

# Analysis of toxicity effects of Di-(2-ethylhexyl) phthalate exposure on human bronchial epithelial 16HBE cells

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**Abstract** Recent studies have indicated that Di-(2-ethylhexyl) phthalate (DEHP), the most commonly used plasticizer in daily-life products, could be dispersed in indoor air and induce human exposure via inhalation. DEHP has been reported to have effects on the respiratory system in both animal and human researches. The toxicity effects of DEHP exposure on cell proliferation, cell cycle progression, apoptosis, global DNA methylation and the expression levels of DNA methyltransferases (DNMTs) were investigated in this study, using human epithelial cell line 16HBE as an in vitro model. Cells were treated with DEHP at doses of 0, 0.125, 0.5 and 2 mmol/L for 48 h. Cell proliferation, cell cycle and apoptosis were tested by

MTT assay and flow cytometer, respectively. The obtained results showed decreased living cell number and cell viability following DEHP exposure at the dose of 2 mmol/L. DEHP also inhibited the cell cycle progression of G1 phase and induced a significant increase in cell apoptosis in 16HBE cells. DEHP exposure could induce cell proliferation inhibition in 16HBE cells via the blocking of cell cycle progression and accelerated cell apoptosis. In addition, decreased global DNA methylation levels and expression levels of DNMTs were observed in DEHP-treated groups which revealed possible epigenetic effects of DEHP.

**Keywords** Apoptosis · Cell cycle · DEHP · DNA methylation · Human bronchial epithelial 16HBE cell

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Yue Ma and Yinsheng Guo have contributed equally to this work.

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## Abbreviations

DEHP	Di-(2-ethylhexyl) phthalate
DMSO	Dimethyl sulfoxide
DNMT1	DNA methyltransferase 1
FBS	Fetal bovine serum
MEHP	Mono-2-ethylhexyl phthalate
MEM	Minimum essential medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
PBS	Phosphate buffer saline
PI	Prodidium iodide
PVC	Polyvinyl chloride
QPCR	Quantitative real-time PCR

## Introduction

Currently, Di-(2-ethylhexyl) phthalate (DEHP) is used as a polyvinyl chloride (PVC) plasticizer in a wide range of consumer products, including vinyl floors, wall covering materials, cosmetics and toys (Cadogan and Howick 1996). As it is not covalently bound to the plastic matrix, DEHP may migrate from PVC materials and be emitted to the air and indoor dust in the environment (Wormuth et al. 2006). Airborne DEHP is present at detectable levels across the surface of Earth (Green et al. 2005). As a result of the wide distribution of DEHP in the environment, DEHP exposure is ubiquitous and inhalation is an important route of human exposure to DEHP during its production and use (Hoppin et al. 2004).

The prevalence of respiratory diseases is increasing. Epidemiological studies have investigated the association between phthalates exposure and respiratory symptoms in both occupational and residential environments (Bornehag et al. 2004; Hoppin et al. 2004, 2013; Jaakkola et al. 2000; Tuomainen et al. 2006). With respect to human exposure, DEHP is the most commonly used phthalate accounting for approximately 50% of total global phthalate consumption. DEHP is widespread in the environment and has been shown to affect the respiratory system. A nested case–control study showed a dose–response relationship between DEHP concentration in indoor dust and wheezing in Bulgarian children (Kolarik et al. 2008). DEHP exposure during pregnancy has been shown to disrupt the proper timing of epithelial cell proliferation in rat pups (Rosicarelli and Stefanini 2009). Mono-2-ethylhexyl phthalate (MEHP), a degradation product of DEHP, has been shown to promote an inflammatory response in cultured human lung epithelial cell line A549 (Jepsen et al. 2004), and to exert a toxic effect on the monocytic cell line THP-1 (Glue et al. 2002).

Respiratory disease involves the interaction of respiratory tract epithelial cells, such as 16HBE cells. Therefore, it can be inferred that DEHP may have toxic effects on human respiratory tract epithelial cells. The cell cycle and apoptosis are the basis for growth and development in all living organisms. Disturbed cell cycle and apoptosis are common toxic reactions induced by exposure to xenobiotic chemicals exposure. However, there has been no research on the toxicity of DEHP on 16HBE cells yet.

Additionally, epigenetic alternations in respiratory tract cells have been found to be important modulators of environmental exposure in respiratory tract diseases (Nicodemus-Johnson et al. 2016). The role of epigenetic modifications in human respiratory diseases is identified by multiple studies showing changed global DNA methylation levels in tissues or cells (Qiu et al. 2012; Stefanowicz et al. 2012). Therefore, identifying DEHP mediated genome methylation changes may advance our understanding of the underlying epigenetic mechanisms that promote DEHP mediated pathogenesis.

In this study, we characterize the toxicity effects of DEHP exposure on 16HBE cells. Cell proliferation, cell cycle progression and cell apoptosis of 16HBE cells were determined. Furthermore, to explore the possible epigenetic toxicity of DEHP on 16HBE cells, the levels of global DNA methylation and the expression levels of DNA methyltransferases were also determined in both DEHP-treated groups and the control.

## Materials and methods

### Chemicals

DEHP (67,261, purity  $\geq 99.5\%$ ) and dimethyl sulfoxide (DMSO) were both purchased from Sigma Chemical (St. Louis, MO, USA). DEHP (100 mg) was dissolved in 1 mL DMSO and stored at 4 °C as a stock solution. Working solutions were freshly prepared each day of treatment, and DMSO was adjusted to a final concentration of 0.1% in each well.

### Cell culture and reagents

The human bronchial epithelial cell line 16HBE was kindly provided by Prof. D.C. Gruenert (University of California, San Francisco, CA, USA). The 16HBE cells were maintained at 37 °C in minimum essential medium (MEM, Gibco, Bleiswijk, The Netherlands) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Gibco) and 100  $\mu\text{g}/\text{mL}$  streptomycin (Gibco) in an incubator with 5% (v/v)  $\text{CO}_2$ . The medium was changed every 48 h.

### Observation of cell morphology

After treatment with different concentrations of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%)

for 48 h, the morphology of 16HBE cells was observed on an inverted light microscope fitted with phase contrast optics. A well of live cells for each treatment was photographed and the process was repeated for two separate plates. Morphometric analysis was performed for all images using ImagePro Plus 6.0 software (Media Cybernetic, Rockville, MD, USA). The cells were counted by manual tagging to determine the cell number, which was averaged for each image pair.

### Cell viability assay

Cell viability of 16HBE cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Twentyman and Luscombe 1987). Briefly, the 16HBE cells were seeded in 96-well plates at the density of  $4 \times 10^4$  cells/mL. After cultured for 24 h, the 16HBE cells were treated with different doses of DEHP (0.25, 0.5, 1, 2, 4, 8, 10, 12, 14 and 16 mmol/L) or vehicle (DMSO, 0.1%) in MEM medium containing 10% (v/v) FBS for 48 h. Then, MTT (5 mg/mL) was added into the medium following incubation for 4 h at 37 °C. The MTT medium was discarded and the 16HBE cells were lysed in 150  $\mu$ l of DMSO each well. The optical density (OD) at 490 nm was measured using a Microplate Reader (Biotek ELx800, Winooski, VT, USA). Cell viability (%) expressed as the percentage of absorbance values at each dose compared to the vehicle control, was calculated by  $[\text{OD}_{\text{DEHP}} - \text{OD}_{\text{blank}}] / [\text{OD}_{0.1\% \text{ DMSO}} - \text{OD}_{\text{blank}}] \times 100\%$ . The blank is the well which contains culture medium but no cells. Based on these results above, treatments were performed with DEHP at 0, 0.125, 0.5 and 2 mmol/L in the subsequent experiments.

### Cell cycle analysis

The cell cycles of 16HBE cells were analyzed by a flow cytometer (BD, FACS, Calibur, Franklin, Lakes, NJ, USA). After 16HBE cells were treated with different concentrations of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h, cells were collected by centrifugation at 800 g for 5 min and washed twice with phosphate buffer saline (PBS). The cell pellet was resuspended with 70% ethanol on ice for 2 h. Then, the ethanol was removed and cells were resuspended in

PBS containing propidium iodide (PI, 20  $\mu$ g/mL; Sigma) and Rnase A (100  $\mu$ g/mL; Qiagen, Valencia, CA, USA) with incubation for 30 min at 37 °C. Fluorescence of 1000 cells was measured for each sample. The cell distribution among different G0/G1, G2/M and S phases was analyzed with ModFit software (Becton-Dickinson, Brea, CA, USA).

### Cell apoptosis analysis

The 16HBE cells were analyzed with an Annexin V-FITC/PI detection kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Cells were planted in 6-well plates ( $1 \times 10^6$  cells/mL) and treated with different concentrations of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. Then, the cells were resuspended in binding buffer and incubated with Annexin V-FITC solution and PI solution for 15 min at room temperature. The proportions of apoptotic cells and normal cells were analyzed in the flow cytometer.

### Analysis of global DNA methylation

Genomic DNA from 16HBE cells was extracted using a Wizard Genomic DNA purification Kit (Promega, Madison, WI, USA). Global DNA methylation was determined by a MethylFlash Methylated DNA Quantification Kit (Epigentek, Brooklyn, NY, USA) according to the manufacturer's instructions.

### RNA isolation and quantitative real-time PCR (Q-PCR)

Total RNA was isolated from 16HBE cells after treatment with different concentrations of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription for cDNA synthesis was performed using Revert Aid First Strand cDNA Synthesis Kits (Fermentas, Hanover, MD, USA). Q-PCR was performed with Power SYBR Green PCR Master Mix reagents (Applied Biosystems, Framingham, MA, USA) on the 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated by the  $2^{-\Delta\Delta C_t}$  method with the housekeeping gene GAPDH as an internal control. Primer sequences for

**Table 1** Primer sequences for real-time PCR

Gene	Primer sequence (5′–3′)	Accession number
GAPDH	Forward TCTGACTTCAACAGCGACACC	NM_001256799.2
	Reverse CTGTTGCTGTAGCCAAATTCGT	
Dnmt1	Forward ACGACCCTGACCTCAAATAT	NM_001130823.2
	Reverse CCATTAACACCACCTTCAAGA	
Dnmt3a	Forward CCACCGGCCATACGGT	NM_001320892.1
	Reverse ACCATCCTTCCTGGGACCTG	
Dnmt3b	Forward CCCCTCAAACCCATTCCCTC	NM_001207055.1
	Reverse GCATCCGTCATCTTTCAGCC	

Q-PCR for DNA methyltransferase 1 (DNMT1), DNMT3a, DNMT3b and GAPDH are listed in Table 1.

### Statistical analysis

Data are expressed as mean  $\pm$  SD. Statistical analysis was carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). To determine statistical significance, the values were compared with one-way ANOVA followed by Dunnett's test. A  $P$  value  $<0.05$  was considered to be statistically significant.

## Results

DEHP decreased the living cell number and cell viability in 16HBE cells

The number of cells in each image was counted by manual tagging after treatment with different concentrations of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. Living cells counted in images are shown in Fig. 1a, b. The number of 16HBE cells was slightly lower in DEHP exposure groups (0.125–2 mmol/L). The difference was significant only at the dose of 2 mmol/L compared to control ( $P < 0.05$ ). Normal cells in control group were observed to be epithelial-like, flat and adherent. However, some cells in the 2 mmol/L DEHP-treated group were observed to be round, small and suspended. Moreover, the number of suspended cells was increased in the cell culture medium.

To determine the cytotoxic effects of DEHP exposure on 16HBE cells, cell viability in response to DEHP treatment for 48 h was tested by MTT. As exhibited in Fig. 1c, 16HBE cell viability was

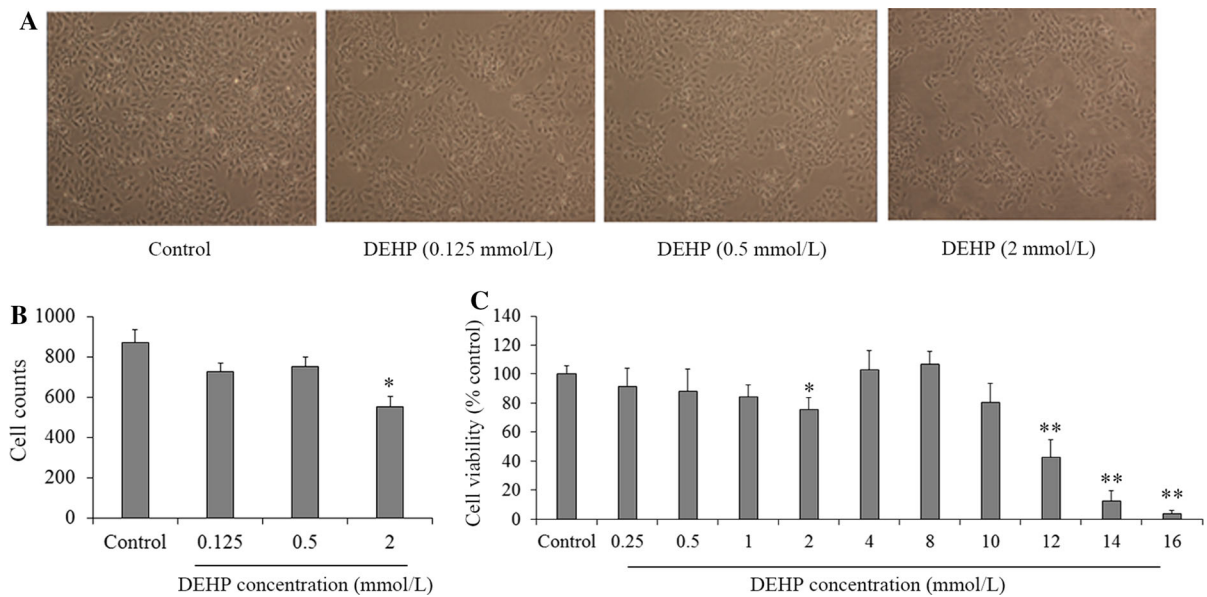
significantly inhibited by DEHP exposure at the dose of 2 mmol/L ( $P < 0.05$ ) and markedly inhibited at the doses of 12, 14 and 16 mmol/L ( $P < 0.01$ ). There is a decreasing trend generally in 16HBE cell viability with the increasing doses of DEHP. However, significant changes in the cell viability were not observed at doses of 4 mmol/L ( $1.06 \pm 0.22$ ,  $P > 0.05$ ) and 8 mmol/L ( $1.10 \pm 0.13$ ,  $P > 0.05$ ) compared to the control ( $1.03 \pm 0.06$ ). Moreover, at 10 mmol/L, the cell viability was slightly but not significantly decreased ( $0.83 \pm 0.25$ ,  $P = 0.056$ ). These results indicate that DEHP exposure induces cytotoxic effects at relatively high doses.

DEHP inhibited cell cycle progression of G1 phase and increased the percentage of cells at the G2/M and S phase

In order to investigate the effects of DEHP exposure on cell cycle progression in 16HBE cells, the cell cycle was determined after treatment with different doses of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. The cell cycle distribution profiles were recorded by flow cytometer. As shown in Fig. 2a, b, DEHP treatment significantly decreased the percentage of cells in the G0/G1 phase at the dose of 2 mmol/L compared to the controls ( $P < 0.05$ ). Significant increases in the percentage of cells at the G2/M and S phase were observed in DEHP treatment group at the dose of 2 mmol/L ( $P < 0.05$ ). These results indicate that DEHP induces cell cycle progression disruption. In addition, DEHP exposure might decrease 16HBE cell proliferation by disrupting cell cycle progression of G1 phase.

DEHP increased the proportions of apoptotic cells

DEHP induced apoptosis in 16HBE cells was determined by a flow cytometer after cells were treated with



**Fig. 1** Morphometric analysis of the response of 16HBE cells exposed to DEHP and effects of DEHP on cell viability. **a** Typical images used for analysis the effect of DEHP exposure on 16HBE cell number. Images of live cells were captured at  $\times 200$ . **b** Cell counts determined using manual tagging in ImagePro Plus 6.1 for cells exposed to vehicle control and different doses of DEHP. **c** Cell viability was determined by

MTT assay after treatment with different concentrations of DEHP (0.25, 0.5, 1, 2, 4, 8, 10, 12, 14 and 16 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. The value of DMSO control was set to 100%. Values are mean  $\pm$  SD. The asterisks indicate a significant difference between DMSO control and DEHP-treated cells. \* $P < 0.05$  and \*\* $P < 0.01$  compared to controls

different doses of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. Figure 3 shows DEHP-induced apoptosis in 16HBE cells determined by Annexin V-FITC/PI staining assay. Compared to the controls, the proportion of normal living cells was significantly decreased ( $P < 0.05$ ) in DEHP-treated groups at all doses as shown in Fig. 3c. The proportions of apoptotic cells were significantly increased after DEHP exposure as shown in Fig. 3b, d ( $P < 0.05$ ).

DEHP decreased the levels of global DNA methylation and the expression levels of Dnmts in 16HBE cells

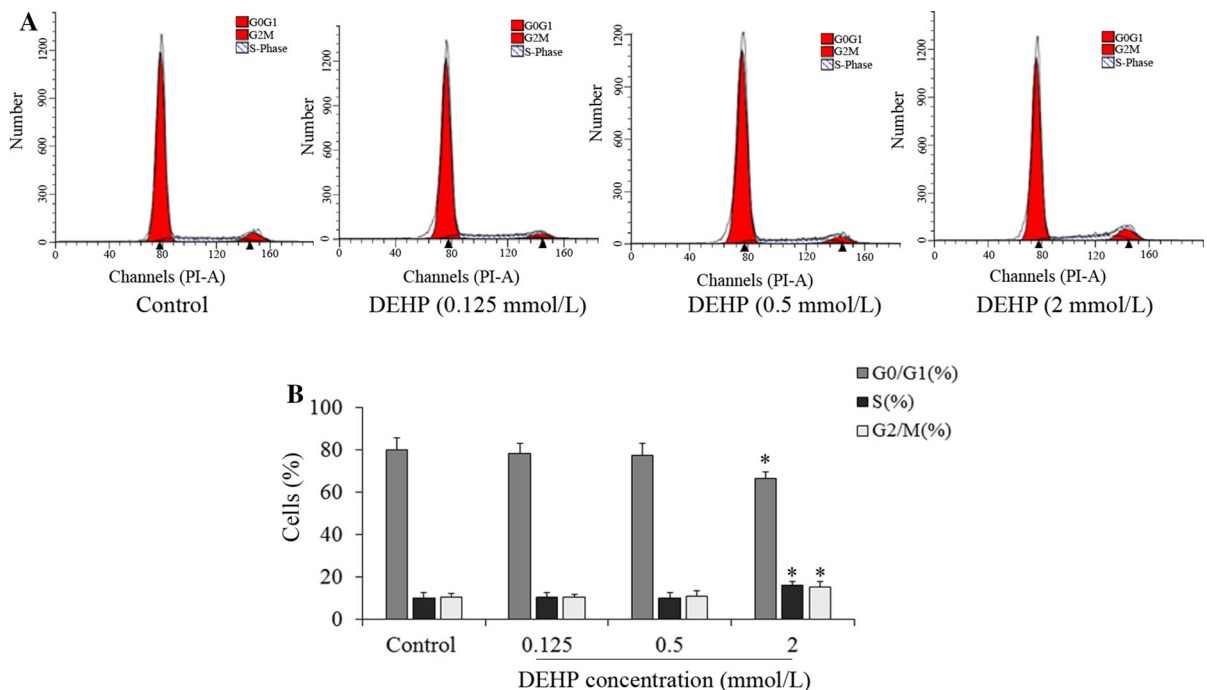
The degree of global DNA methylation of 16HBE cells was determined after treatment with different concentrations of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. As shown in Fig. 4a, the degree of global DNA methylation of 16HBE cells were slightly decreased after exposure to DEHP at 0.125 ( $P = 0.075$ ) and 0.5 mmol/L ( $P = 0.063$ ). The decrease was significant only at the concentration of

2 mmol/L compared to the control ( $P < 0.05$ ). The obtained results indicated that DEHP exposure could induce significant decrease of global DNA methylation in 16HBE cells.

To identify whether DNA methylation is affected by DEHP treatment, the mRNA expression levels of *Dnmt1*, *Dnmt3a* and *Dnmt3b* were determined by Q-PCR after 16HBE cells were treated with different doses of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. Results are shown in Fig. 4b. In the DEHP-treated groups, the expression levels of *Dnmt1* and *Dnmt3b* were decreased at all doses (0.125, 0.5 and 2 mmol/L,  $P < 0.05$ ). However, there was no significant change in the expression level of *Dnmt3a*. The results indicate that DEHP affects the expression level of DNMTs and influences the global DNA methylation of 16HBE cells.

## Discussion

DEHP as the most commonly used phthalate and PVC plasticizer has been shown to have effects on the



**Fig. 2** Effects of DEHP on cell cycle progression in 16HBE cells. The 16HBE cells were treated with different doses of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. **a, b** Show the cell cycle distribution examined by flow cytometry. Results were expressed as the percentage of cells in

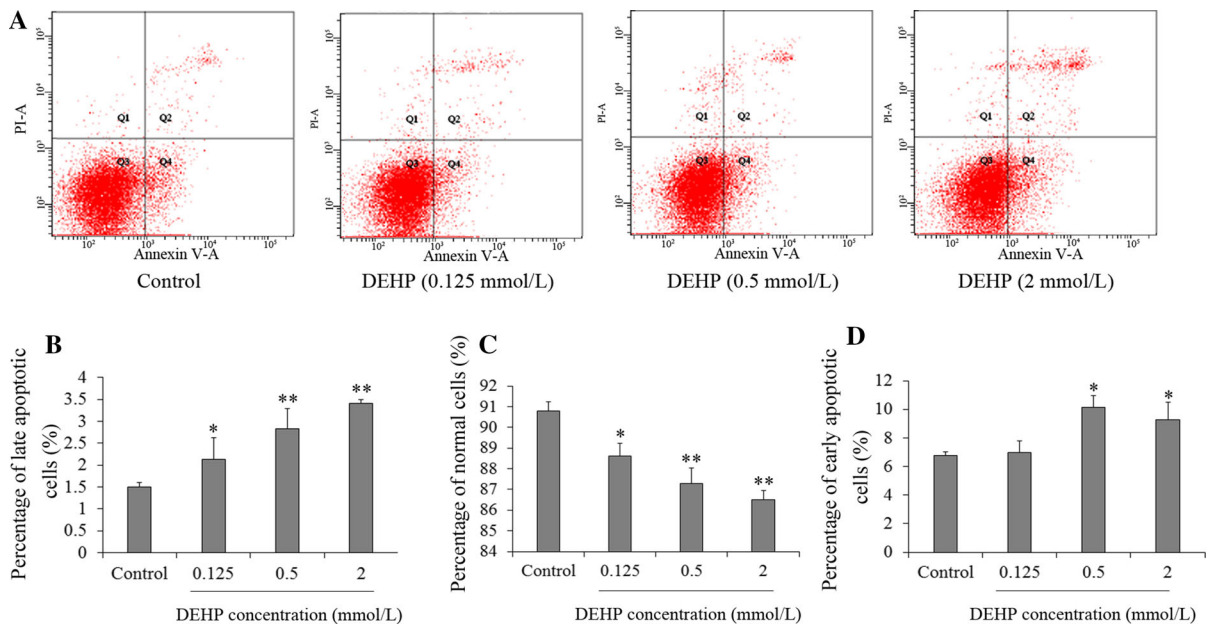
G0/G1, S and G2/M phase of the cell cycle. Values are presented as mean  $\pm$  SD of three independent experiments. The *asterisk* indicates a significant difference between DMSO control and DEHP-treated cells. \* $P < 0.05$  compared to controls

respiratory system via inhalation (Kolarik et al. 2008). Phthalates-induced respiratory diseases involve the interaction of respiratory tract epithelial cells (Glue et al. 2002; Jepsen et al. 2004). Whether DEHP exposure could cause toxic reactions in human bronchial epithelial 16HBE cells is still unexplored. The present study provides the first evidence that DEHP exposure can produce toxic effects in cultured 16HBE cells.

Decreased living cell number and cell viability were observed after treatment with DEHP at the dose of 2 mmol/L. However, the cell viability of 16 HBE cells was slightly increased in the 4 mmol/L ( $1.06 \pm 0.22$ ,  $P > 0.05$ ) and 8 mmol/L ( $1.10 \pm 0.13$ ,  $P > 0.05$ ) groups and decreased in the 10 mmol/L group ( $0.83 \pm 0.25$ ,  $P = 0.056$ ) compared to the control ( $1.03 \pm 0.06$ ). DEHP is an organic compound which barely dissolves in water. The solubility of DEHP in water is 0.00003% (23.8 °C). As the DEHP application solutions were obtained by diluting the DEHP stock solution (dissolved in DMSO) in the cell culture medium, the

solubility of DEHP might be decreased with the decreasing volume of DMSO and increasing volume of cell culture medium. At doses of 4–10 mmol/L, the DEHP in the cell culture medium might reach lowest dissolving capacity. Thus, the effects of DEHP on the cell viability of 16HBE cells came to a plateau with the increase of concentration.

To investigate the possible reasons for decreased living cell number and cell viability, the cell cycle of 16HBE cells was determined. From observations of cell cycle distribution, we found that DEHP (2 mmol/L) induced a decreased percentage of cells at G0/G1 phase and increased the percentage of cells at S and G2/M phases. The G1 phase, also called the growth phase is susceptible to external stimulus and is variable. The transition from G1 phase to S phase, also known as the restriction point is a rate-limiting step in the cell cycle (Robbins and Ramzi 2004). This transition is where the cell checks whether it has enough raw materials to fully replicate its DNA. Unhealthy or malnourished cells will get stuck at this checkpoint. The increase of S phase cells is maybe due



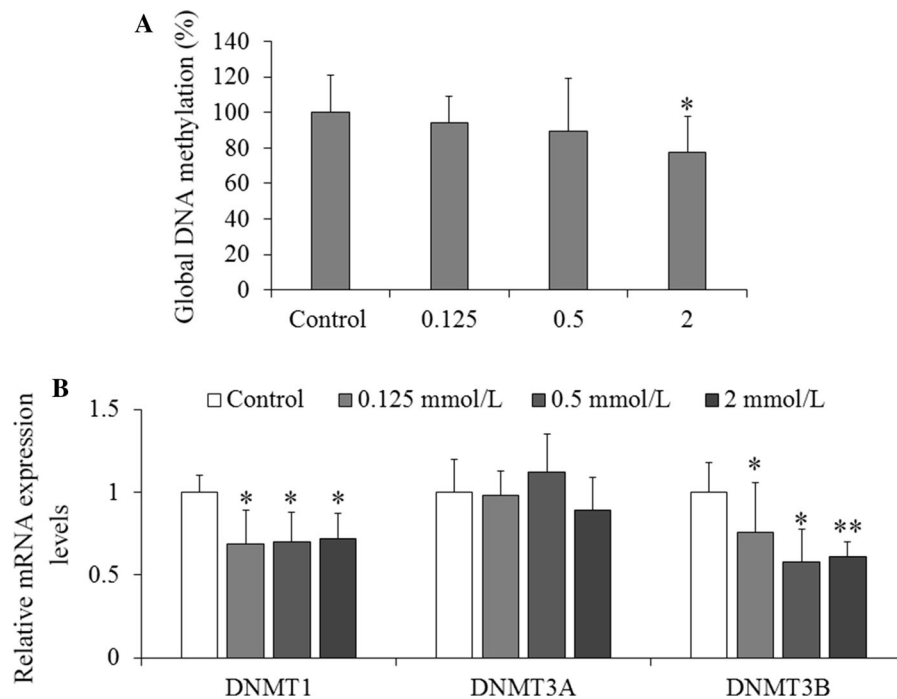
**Fig. 3** Effects of DEHP on cell apoptosis in 16HBE cells. 16HBE cells were treated with different doses of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. **a** Show the flow cytometric plots of normal cells and apoptotic cells stained by Annexin V-FITC/PI. Q1, necrotic cells; Q2, late apoptotic cells; Q3, normal cells; Q4 early apoptotic cells. **b–d**

Show the proportion of apoptotic cells and normal cells. Values are presented as mean  $\pm$  SD of three independent experiments. The *asterisks* indicate a significant difference between DMSO control and DEHP-treated cells. \* $P < 0.05$  and \*\* $P < 0.01$  compared to controls

to S phase arrest, acceleration of the transition from G1 to S phase or both of them (Zhao et al. 2008). This was the same as the G2/M phase. The increase of G2/M phase cells is maybe due to G2/M arrest and/or acceleration of the transition from S to G2 phase. Cell cycle checkpoints at G2/M is found critical in maintaining DNA integrity which is associated with cell proliferation and apoptosis (Pietenpol and Stewart 2002). Besides, by using parameter *Exit%* DES was proven to arrest cell cycle by accelerating the transition from G1 to S phase and arresting S and G2/M progression with increased S and G2/M phase in 16HBE cells (Zhao et al. 2013). Further study is required to determine the type of the disruption after DEHP exposure (cell cycle arrest or acceleration). Therefore, this alteration of cell cycle distribution indicates that the cell cycle progression of 16HBE cells is disrupted by DEHP. DEHP might decrease 16HBE cell proliferation by inhibiting cell cycle progression at the G1 phase.

Besides the inhibition of cell cycle progression, accelerated cell apoptosis is another factor decreasing living cell number and cell viability. Cell apoptosis plays an important role in the pathophysiology of

respiratory diseases and pulmonary inflammation (El Kebir et al. 2012; Pierce et al. 2007). Flow cytometry is the most common method used to verify cell apoptosis (Guo et al. 2015). Exploring whether DEHP leads to apoptosis in 16HBE cells could contribute to further understanding of DEHP-induced respiratory diseases. This study provides the first evidence that DEHP induces apoptosis in human bronchial epithelial 16HBE cells in vitro. The proportion of apoptotic cells (both late and early apoptotic) was increased after exposure to DEHP. Apoptosis is a stage-dependent process from its induction to early and late stage apoptosis. During the early stage apoptosis, cell shrinkage and pyknosis are visible by light microscopy. Phosphatidylserin (PS) which is cytosolic in normal cells translocates to the extracellular portion in early apoptotic cells. Annexin V can be used for PS detection. In addition, early stage of apoptotic cells can be rescued from the apoptotic program if the apoptotic stimulus is removed. Late apoptotic cells can be identified by detecting the DNA fragmentation. Due to the increase of cell membrane permeability, PI could penetrate the cell membrane and stain the nuclei at the late stage of apoptosis. Thus, a two color stain



**Fig. 4** Effects of DEHP on the global DNA methylation and mRNA expression levels of *Dnmt1*, *Dnmt3a* and *Dnmt3b* in 16HBE cells. 16HBE cells were treated with different doses of DEHP (0.125, 0.5 and 2 mmol/L) or vehicle (DMSO, 0.1%) for 48 h. **a** Show the global DNA methylation levels of 16HBE cells. **b** Show the mRNA expression levels of *Dnmt1*, *Dnmt3a*

and *Dnmt3b* in 16HBE cells. Values are presented as mean  $\pm$  SD of three independent experiments. The asterisks indicate a significant difference between DMSO control and DEHP-treated cells. \* $P < 0.05$  and \*\* $P < 0.01$  compared to controls

using Annexin V-FITC/PI can be used to distinguish and determine early and late apoptotic cells (Elmore 2007). Phthalates induced toxic effects in human bronchial epithelial cells and pulmonary cells have lately raised concerns. Monophthalates have been reported to possess adjuvant effects in THP-1 cells and peripheral blood mononuclear cells (Glue et al. 2002). Xenobiotic toxicants such as diethyl sulfate have been reported to induce 16HBE cell proliferation arrest via cell cycle disruption and cell apoptosis (Zhao et al. 2013). Manganese and manganese chloride caused pulmonary edema and impaired function and have also been found to be associated with activated apoptosis in 16HBE cells and a disturbed cell cycle in human lung carcinoma A549 cells (Zhang et al. 2013; Zhao et al. 2008).

Changes in global DNA methylation patterns have been shown to play a role in respiratory tract diseases (Qiu et al. 2012; Stefanowicz et al. 2012). Moreover, global DNA hypomethylation occurs early in tumorigenesis and is associated with transcriptional activation,

leading to chromosomal instability and aberrant expression of genes (Portela and Esteller 2010). However, there has been no research on the epigenetic toxicity of DEHP in 16HBE cells. In this study, we first discovered that the degree of global DNA methylation and the expression levels of *Dnmt1* and *Dnmt3b* were decreased in DEHP-treated groups. The methylation of mammalian genomic DNA is catalyzed by DNA methyltransferases, which encompasses DNMT1, DNMT3a and DNMT3b. DNMT1 has maintenance as well as de novo methyltransferase activity (Jiang et al. 2008). Evidence indicates that DNMT3B could methylate hemimethylated and unmethylated CpG sites and also possess maintenance functions (Chen et al. 2003). Furthermore, DNMT3b may contribute to the global hypomethylation of the tumor. Therefore, the decreased expression levels of *Dnmt1* and *Dnmt3b* suggest that DEHP might influence the DNA methylation patterns of 16HBE cells. The global DNA methylation of 16HBE cells was determined by calculating the amount and percentage of methylated DNA (5-mC) in total



DNA using a colorimetric assay. As reported, DEHP could also induce hypermethylation in promoter regions of genes which were associated with modified *Dnmt1* and *Dnmt3b* expression levels (Sekaran and Jagadeesan 2015). Thus we speculated that, the decreasing but not statistically significant trends in global DNA methylation may be due to the potential hypermethylation in genes promoter regions induced by DEHP exposure and down-regulated expression of *Dnmt1* and *Dnmt3b*. In addition, the reduction of *Dnmt1* and *Dnmt3b* expression were triggered by DEHP exposure at the first time. Inactivation of DNA methyltransferases has been shown to cause global demethylation (Bestor 2000). The reduction in global DNA methylation is probably to be functionally linked to the decrease in *Dnmt1* and *Dnmt3b* expression. Moreover, decreased in *Dnmt1* and *Dnmt3b* expression may cause demethylation in specific areas of the genome which may not be detectable by a global measurement of DNA methylation. This may explain why *Dnmt1* and *Dnmt3b* expression appears to be more sensitive to DEHP exposure compared to global methylation measurements. It is also possible that the time of exposure used in our study was not sufficient for detectable global DNA hypomethylation at exposure to relatively low doses of DEHP. Indeed, similar results are reported in a study which indicates BPA exposure decreases *Dnmt1* transcription at doses of 0.01, 0.1 and 1 mg/L and reduces global DNA methylation at doses of 1 mg/L in zebrafish ovaries (Laing et al. 2016). In summary, DNMT1, DNMT3a, DNMT3b and MBD2 play important roles in epigenetic changes in 16HBE cells induced by DEHP exposure.

Evidence for the toxicity effects induced by DEHP exposure is provided in this study. The results suggest that DEHP inhibits cell proliferation and induces apoptosis in 16HBE cells resulting in a decreased living cell number and decreased cell viability. Furthermore, DEHP was found to have epigenetic toxicity. Concerns about possible epigenetic mechanisms should be raised associated with DEHP exposure in respiratory tissues or cells.

## Conclusions

In this study, we have demonstrated that DEHP inhibited proliferation and caused decreased living cell number and decreased cell viability in 16HBE

cells. Moreover, the present study indicated that DEHP induced apoptosis in 16HBE cells. And decreased global DNA methylation and expression levels of *Dnmt1* and *Dnmt3b* after treatment with DEHP provided that DEHP had epigenetic toxicity in 16HBE cells.

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## Compliance with ethical standards

**Conflict of interest** The authors have no conflict of interest.

## References

- Bestor TH (2000) The DNA methyltransferases of mammals. *Hum Mol Genet* 9:2395–2402
- Bornehag CG, Sundell J, Weschler CJ, Sigsgaard T, Lundgren B, Hasselgren M, Hagerhed-Engman L (2004) The association between asthma and allergic symptoms in children and phthalates in house dust: a nested case-control study. *Environ Health Perspect* 112:1393–1397
- Cadogan DF, Howick CJ (1996) Encyclopedia of chemical technology, vol. In: Othmer K (ed) Plasticizers. Wiley, New York
- Chen T, Ueda Y, Dodge JE, Wang Z, Li E (2003) Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol Cell Biol* 23:5594–5605
- El Kebir D, Gjorstrup P, Filep JG (2012) Resolvin E1 promotes phagocytosis-induced neutrophil apoptosis and accelerates resolution of pulmonary inflammation. *Proc Natl Acad Sci USA* 109:14983–14988. doi:10.1073/pnas.1206641109
- Elmore S (2007) Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35:495–516. doi:10.1080/01926230701320337
- Glue C, Millner A, Bodtger U, Jinquan T, Poulsen LK (2002) In vitro effects of monophthalates on cytokine expression in the monocytic cell line THP-1 and in peripheral blood mononuclear cells from allergic and non-allergic donors. *Toxicol in vitro: an international journal published in association with BIBRA* 16:657–662
- Green R, Hauser R, Calafat AM, Weuve J, Schettler T, Ringer S, Huttner K, Hu H (2005) Use of di(2-ethylhexyl) phthalate-containing medical products and urinary levels of mono(2-ethylhexyl) phthalate in neonatal intensive care unit infants. *Environ Health Perspect* 113:1222–1225
- Guo Y, Ji J, Wang W, Dong Y, Zhang Z, Zhou Y, Chen G, Cheng J (2015) Role of endoplasmic reticulum apoptotic pathway in testicular Sertoli cells injury induced by carbon disulfide. *Chemosphere* 132:70–78. doi:10.1016/j.chemosphere.2015.02.058

- Hoppin JA, Ulmer R, London SJ (2004) Phthalate exposure and pulmonary function. *Environ Health Perspect* 112:571–574
- Hoppin JA, Jaramillo R, London SJ, Bertelsen RJ, Salo PM, Sandler DP, Zeldin DC (2013) Phthalate exposure and allergy in the U.S. population: results from NHANES 2005–2006. *Environ Health Perspect* 121:1129–1134. doi:[10.1289/ehp.1206211](https://doi.org/10.1289/ehp.1206211)
- Jaakkola JJ, Verkasalo PK, Jaakkola N (2000) Plastic wall materials in the home and respiratory health in young children. *Am J Public Health* 90:797–799
- Jepsen KF, Abildtrup A, Larsen ST (2004) Monophthalates promote IL-6 and IL-8 production in the human epithelial cell line A549. *Toxicology in vitro: an international journal published in association with BIBRA* 18:265–269. doi:[10.1016/j.tiv.2003.09.008](https://doi.org/10.1016/j.tiv.2003.09.008)
- Jiang G, Xu L, Song S, Zhu C, Wu Q, Zhang L, Wu L (2008) Effects of long-term low-dose cadmium exposure on genomic DNA methylation in human embryo lung fibroblast cells. *Toxicology* 244:49–55. doi:[10.1016/j.tox.2007.10.028](https://doi.org/10.1016/j.tox.2007.10.028)
- Kolarik B, Naydenov K, Larsson M, Bornehag CG, Sundell J (2008) The association between phthalates in dust and allergic diseases among Bulgarian children. *Environ Health Perspect* 116:98–103. doi:[10.1289/ehp.10498](https://doi.org/10.1289/ehp.10498)
- Laing LV, Viana J, Dempster EL, Trznadel M, Trunkfield LA, Uren Webster TM, van Aerle R, Paull GC, Wilson RJ, Mill J, Santos EM (2016) Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription, and reduces global DNA methylation in breeding zebrafish (*Danio rerio*). *Epigenetics* 11:526–538. doi:[10.1080/15592294.2016.1182272](https://doi.org/10.1080/15592294.2016.1182272)
- Nicodemus-Johnson J, Naughton KA, Sudi J, Hogarth K, Nau-rekas ET, Nicolae DL, Sperling AI, Solway J, White SR, Ober C (2016) Genome-wide methylation study identifies an IL-13-induced epigenetic signature in asthmatic airways. *Am J Respir Crit Care Med* 193:376–385. doi:[10.1164/rccm.201506-1243OC](https://doi.org/10.1164/rccm.201506-1243OC)
- Pierce JD, Pierce J, Stremming S, Fakhari M, Clancy RL (2007) The role of apoptosis in respiratory diseases. *Clin Nurse Spec CNS* 21:22–28 **quiz 29–30**
- Pietenpol JA, Stewart ZA (2002) Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. *Toxicology* 181–182:475–481
- Portela A, Esteller M (2010) Epigenetic modifications and human disease *Nature biotechnology*. *Nat Biotech* 28:1057–1068. doi:[10.1038/mbt.1685](https://doi.org/10.1038/mbt.1685)
- Qiu W, Baccarelli A, Carey VJ, Boutaoui N, Bacherman H, Klanderman B, Rennard S, Agusti A, Anderson W, Lomas DA, DeMeo DL (2012) Variable DNA methylation is associated with chronic obstructive pulmonary disease and lung function. *Am J Respir Crit Care Med* 185:373–381. doi:[10.1164/rccm.201108-1382OC](https://doi.org/10.1164/rccm.201108-1382OC)
- Robbins SLC, Ramzi S (2004) *Pathological basis of disease*. Saunders, Philadelphia
- Rosicarelli B, Stefanini S (2009) DEHP effects on histology and cell proliferation in lung of newborn rats. *Histochem Cell Biol* 131:491–500. doi:[10.1007/s00418-008-0550-4](https://doi.org/10.1007/s00418-008-0550-4)
- Sekaran S, Jagadeesan A (2015) In utero exposure to phthalate downregulates critical genes in Leydig cells of F1 male progeny. *J Cell Biochem* 116:1466–1477. doi:[10.1002/jcb.25108](https://doi.org/10.1002/jcb.25108)
- Stefanowicz D, Hackett TL, Garmaroudi FS, Günther OP, Neumann S, Sutanto EN, Ling KM, Kobor MS, Kicic A, Stick SM, Paré PD, Knight DA (2012) DNA methylation profiles of airway epithelial cells and PBMCs from healthy, atopic and asthmatic children. *PLoS ONE* 7:e44213. doi:[10.1371/journal.pone.0044213](https://doi.org/10.1371/journal.pone.0044213)
- Tuomainen A, Stark H, Seuri M, Hirvonen MR, Linnainmaa M, Sieppi A, Tukiainen H (2006) Experimental PVC material challenge in subjects with occupational PVC exposure. *Environ Health Perspect* 114:1409–1413
- Twentyman PR, Luscombe M (1987) A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer* 56:279–285
- Wormuth M, Scheringer M, Vollenweider M, Hungerbühler K (2006) What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? *Risk Anal* 26:803–824. doi:[10.1111/j.1539-6924.2006.00770.x](https://doi.org/10.1111/j.1539-6924.2006.00770.x)
- Zhang L, Sang H, Liu Y, Li J (2013) Manganese activates caspase-9-dependent apoptosis in human bronchial epithelial cells. *Hum Exp Toxicol* 32:1155–1163. doi:[10.1177/0960327112470272](https://doi.org/10.1177/0960327112470272)
- Zhao P, Zhong W, Ying X, Yuan Z, Fu J, Zhou Z (2008) Manganese chloride-induced G0/G1 and S phase arrest in A549 cells. *Toxicology* 250:39–46. doi:[10.1016/j.tox.2008.05.016](https://doi.org/10.1016/j.tox.2008.05.016)
- Zhao P, Fu J, Yao B, Hu E, Song Y, Mi L, Li Z, Zhang H, Jia Y, Ma S, Chen W, Zhou Z (2013) Diethyl sulfate-induced cell cycle arrest and apoptosis in human bronchial epithelial 16HBE cells. *Chem Biol Interact* 205:81–89. doi:[10.1016/j.cbi.2013.06.014](https://doi.org/10.1016/j.cbi.2013.06.014)