/mg of protein in cells treated with DEHP at 32.5, 65, and 130 μM respectively.

CAT decomposes the H_2O_2 derived from the SOD activity to water and oxygen. The results show a marked increase in CAT activity. This increased activity passes from 32.5 ± 4.73 μmol H_2O_2 /min/mg of protein in control group to 46.55 ± 5.73 μmol H_2O_2 /min/mg of protein, 80.17 ± 11.71 μmol H_2O_2 /min/mg of protein, and 147.53 ± 10.2 μmol H_2O_2 /min/mg of protein in HCT 116 cells treated with 32.5, 65, and 130 μM of DEHP, respectively (Fig. 3b).

DEHP induces glutathione depletion in HCT 116 cells

Reduced glutathione plays an important role in antioxidant defense thanks to its nucleophilic and reducing properties. Under normal conditions, the overproduction of ROS is neutralized by reduced glutathione which leads to its depletion

Fig. 3 Effects of DEHP on superoxide dismutase and catalase activities measured in HCT 116 cells exposed to DEHP (40, 65, and 130 μM) for 24 h. **(a)** Superoxide dismutase activity was calculated as the amount of protein that caused 50% pyrogallol autoxidation inhibition. **(b)** Catalase activity was determined by the hydrolysis of H_2O_2 . Data are expressed as the mean $\pm$ SD of three separate experiments. ** $P < 0.01$ and *** $P < 0.001$ values are significantly different from control

DEHP alters antioxidant enzyme activities in HCT 116 cells (SOD and CAT)

inside the cell. Thus, we tested the effect of DEHP on glutathione modulation in HCT 116 cells. Treatment with DEHP for 24 h showed a decrease in the GSH/GSSG ratio (from GSH depletion) as compared to the control group. The GSH/GSSG ratio changed from 76.67 ± 6.19 in control group to 57.23 ± 2.99 , 29.87 ± 2.32 , and 15.30 ± 3.04 in HCT 116 cells treated with DEHP at 32.5, 65, and 130 μM , respectively (Fig. 4a).

DEHP inhibits glutathione reductase activity in HCT 116 cells

Glutathione reductase (GR) is responsible for maintaining glutathione on its reduced form, for that we have evaluated the effect of DEHP on the activity of this enzyme after 24 h of exposure. Data in Fig. 4b show a decrease in the GR activity following treatment with DEHP. This decreased activity passes from 15.98 ± 1.01 nmol NADPH oxidized/min/mg of protein in control group to 14.43 ± 1.77 nmol NADPH oxidized/min/mg of protein, 11.22 ± 0.63 nmol NADPH oxidized/min/mg of protein, and 7.30 ± 0.50 nmol NADPH oxidized/min/mg of protein in cells treated with DEHP at 32.5, 65, and 130 μM , respectively.

Fig. 4 DEHP induces glutathione depletion and affects the glutathione reductase activity in HCT 116 cells exposed to 32.5, 65, and 130 μM for 24 h. **(a)** The glutathione depletion is determined by measuring the ratio GSH/GSSG. **(b)** The glutathione reductase activity was determined as the amount of NADPH used during the reaction. Data are expressed as the mean $\pm$ SD of three separate experiments. $**P < 0.01$ and $***P < 0.001$ values are significantly different from control

DEHP inhibits dehydrogenase activities in the oxidative phase of the pentose phosphate pathway

Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) are the main routes of NADPH biosynthesis which is used by the GR as a cofactor to maintain the reduced form of glutathione.

Figure 5a shows a dose-dependent effect on dehydrogenase activities following cell treatment with DEHP for 24 h. In fact, this phthalate was able to decrease both G6PD (Fig. 5b) and 6PGD (Fig. 5c) activities. G6PD activity passes from 4.18 ± 0.19 nmol NADPH/mg of protein in control group to 3.75 ± 0.22 nmol NADPH/mg of protein, 3.00 ± 0.06 nmol NADPH/mg of protein, and 2.04 ± 0.38 nmol NADPH/mg of protein in HCT 116 cells treated with DEHP at 32.5, 65, and 130 μM , respectively.

6PGD activity passes from 21.10 ± 1.17 nmol NADPH/mg of protein in control group to 19.91 ± 0.82 nmol NADPH/mg of protein, 15.39 ± 0.54 nmol NADPH/mg of protein, and 11.93 ± 1.43 nmol NADPH/mg of protein when cells were treated with DEHP at 32.5, 65, and 130 μM , respectively.

DEHP induced glutathione depletion in HCT 116 cells

DEHP inhibits glutathione reductase activity in HCT 116 cells

DEHP inhibits dehydrogenase activities in the oxidative phase of the pentose phosphate pathway in HCT 116 cells

Fig. 5 (a) Effect of DEHP on total dehydrogenase enzymes from the pentose phosphate pathway activities. (b) Effect of DEHP on glucose-6-phosphate dehydrogenase activity (c) Effect of DEHP on 6-phosphogluconate dehydrogenase activity. HCT 116 cells were exposed to DEHP (32.5, 65, and 130 μM) for 24 h. Glucose dehydrogenase

activities were determined as the amount of NADP reduced formed during the reaction. Data are expressed as the mean $\pm$ SD of three separate experiments. ** $P < 0.01$ and *** $P < 0.001$ values are significantly different from control

DEHP induces loss of mitochondrial membrane potential in HCT 116 cells

To determine the nature of cell death provoked by DEHP after 24 h, HCT 116 cells were stained with rhodamine-123 to evaluate the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the protein levels of Bax and Bcl2 that control the intrinsic apoptotic pathway and were also evaluated by Western blot (Fig. 6a and b). Following DEHP treatment, the uptake of the rhodamine-123 was reduced in HCT 116 cells in a dose-dependent manner, demonstrating that DEHP causes a significant decrease in $\Delta\Psi_m$ (Fig. 6a). This loss of $\Delta\Psi_m$ was associated with the overexpression of proapoptotic protein, Bax (Fig. 6b), and the decrease in the anti-apoptotic protein, Bcl2 (Fig. 6b).

DEHP induces DNA fragmentation

We next tested if this phthalate induced DNA damage, one of apoptotic hall markers. We used the alkaline comet assay which is very sensible to analyze DNA fragmentations in

individual cells. As shown in Fig. 6c, the total score of damaged DNA was about 158.5 ± 4.94 , 174 ± 8.48 , and 214 ± 14.14 when HCT 116 cells were treated with 32.5, 65, and 130 μM DEHP, respectively, for 24 h, as compared to a total score of 144.5 ± 6.36 in control cells.

Discussion

Phthalates like DEHP remain irreplaceable as plasticizers that are used to impart flexibility and elasticity to polyvinyl chloride products. People are inevitably exposed to phthalates through environmental contamination. Exposure to DEHP was associated with a variety of toxicological outcomes in mammals such as reproductive toxicity (Wang et al. 2017), neurotoxicity (You et al. 2018), carcinogenicity (Chen et al. 2017), hepatotoxicity (Zhang et al. 2018), and lipid metabolism disruption (Pradhan et al. 2018).

In this study, we focused on the harmful effect of this plasticizer on its first exposure route, the intestinal tract, since the metabolism of this phthalate is partly in the intestinal cells. For

DEHP induces loss of mitochondrial apoptosis

Fig. 6 (a) DEHP induces loss of mitochondrial transmembrane potential. (b) DEHP induces apoptosis. (c) DEHP induces DNA fragmentation. Cells were treated with DEHP (32.5, 65, and 130 μM) for 24 h. The mitochondrial potential was assessed by measuring the uptake of rhodamine-123. Apoptosis markers Bax and Bcl2 were analyzed by

Western blot. β-Actin was used as a loading control. *RD* relative density as described in the Material and Methods and DNA fragmentation were analyzed by the comet assay. Data are expressed as the mean ± SD of three separate experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ values are significantly different from control

this reason, HCT 116 cells were chosen as a cell model derived from the large intestine, and we aimed to test whether the DEHP-induced cytotoxicity on HCT 116 was mediated by an oxidative stress. In fact, the MEHP, the first metabolite of the DEHP, was metabolized by CYP-dependent oxidation and dealkylation in the human intestine. Human CYP2 (2C9 1, 2C9 2, 2C9 3) and 3A4 were the major CYP enzymes toward MEHP (Choi et al. 2012). hCYP2C9 is low expressed in the human intestine (15% of total intestinal CYP content), assures the oxidative metabolism of MEHP, and produces 5-OH MEHP, 5-carboxy MMHP, and 5-oxo MEHP. Moreover, CYP3A4, with its high expression level in the human intestine (80% of total intestinal CYP content), they have a predominant catalytic efficiency for PA production dealkylated from MEHP. hCYP2C9 1 and 3A4 might play a considerably important role in MEHP metabolism, especially at its low concentrations that represent environmentally relevant exposure concentrations. It has been estimated that humans are exposed to DEHP at concentrations ranging from 10 mg/kg in cream; 25 mg/kg in cheese; 30 to 70 mg/kg in fruit juice, edible oil, meat, and poultry; and 158 mg/kg in oily foods in jars (Cao 2010). Exposure among individuals may vary considerably

because of the wide variety of products into which DEHP is incorporated. The estimated average daily adult dose from the consumption of commodities highly likely to be contaminated (such as milk, cheese, margarine) is about 200 μg/day (Directorate for Health Sciences 1985; Tickner et al. 2001). This quantity is higher than those used in our work that represents 12.7, 25.38, and 50.77 μg, respectively.

We started our work with a study of cell viability by the MTT test which indicated that DEHP treatment significantly decreased the viability of HCT 116 cells in a dose-dependent manner. This observed cytotoxicity may be the result of different biochemical alterations and various cellular dysfunctions in response to DEHP treatment.

Then, we evaluate the expression of Hsp70 protein, being an early marker of oxidative stress and cytotoxicity (El Golli et al. 2006), and we have found that DEHP induced an over-expression of this protein. In order to test the ability of DEHP to generate free radicals, ROS production was measured using DCFH-DA as a fluorescent probe in cultured cells. We have demonstrated an important increase in the production of the fluorescent DCF indicating a significant ROS generation that can lead to a disturbance of redox status balance in cells. In

agreement with our studies, a significant increase in ROS generation was observed in the following DEHP treatment both in vivo and in vitro (Bahrami et al. 2018; Zhao et al. 2018; Sun et al. 2018).

It is well known that excess generation of free radicals can alter cell membranes and increase cell membrane permeability (Giorgi et al. 2012). Besides, it was reported that the subsequent consequence of DEHP-induced ROS generation was the induction of lipid peroxidation in rat testis (Suna et al. 2007), in pancreatic β cells (She et al. 2017), and in HepG2 cells (Zhang et al. 2017). Interestingly and as evidenced by the measurement of malondialdehyde (MDA), the end product of lipid peroxidation, and the significant indicator of the lipid peroxidation, we showed that DEHP induced a significant increase in MDA levels in a dose-dependent manner.

To counteract the harmful effects of ROS, cells were used as an antioxidant defense system composed by enzymatic and nonenzymatic antioxidants acting as free radical scavengers to protect cells from oxidative damage (Abdollahi et al. 2004). Among which SOD and CAT are considered as first line of defense that protect cell macromolecules from oxidative damage. In fact, SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide that will be converted to water by the activity of CAT and GPx (Peixoto et al. 2004; Sharma and Sangha 2014). In the present work, compared with those of the control group, the SOD and CAT activities increased significantly in cells treated with DEHP. This could be explained by an increase in $O_2^{\bullet-}$ production, which prompted an increase in SOD activity to convert it to H_2O_2 and water through the CAT activity. In accordance with our study, a significant increase in SOD and CAT activities was observed in the liver of rats treated with DEHP (Erkekoglu et al. 2014). Antioxidant status in HCT 116 cells treated with DEHP was also investigated through quantification of reduced GSH (GSH) and oxidized (GSSG) content. We found that treatment with DEHP resulted in a significant decrease in the GSH/GSSG ratio which is related to a decrease in GSH and an increase in GSSG concentrations. Changes in the redox state of glutathione were in agreement with the oxidative changes already observed after treatment with DEHP. Similarly, a significant decrease in GSH content was observed in vivo and in vitro following DEHP treatment (She et al. 2017; Zhao et al. 2018). GSH regeneration by the glutathione reductase (GR) is essential to maintain the cellular redox balance. Here, we report that DEHP induced a decrease in the GR activity. Interestingly, similar results were observed by Abdel-Kawi et al. (2016), who showed that the DEHP treatment induced oxidative testicular injuries in male albino rats and decreased the GR activity. It is known that for the reduced GSH regeneration, the GR needs the NADPH as a cofactor (De Freitas-Silva et al. 2017). This data led us to test the hypothesis whether the observed decrease in GR activity could be a result of the decrease in NADPH rate.

Based on this idea, it is proved that the change in the G6PDH activity, which is responsible for the regeneration of NADPH under the pentose cycle, can modify the content of this cofactor (Tian et al. 1999). As expected, our work has shown that DEHP decreased the activity of this enzyme which could explain the decrease of the NADPH content and, by the end, disturb the redox homeostasis by decreasing the GSH content.

On the other hand, we measured the transmembrane mitochondrial potential ($\Delta\psi_m$). In fact, a high $\Delta\psi_m$ is necessary for the cellular integrity maintenance, given the specific activities of the mitochondria, namely, cellular bioenergetics, urea cycle, as well as fats and amino acid catabolism (Song et al. 2017). An increase in ROS production is an upstream event for the mitochondrial reduction membrane potential which acts as a potent mediator in cell death.

It is largely known that the cell death process is produced in cells through the intrinsic or the extrinsic pathway (Loreto et al. 2014). Here, we suggest that DEHP induces apoptosis by triggering the mitochondrial intrinsic pathway. This observation was supported by the decrease in the $\Delta\psi_m$ provoked by DEHP. Moreover, the intrinsic apoptotic pathway is often controlled by the Bcl-2 family proteins which are essential modulators of $\Delta\psi_m$ (Giorgi et al. 2012). In order to determine whether Bax, a proapoptotic protein, and Bcl2, an anti-apoptotic protein of Bcl-2 family, were implicated in DEHP-induced apoptosis, their expression levels were quantified by Western blot. Our results showed that DEHP treatment increased Bax and decreased Bcl2 expressions. These findings confirm that DEHP induces an apoptotic cell death as described by Sun et al. (2018) who demonstrated that this phthalate induced apoptosis in mouse Leydig cells, as revealed by significant downregulation of Bcl-2 and upregulation of Bax proteins.

In addition, significant fragmentation of DNA was clearly observed by the comet test. Similar results have been reported in HEK293 cells treated with DEHP, which showed that this phthalate induced oxidative DNA damage (Wang et al. 2015).

Conclusion

In conclusion, our work gives new information on molecular mechanism of the cytotoxicity induced by the commonly used phthalate DEHP. We show that this phthalate induces an overproduction of ROS that elucidates its toxic effect in intestine cells. Generated ROS was related to the inhibition of glutathione regeneration through the inhibition of the enzymes involved in the oxidative phase of the pentose phosphate pathway. ROS overgeneration activates then the mitochondrial pathway of apoptosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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