

# Analysis of the *in vitro* effects of di-(2-ethylhexyl) phthalate exposure on human uterine leiomyoma cells

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**Abstract.** Uterine leiomyoma is the most common benign tumor type of the female reproductive tract. Despite its high prevalence, the exact pathogenesis of the benign tumor remains unknown. In the present study, the effects of di-(2-ethylhexyl) phthalate (DEHP) on the proliferation and apoptosis rates and expression of inflammatory proteins in human leiomyoma cells were evaluated. The effects of DEHP on cell viability were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The effects on apoptosis were evaluated by western blotting, TUNEL assay and Annexin V staining. Western blotting was also performed to evaluate the expression of inflammatory proteins. It was observed that DEHP-treated leiomyoma cells had higher viability, as well as proliferating cell nuclear antigen and B-cell lymphoma 2 protein expression, and lower apoptosis rates compared with the untreated controls. Additionally, hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and cyclooxygenase-2 (COX-2) expression increased in human leiomyoma cells following DEHP treatment. In conclusion, DEHP promoted cell viability and anti-apoptotic protein expression and induced HIF-1 $\alpha$  and COX-2 expression in human leiomyoma cells. These results suggested that DEHP may disrupt mechanisms underlying various processes in human leiomyoma cells. Furthermore, the current study revealed a basic mechanism of action of DEHP in human leiomyoma cells. Further research on the effects of various endocrine disruptors on the pathogenesis of uterine leiomyoma during early development may reveal strategies to prevent this disease.

## Introduction

Uterine leiomyoma, the most common benign tumor type of the female reproductive tract, has a reported prevalence of

20-50% in women of reproductive age (1,2). It is associated with high rates of gynecological morbidity, including dysmenorrhea, infertility, menometrorrhagia and pelvic pain (3). Numerous factors are likely to influence fibroid growth, and uterine muscle cells may be particularly vulnerable to growth abnormalities. In premenopausal women, the uterus is regulated in part by estrogen, a hormone broadly associated with cell growth (4,5). Uterine leiomyoma is common; >80% of African American females and 70% of white females develop uterine fibroids detectable on ultrasound in the USA (6). The risk of uterine leiomyoma is influenced by age, ethnicity, caffeine intake, number of pregnancies, endogenous hormone levels, obesity and genetic factors (7).

Environmental endocrine disruptors comprise of numerous natural and synthetic compounds that have the potential to interfere with the normal endocrine system of animals, including humans; they include environmental estrogens, which mimic the action of natural estrogen in the body (8). Uterine leiomyoma is a gynecological disease characterized by estrogen dependence (9); however, the association between exposure to environmental endocrine disruptors and the disease remains unclear.

Di-(2-ethylhexyl) phthalate (DEHP) is the most common environmental endocrine disruptor (10-12). DEHP is widely used in consumer products, including food packaging, medical devices and toys, to improve the flexibility and durability of polyvinyl chloride-based plastics. DEHP is not covalently bound to the plastic matrix and maybe released from its substrate into the environment (13). As a widespread environmental pollutant and an endocrine disruptor, DEHP is a serious concern due to its potential toxic effects, including reproductive toxicity (14), neurotoxicity (15) and carcinogenicity (16). Recent epidemiological evidence suggests that women have an increased exposure profile to phthalates compared with men, as they are present in a number of beauty products, including skin lotions, perfumes and nail products, which raises concerns about their potential health hazards (17,18). In animals, the reproductive and developmental toxicities are exerted through similar mechanisms in the two sexes, but the toxicity seems to occur at an older age in females compared with males (18).

Hypoxia inducible factor-1 (HIF-1) is a heterodimeric transcription factor comprising a basic helix-loop-helix/PAS domain, and includes the subunits HIF-1 $\alpha$  and aryl hydrocarbon receptor nuclear translocator (also known as HIF-1 $\beta$ ) (19).

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The availability of HIF-1 is determined primarily by HIF-1 $\alpha$ , which is regulated at the protein level in an oxygen-sensitive manner (20). HIF-1 $\alpha$  has been demonstrated to mediate angiogenesis, cell proliferation, apoptosis and migration (21). By contrast, HIF-1 $\beta$  is stably expressed (20). HIF-1 has been reported to enhance cyclooxygenase-2 (COX-2) expression by interacting with functional hypoxia response elements in the COX-2 promoter region (22). COX proteins (COX-1 and COX-2) catalyze the synthesis of prostaglandins from arachidonic acid. While COX-1 is expressed constitutively in most of the tissues and appears to be responsible for housekeeping functions, COX-2 is transcriptionally induced by pro-inflammatory stimuli (23). COX-2 is upregulated in numerous types of malignancy (24,25) and favors malignant growth by stimulating proliferation and angiogenesis (26) via multiple pathways, including the mitogen-activated protein kinase and NF- $\kappa$ B pathways, in different cell types (27).

To the best of our knowledge, the mechanisms underlying DEHP action in human leiomyoma cells have not been studied previously. In the present study, the effect of phthalate exposure on the pathogenesis of uterine leiomyoma was investigated. It was identified that DEHP enhances proliferative activity and blocks apoptosis of leiomyoma cells, and induces the expression of HIF-1 $\alpha$  and COX-2.

## Materials and methods

**Chemicals.** DEHP was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

**Human uterine leiomyoma cells.** Human uterine leiomyoma cells (GM10964) were purchased from the Coriell Institute for Medical Research (Camden, NJ, USA) and maintained in minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with MEM vitamins solution (100X; cat. no. 1112005), MEM amino acids (50X; cat. no. 11130036), MEM non-essential amino acids (100X; cat. no. 11140050) and L-glutamine (2 mM; all Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 95% humidity with 5% carbon dioxide, as described previously (28).

**Cell viability assay.** Cell viability was analyzed using a MTS assay (CellTiter 96<sup>®</sup>Aqueous Cell Proliferation Assay kit; Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

Briefly, the cells were cultured for 24 and 48 h and 20  $\mu$ l/well MTS solution was added to the samples in 100  $\mu$ l culture medium. The cells were subsequently incubated at 37°C for 4 h and the absorbance was measured using a microplate reader at 490 nm.

**Concentration of DEHP.** In previous case-control studies, serum concentrations of DEHP were reported to range from 1.5 to 6.2  $\mu$ M (29,30). The concentrations of DEHP used in the present study were determined using a concentration-response curve. Briefly, leiomyoma cells were exposed for 48 h to increasing concentrations of DEHP (from 0.0 to 6.0  $\mu$ M). After 48 h, cell survival was analyzed by MTT assay according to the aforementioned method (data not shown). Concentrations of DEHP >1.5  $\mu$ M significantly decreased the percentage of

live cells in the MTT assay. Therefore, for the majority of experiments, concentrations of DEHP ranging from 0.0 to 1.0  $\mu$ M were selected. Concentrations of 0.01 and 1  $\mu$ M DEHP were regarded as 'low' and 'high' levels of DEHP exposure, respectively.

**TUNEL assay.** In order to confirm apoptosis by identifying apoptotic bodies in human uterine leiomyoma cells, a TUNEL assay was performed using an *in situ* cell death detection kit (cat. no. 1684795; Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells (5x10<sup>4</sup>/per well) were fixed using 4% formaldehyde for 40 min at room temperature. This was followed by multiple rinses in PBS and permeabilization in 0.2% Triton X-100 solution on ice for 5 min. Subsequently, 50  $\mu$ l TUNEL reaction mixture was added on coverslips before being incubated for 60 min at 37°C in a dark, humidified chamber. Finally, the coverslips were incubated with 4',6-diamidino-2-phenylindole (DAPI, 2  $\mu$ g/ml; Sigma-Aldrich; Merck KGaA) for 20 min at room temperature and mounted with VECTASHIELD Antifade Mounting medium (cat. no. H-1000; Vector Laboratories Ltd., Peterborough, UK). The coverslips were examined with an LSM 510 confocal microscope (Zeiss GmbH, Jena, Germany) and counted in three fields of view. Data were expressed as the ratio of TUNEL-positive cells to total nuclei.

**Annexin V staining and flow cytometry.** To determine the apoptosis rate, cells were incubated in culture medium containing 0, 0.1 and 1  $\mu$ M DEHP for 48 h and stained with Annexin V-fluorescein isothiocyanate (FITC), according to the manufacturer's protocol (Molecular Probes; Thermo Fisher Scientific, Inc.). Approximately 1x10<sup>5</sup> cells were harvested and washed with phosphate-buffered saline. Cells were then resuspended in 100  $\mu$ l Annexin V binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl<sub>2</sub>, pH 7.4), incubated with 5  $\mu$ l of Annexin V-FITC for 15 min at room temperature, and counterstained with propidium iodide (PI; final concentration, 1  $\mu$ g/ml) for 10 min at room temperature. Following the incubation period, the cells were diluted with 190  $\mu$ l Annexin V binding buffer. Cells were analyzed by flow cytometry using a Becton-Dickinson FACScan flow cytometer with Cell Quest 3.1 software (BD Biosciences, Franklin Lakes, NJ, USA).

**Western blot analysis.** The cells (1x10<sup>6</sup>/per ml) were resuspended in a radioimmunoprecipitation buffer (50 mM Tris; pH 8.0; Cell Signaling Technology, Inc., Danvers, MA, USA) containing a protease inhibitor cocktail (cOmplete<sup>™</sup> Mini Protease Inhibitor Tablet; Roche Diagnostics GmbH). The protein concentration was measured in the supernatant using a Pierce BCA Protein Assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). A total of 40  $\mu$ g of protein was loaded per lane, separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (cat. no. LC2009; Thermo Fisher Scientific, Inc.). Following transfer the membranes were blocked for 2 h at room temperature with 5% skimmed milk in Tris buffered saline-Tween-20 (TBST; 20 mM Tris, 500 nM NaCl, 0.1% Tween-20; pH 7.5). The membranes were incubated with primary antibodies against proliferating cell nuclear antigen (PCNA; cat. no. 13110; 1:1,000), B-cell lymphoma 2 (Bcl-2; cat. no. 2872; 1:1000), HIF-1 $\alpha$  (cat. no. 14179; 1:1,000), COX-2 (cat. no. 12282; 1:1,000)

or  $\beta$ -actin (cat. no. 4970; 1:5,000; all Cell Signaling Technology, Inc.) at 4°C. Following three washes with TBST, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-IgG antibodies (cat. no. 65-6120; 1:5,000; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at room temperature and visualized using a Pierce enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.). Densitometric quantification of the protein density bands was achieved using ImageJ software (version 1.29x, National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** The Kolmogorov-Smirnov test was performed to evaluate whether data were normally distributed. If this was the case, continuous variables were compared using two-sample Student's t-tests or, with three groups, analysis of variance followed by Fisher's least significant difference post-hoc test for pairwise comparisons. If data were not normally distributed, the variables were compared using the Mann-Whitney U test or the Kruskal-Wallis test followed by the Mann-Whitney U test with Bonferroni correction, depending on whether two or three groups were considered, respectively. Statistical analysis was performed on SPSS 14.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean  $\pm$  standard deviation of separate experiments (n=3). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Viability of human leiomyoma cells following DEHP exposure.** To assess the influence of DEHP exposure on cell viability, an MTT assay was conducted using leiomyoma cells treated for 24 or 48 h with DEHP (0, 0.01 and 1  $\mu$ M). Exposure of leiomyoma cells to 0.01 or 1  $\mu$ M DEHP for 24 and 48 h led to higher viability compared with the untreated controls. Viability of leiomyoma cells was significantly higher after 24 h exposure to 1  $\mu$ M DEHP (P<0.05) and 48 h exposure to 0.01 or 1  $\mu$ M DEHP (both P<0.05) compared with control cells (Fig. 1). To further examine the effects of DEHP on cell proliferation, PCNA protein levels were determined by western blot analysis. PCNA expression was significantly higher in leiomyoma cells following treatment with 1  $\mu$ M DEHP for 24 h (P<0.05) and 0.01 and 1  $\mu$ M DEHP for 48 h (both P<0.05) compared with control cells (Fig. 2).

**Effects of DEHP on the apoptosis of human leiomyoma cells.** To examine if the increased viability could be attributed to an anti-apoptotic pathway, Bcl-2 expression levels were determined by western blotting. Bcl-2 expression was significantly higher in leiomyoma cells after 24 h exposure to 1  $\mu$ M DEHP (P<0.05) and 48 h exposure to 0.01 or 1  $\mu$ M DEHP (both P<0.05), as compared with control cells (Fig. 3A).

To assess if DEHP caused apoptosis, apoptotic cells were detected by TUNEL assay. The rate of apoptosis was assessed in cells treated with 0, 0.01 and 1  $\mu$ M DEHP for 48 h by confocal microscopy. The number of apoptotic cells was significantly lower after 48 h exposure to 0.01 or 1  $\mu$ M DEHP compared with the control group (both P<0.05; Fig. 3B).

To assess if DEHP had an anti-apoptotic effect, the percentage of apoptotic cells was determined by Annexin V and

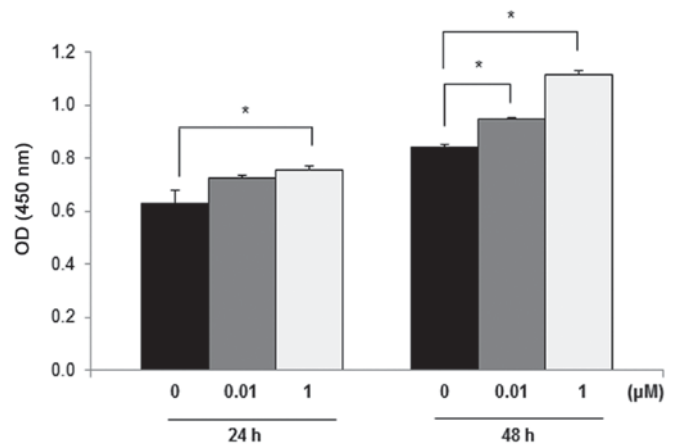


Figure 1. Viability of human leiomyoma cells following exposure to DEHP. Cells were exposed to 0, 0.01 or 1  $\mu$ M DEHP for 24 or 48 h. Data are presented as the mean  $\pm$  standard deviation. \*P<0.05. DEHP, di-(2-ethylhexyl) phthalate; OD, optical density.

PI staining. The rate of apoptosis was assessed in leiomyoma cells treated with 0, 0.01 and 1  $\mu$ M DEHP for 48 h by flow cytometry analysis. The rate of late apoptosis in the control group was 16.4 $\pm$ 4.7%, indicating that apoptosis was the primary cell death mechanism in leiomyoma cells in the control group. Compared with the control group, the apoptosis rate of leiomyoma cells was significantly inhibited when exposed to 0.1  $\mu$ M DEHP (rate of late apoptosis, 7.2 $\pm$ 3.1%; P<0.05) or 1  $\mu$ M DEHP (rate of late apoptosis, 4.6 $\pm$ 0.7%; P<0.05; Fig. 3C).

**Effects of DEHP on HIF-1 $\alpha$  and COX-2 expression in human leiomyoma cells.** Western blot analysis was performed to determine the effects of DEHP exposure on HIF-1 $\alpha$  and COX-2 expression. In leiomyoma cells, HIF-1 $\alpha$  and COX-2 expression was significantly increased following exposure to 1  $\mu$ M DEHP for 48 h compared with control cells (P<0.05). However, HIF-1 $\alpha$  and COX-2 expression did not significantly increase following exposure to DEHP for 24 h compared with control cells (Fig. 4).

## Discussion

The present study demonstrated that *in vitro* DEHP treatment leads to increased viability, proliferation and anti-apoptotic protein expression in human leiomyoma cells. Furthermore, HIF-1 $\alpha$  and COX-2 expression following DEHP treatment was higher in human leiomyoma cells compared with control cells. These *in vitro* results suggest that exposure to phthalates may serve a function in the pathogenesis of uterine leiomyoma.

DEHP is the most common plasticizer in polyvinyl chloride-containing plastics. Furthermore, DEHP is an endocrine disruptor that is able to alter sexual differentiation and energy metabolism (10-12). Given its chemical structure, DEHP easily enters food, air and even the human body. Approximately 7.3% of mono-ethylhexyl phthalate and 66.9% of oxidative metabolites are excreted in urine by the human body; however, 25.8% of DEHP bioaccumulates in the body (31). In addition, the direct effects of DEHP are not well-characterized. The toxicity potential of DEHP remains controversial. The presence of DEHP has been reported in various human cell types (32-35). DEHP could promote proliferation of breast cancer cells and

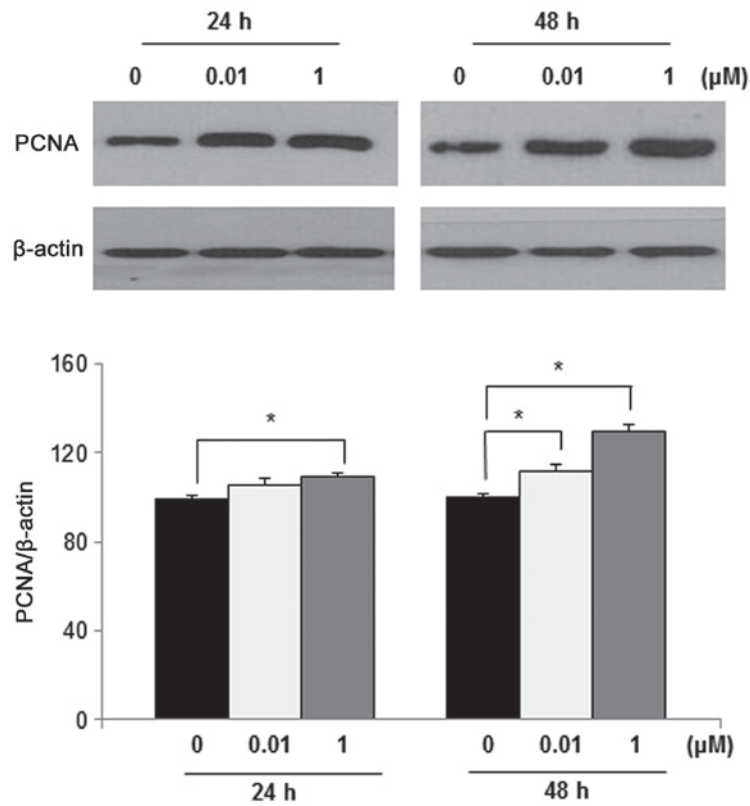


Figure 2. Effects of *in vitro* DEHP treatment on proliferation of human leiomyoma cells. PCNA expression levels in human leiomyoma cells were measured by western blot analysis. Western blot data are expressed as percentages, with cells treated with vehicle normalized to 100%. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ . DEHP, di-(2-ethylhexyl) phthalate; PCNA, proliferating cell nuclear antigen.

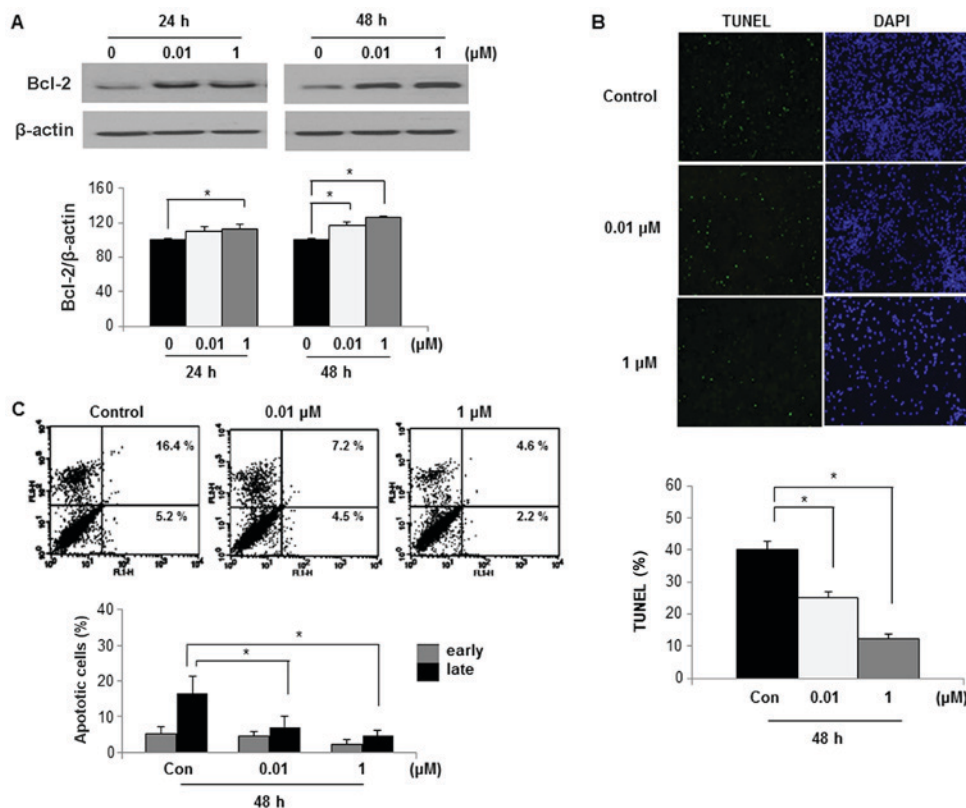


Figure 3. Effects of *in vitro* DEHP treatment on anti-apoptotic and apoptotic protein expression in human leiomyoma cells. (A) Bcl-2 expression levels in human leiomyoma cells were measured by western blot analysis. Western blot data are expressed as percentages, with cells treated with vehicle normalized to 100%. (B) Detection of apoptosis in human leiomyoma cells by TUNEL assay (magnification, x200). Histograms present the average number of TUNEL-positive cells as a percentage for each group. (C) Annexin V flow cytometry assay was performed to visualize the extent of programmed cell death in the control group and groups treated with DEHP. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ . DEHP, di-(2-ethylhexyl) phthalate; Bcl-2, B-cell lymphoma 2.

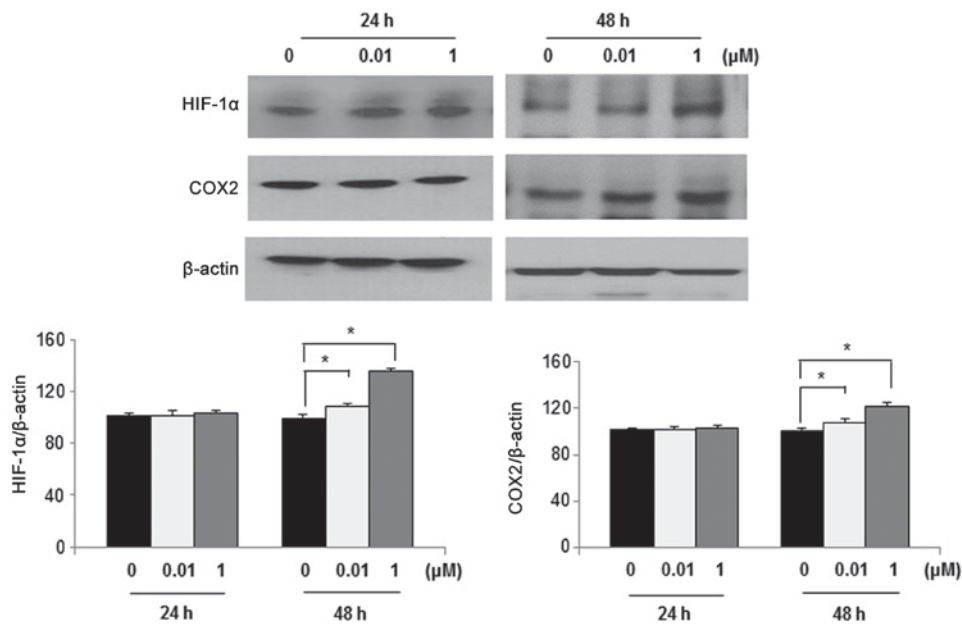


Figure 4. Effects of *in vitro* DEHP treatment on inflammation in human leiomyoma cells. HIF-1 $\alpha$  and COX-2 expression levels in human leiomyoma cells were measured by western blot analysis. Western blot data are expressed as percentages, with cells treated with vehicle normalized to 100%. Data are presented as the mean  $\pm$  standard deviation. \*P<0.05. DEHP, di-(2-ethylhexyl) phthalate; HIF-1 $\alpha$ , hypoxia inducible factor 1 $\alpha$ ; COX-2, cyclooxygenase-2.

induce necrosis of keratinocytes (32,33). DEHP exposure was observed to promote invasion of neuroblastoma cells and increase the growth rate of hepatic carcinoma cells (34,35). Furthermore, phthalates enhance pro-inflammatory cytokine production *in vitro* (36) and may contribute to pro-inflammatory processes, with the potential to interact with other risk factors, as revealed in clinical case reports (37,38). Given that uterine leiomyoma is the most common gynecological disease, it is necessary to determine whether exposure to phthalate is associated with leiomyoma risk. A previous study suggested a possible association of increased urinary level of phthalate metabolites with the risk of uterine leiomyoma (39). However, to the best of our knowledge, no study on the effects of phthalate on human leiomyoma cells has been reported.

In the present study, it was identified that *in vitro* DEHP treatment leads to increased viability and elevated PCNA and Bcl-2 expression in leiomyoma cells. PCNA localizes in the nucleus of proliferating cells (40) and serves as a cofactor for DNA replication and repair, and cell cycle regulation (41,42). Bcl-2 family members are important regulators of programmed cell death and act as inhibitors of apoptosis (43-45). These findings suggest that DEHP may cause an imbalance in cellular proliferation and apoptosis, which drives the pathogenesis of uterine leiomyoma (46-48).

Furthermore, the present study revealed that HIF-1 $\alpha$  and COX-2 expression following DEHP treatment may elicit inflammation. The current results indicated that DEHP exposure increased the expression levels of HIF-1 $\alpha$  and COX-2 after 48 h. These findings suggest that chronic continuous exposure to DEHP may be a critical factor for leiomyoma cells to experience persistently enhanced inflammation. The results of this study indicate that continual exposure to DEHP may induce HIF-1 $\alpha$  and COX-2 expression and may enhance proliferation ability, which is consistent with previous similar studies (49,50). Elevated expression of inflammatory proteins

could elicit various effects, including the promotion of tumor progression, by inducing proliferation and resistance to apoptosis (51). HIF-1 $\alpha$  and COX-2 expression was indicated to be increased by DEHP treatment, suggesting that DEHP may serve a critical function in inflammation of human leiomyoma cells. Since the effects of DEHP following inhibition of inflammation are not yet known, these effects of DEHP on human leiomyoma cells should be studied in the future.

In conclusion, DEHP promoted cellular viability and anti-apoptotic protein expression and induced HIF-1 $\alpha$  and COX-2 expression in human leiomyoma cells. These results suggest that DEHP may disrupt mechanisms underlying various processes in human leiomyoma cells. Furthermore, the present study reveals a basic mechanism of action of DEHP in human leiomyoma cells. Further research on the effects of various endocrine disruptors on the pathogenesis of uterine leiomyoma during early development may reveal strategies to prevent this disease. Further *in vivo* study will be necessary to confirm these findings, since the current results were based solely on an *in vitro* model.

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#### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

**Author's contributions**

JHK conceived and designed the experiments, performed the experiments, analyzed the data and wrote the paper.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The author declares that they have no competing interests.

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