

# Morphological Alterations in Human Skeletal Muscle Cells Exposed to di-(2-Ethylhexyl) Phthalate (dehp) in Primary Cell Culture Conditions

Alteraciones Morfológicas en Células Musculares Esqueléticas Humanas Expuestas a Ftalato de di-(2-etilhexilo) (dehp) en Condiciones de Cultivo Celular Primario

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**SUMMARY:** Di-(2-ethylhexyl) phthalate (DEHP) is among the most common plasticizer additives that humans are in contact with daily. DEHP can be released from plastic and enter the human body, whereby it is metabolized and transformed into oxidative hydrophilic molecules. Clinical follow-ups in patients exposed to this phthalate and investigations in cultures of several cell types have provided information on its effects. For example, it is associated with inhibition of diploid human cell development and morphological changes in cultured germ cells. Although skeletal muscle represents around 50 % of the human body mass, knowledge about the effects of DEHP on this tissue is poor. Cultured skeletal muscle cells were exposed to DEHP (1 mM) for 13 days with the aim of exploring and evaluating some of the potential morphological effects. Three culture development parameters and nine cell characteristics were monitored during the bioassay. At 13 days, growth area, cell viability, and concentration of total proteins were lower in DEHP exposed than in control cells. Cell width and area, as well as the diameter of the nucleus and nucleolus, were greater in exposed cells than in control cells. These are interpreted as signs of cytotoxicity and suggest potential adverse effects on the development of skeletal muscle cells from DEHP exposure, as reported for other cell types.

**KEY WORDS:** Emerging pollutants; Morphometry; Phthalates; Plasticizers; Toxicity.

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## INTRODUCTION

Human beings are exposed daily to plasticizer additives, such as di-(2-ethylhexyl) phthalate (DEHP). The amount of DEHP added to commonly used plastics is variable (Agency for Toxic Substances and Disease Registry, 2022; National Center for Biotechnology Information, 2022). DEHP does not bind covalently to plastic, so it can be released relatively easily and enter organisms through dermal contact, inhalation, with food or medical treatments (Agency for Toxic Substances and Disease Registry, 2022;

National Center for Biotechnology Information, 2022). DEHP is ubiquitous (Wang *et al.*, 2019) and is the phthalate found in the highest frequency and concentration in food due to contact with plastics from preparation to packaging (Schechter *et al.*, 2013; Wang *et al.*, 2019). Because of its association with adverse effects on human health, over the last twenty years, its use has been regulated or prohibited for the manufacture of children's toys, water bottles, cosmetics, drugs, and biological products (v.g., CDER,

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2012; Chung *et al.*, 2019; Agency for Toxic Substances and Disease Registry, 2022).

After entering the body, DEHP is metabolized and transformed into mono-(2-ethylhexyl) phthalate (MEHP), remaining within the body for a few hours or days (Hanioka *et al.*, 2016). From the concentration of MEHP in urine, exposure to DEHP has been estimated to be widespread and frequent in the world's population. Up to 80 % of the North American population ingested this phthalate from 1999 to 2006 (Tetz *et al.*, 2013). A daily intake from 3 to 30 mg DEHP kg<sup>-1</sup> day<sup>-1</sup> has been estimated (Kohn *et al.*, 2000), although the record of DEHP in house dust (up to 508 mg kg<sup>-1</sup>) suggests a higher exposure (Becker *et al.*, 2004). It is even considered that hospitalized patients could be exposed to higher DEHP concentrations (Green *et al.*, 2005; Agency for Toxic Substances and Disease Registry, 2022; National Center for Biotechnology Information, 2022).

Numerous epidemiological registries and laboratory investigations link DEHP with adverse effects on the respiratory, renal, circulatory, and hepatic systems (National Center for Biotechnology Information, 2022), alterations in the immune responses (Kimber & Dearman, 2010), and cancer processes (López-Carrillo *et al.*, 2010; Chen *et al.*, 2018), decreased pregnancy rate, increased spontaneous abortions, among others (Hannon & Flaws, 2015). Medical and experimental studies have shown effects on reproductive morphology and hormonal activity, both in females (Hannon & Flaws, 2015) and males (Swan *et al.*, 2005). Because DEHP and other phthalates act like synthetic hormones, they penetrate the endocrine system, altering the physiological functions of endogenous hormones and promoting oxidative stress (Martínez-Razo *et al.*, 2021). This evidence suggests that all cell types can be affected, including the cells that make up muscle tissue. DEHP has been experimentally shown to induce oxidative stress and various metabolic effects in cardiac cells (Posnack *et al.*, 2012; Rowdhwil & Chen, 2018) and disrupt insulin signaling and glucose oxidation in skeletal muscle (Srinivasan *et al.*, 2011). Prenatal exposure to DEHP is reported to inhibit skeletal muscle development, particularly on 6-year-old girls (Lee *et al.*, 2020). However, little is known about the morphological effects that DEHP produce in skeletal muscle cells, even though cell morphology has been a fundamental tool in the diagnosis of lesions and pathologies (Kumar *et al.*, 2017), and as evidence of toxic effects (v.g., Jones *et al.*, 1975; Gray & Beamand, 1984; Komitowski *et al.*, 1986).

Since the 1970's the toxicity of phthalates has been investigated in cultures of different cell types and various species (Autian, 1973). Cell cultures allow bioassays in a

short time, provide better control of variables, and a detailed approach to analyzing the effects (Johns *et al.*, 2015). DEHP is partially soluble in lipoproteic fluids (Agency for Toxic Substances and Disease Registry, 2022; National Center for Biotechnology Information, 2022), and fetal calf serum due to the lipidic content (Freshney, 2015). Although it is reported to have a low solubility in the aqueous phase, under cell culture conditions it can be dissolved from 14 % to 20 % in pure calf serum as a previous step to the elaboration of the cell culture medium, making it available for the different cell pathways (Jones *et al.*, 1975). Using this simple, inexpensive, and repeatable procedure, changes in cell morphology, inhibition of development, and reduction of total protein production have been observed in human cells (Jones *et al.*, 1975; Li *et al.*, 2014). In order to explore the morphological effects that DEHP induces in human skeletal muscle cells, nine cell characteristics, as well as three parameters of cell culture development (growth area, cell viability, and total protein concentration) were analyzed.

## MATERIAL AND METHODS

**Ethical aspects.** This study was conducted in compliance with the principles of both Declaration of Helsinki and laws of Mexico (NOM-007-SSA2-2016; Ley General de Salud, 2020). The informed consent, sampling and research protocols were approved by Coordinación Nacional de Investigación en Salud of Instituto Mexicano del Seguro Social (IMSS; Ref. F-CNIC 2019-174; R 2000-785-008), and Coordinación Nacional de Bioética (CONBIOÉTICA; Ref. B5-61-2800/202000). Prior to sampling, procedures, research purposes, and destination of biological samples were explained to donors, after which they freely expressed their consent.

**Criteria for donor selection.** Skeletal muscle samples were obtained from healthy donors scheduled for cesarean delivery at Hospital General de Zona 1, IMSS, La Paz, Baja California Sur, Mexico. Healthy adult donors whose health was continuously monitored during pregnancy and were scheduled for a routine cesarean section were included. Chronic diseases, urgent cesarean section, or unwillingness to sign the informed consent were reasons for exclusion.

**Sample collection and transport.** Approximately 3 g of rectus abdominis skeletal muscle were taken from each donor (n=5). The tissue was rinsed with 2 % antibiotic solution (penicillin/streptomycin 10,000 U mL<sup>-1</sup>; Gibco™) in phosphate buffer saline (DPBS Gibco™) and immersed in transport solution (1 % penicillin/streptomycin antibiotics a 10,000 U mL<sup>-1</sup>, Gibco™, in 1X DMEM F12 culture medium, Gibco™). Samples were immediately transported to Labo-

ratorio de Estrés Oxidativo at Centro de Investigaciones Biológicas del Noroeste, S. C., in a cold chain and under the appropriate biosafety considerations (NOM-051-SCT2/2011; World Health Organization, 2019).

**Skeletal muscle cell primary culture.** In a laminar flow hood (Class II Type A2, Series E; Esco Labculture™), samples were transferred to sterile Petri dishes and rinsed with DPBS added with penicillin/streptomycin. Muscle tissue was recovered, finely fragmented with scalpel (Freshney, 2015), and placed in cell culture flasks (25 mL, Falcon™) with 2 mL of growth medium (1X DMEM/F12, 15 % v/v fetal calf serum, 10,000 U mL<sup>-1</sup> penicillin/streptomycin, 100X GlutaMAX, 100 mM sodium pyruvate, and 1M HEPES; all Gibco™). All flasks were incubated (Thermo Scientific™, Series 8000) at 37 °C (± 0.5 °C) in 5 % CO<sub>2</sub>, and 95 % (± 5 %) humidity; after 24 h, 1 mL of growth medium was added. The medium was replaced every three days, and all cultures were monitored daily using an inverted microscope (Zeiss Axio, Vert.A1™). When skeletal muscle cells covered most of the available surface (at 90–100 % of confluence), they were recovered by trypsinization, transferred to larger flasks (70 mL, Falcon™), and incubated under the same conditions (Hernández-Almaraz *et al.*, 2022). These new cell cultures were the source for bioassays.

**Bioassays.** To explore the morphological effects that DEHP induces in human skeletal muscle, bioassays were performed. Each bioassay was prepared from a stock of ≥ 15 cell culture flasks (70 mL) in passage 3, cell confluence with 95–100 % coverage, and cell density of ≥ 1.0 × 10<sup>6</sup> cells mL<sup>-1</sup> per flask. All the cells were pooled and distributed in 15 culture flasks by a standard procedure, seeding 4 × 10<sup>5</sup> cells mL<sup>-1</sup> per flask. Ten culture flasks were exposed to DEHP (di-(2-ethylhexyl) phthalate, purity > 99.0 %; Sigma-Aldrich®), and five were maintained with no DEHP added as controls, for 13 days. All flasks were incubated under the same conditions (37°C, 5 % CO<sub>2</sub>, and 95 % humidity), the respective growth medium was changed every three days; cell development and confluence were monitored daily.

The growth medium with DEHP added was prepared following the procedure reported by Jones *et al.* (1975) and used by Li *et al.* (2014). Namely, DEHP was added to fetal calf serum (HyClone™). After 22 h, this solution was added to the growth medium (DMEM-F12, penicillin/streptomycin, HEPES, GlutaMAX, sodium pyruvate). Finally, the mixture was allowed to stand for 22 h before being used in a bioassay. Because DEHP solubilizes up to 20 % within 44 h (Jones *et al.*, 1975; Li *et al.* 2014), theoretical concentrations (0.5, 1, 10, 100, 200, and 1000 mM) were calculated and applied in

preparative bioassays. In order to verify the reproducibility of the results an identify changes in cell morphology, these preparative bioassays were initially monitored for up to 20 days of exposure. However, given that at 13 days contrasting cellular changes, growth inhibition and reduced total protein production were observed this was selected as the bioassay period. Bioassays were performed in triplicate from the sample obtained from each donor. At the end of the bioassay, all cells were recovered, cell density and viability, as well as total protein content were determined.

**Cell development and confluence monitoring.** The growth area occupied by the cells was observed in all culture flasks, and the percentage occupied (coverage) calculated for each. When the cells utilized all the available growth areas and were in close contact, cell confluence (%) was determined (Freshney, 2015).

**Cell density and viability.** At the beginning and end of each bioassay, density and viability of cells were calculated. Briefly, a fraction of the cell suspension was diluted in DPBS and 14 % trypan blue stain (TBS) (0.4 %; Gibco™). After 5 min, 10 mL of this mixture was deposited in a Neubauer chamber (0.0025 μm<sup>2</sup>, bright-line, Marienfeld™). In a light microscope (Zeiss Standard 25, ICS™), both live and dead cells were counted in four quadrants of the camera, under 10 X objective (Louis & Siegel, 2011). Density (Dc) and viability (Vc) were calculated as:

$$\%Vc = (\text{Number of viable cells} / \text{Total number of cells}) * 100$$

$$Dc = (\text{Total number of cells} / \text{Quadrants of the Neubauer camera}) \times 10,000 \times \text{Sample dilution factor (Sample + DPBS + TBS)}$$

**Total protein content.** At the end of each bioassay, the total protein concentration was determined by Bradford's method (Freshney, 2015) adapted for use in a microplate reader (Multiskan™; Thermo Scientific™). Bradford reagent, based on Coomassie blue (Sigma-Aldrich®), was used. A calibration curve with bovine serum albumin (BSA; 5 to 200 mg mL<sup>-1</sup>; ≥ 98 %, Sigma-Aldrich®) was included in each run. Protein content in cell samples was obtained from the linear regression analysis performed using concentration and absorbance data for the calibration curve (Daniel & Cross, 2018).

**Morphometric analysis.** Photomicrographs were obtained with a digital camera (AxioCam, ICc5™) coupled to an inverted microscope with phase contrast illumination (10X, 20X, and 40X objectives). All photomicrographs were obtained in color (32 bpp, RGB), JPEG format, and high

resolution (5 mp; 2,452 × 2,056 pixels). Forty of these photographs were chosen for their image quality. With the public domain software ImageJ 1.53c (Rasband, 2018), the images were transformed to grayscale (8 bpp); brightness and contrast were adjusted manually or with the program's automatic function (Enhance Local Contrast; maximum slope 2, non mask, non fast). All editing was done without altering resolution, scale, or cellular aspects.

Based on the morphology described in muscle cell cultures developed under standard culture conditions (Hernández-Almaraz *et al.*, 2022), nine informative cell characteristics were chosen; namely, shape, length, width, cell area, length of cell projections, the diameter of the nucleus, the diameter of the nucleolus, number of nuclei, and number of nucleoli. These morphological characteristics were measured in 162 cells.

**Statistical analysis.** Morphological measurements, cell viability values, and total protein content were analyzed with descriptive statistics. Data are presented as Q25–Q75 interval and mean ± SD unless otherwise indicated. Normality and homoscedasticity tests were performed with the Lilliefors-Kolmogorov-Smirnov and Levene methods (median as the center of each group), respectively. Outliers were removed (Grubbs Test for two opposite outliers), and data were transformed with the log10 method (Sokal & Rohlf, 2009). Student's t-Test was applied to compare the morphological parameters in control and DEHP-exposed cultures, and between the beginning and end of the bioassay. Significant differences were assumed when  $P < 0.05$  ( $\alpha = 5\%$ ) (Daniel & Cross, 2018). Statistical and graphic analyzes were performed in RStudio 2022.06.0 Build 340 (RStudio Team, 2022).

## RESULTS

Muscle cells presented spherical shape at seeding (propagation stage) and displayed irregular shape (division stage) after adhesion to the substrate and growth. Later, they took a spindle shape (maturation stage). All cells were mononucleated, with a circular to semi-circular nucleus in a central position, each with one to four nucleoli. The global morphometric values are presented in Table I.

In both control cultures and those exposed to DEHP (1 mM), the cell growth area presented 80 % coverage 24 h after starting the bioassays. Cells detached from the substrate or dead were not considered, but spherical cells in the settlement process and cells in the division process were quantified and recorded. Active development was maintained in the control cell cultures throughout the bioassays but not in the cell cultures exposed to DEHP.

Cells exposed to DEHP showed a different morphological appearance than control cells from day 2 (Fig. 1A). Numerous shiny golden droplets, spherical to hemispherical ( $3.2 \pm 1.2 \mu\text{m}$  in diameter), were observed scattered and without apparent order in the cytoplasm of DEHP exposed cells (Fig. 2A). In these cells, the diameter of the nuclei was  $22.2 \pm 4.7 \mu\text{m}$  (19.0–24.5  $\mu\text{m}$ ), being greater ( $p < 0.05$  by Students t-test) than that in the control cells ( $20.0 \pm 3.3 \mu\text{m}$ ; 17.6–21.9  $\mu\text{m}$ ).

The cell growth area gradually increased in all cultures as bioassays progressed. Granular cytoplasm (Fig. 3A), caused by the increase of bright droplets dispersed in

Table I. Morphometry of human skeletal muscle cells in cellular culture, exposed for 13 days to di-(2-ethylhexyl) phthalate (DEHP) 1 mM.

Character	n	Min	Max	Q25	Q75	Median	Mean	(±) SD
Control cells								
Length ( $\mu\text{m}$ )	80	61.5	475.6	166.5	241.1	211.0	209.7	66.0
Width ( $\mu\text{m}$ )	80	12.5	96.0	27.7	64.4	44.1	46.5	21.3
Area ( $\mu\text{m}^2$ )	80	2,082	15,174	4,731	9,932	6,445	7,375	3,466
Projections length ( $\mu\text{m}$ )	80	18.3	187.3	39.2	70.5	58.9	59.9	30.2
Nucleus diameter ( $\mu\text{m}$ )	80	14.1	29.4	18.2	23.1	20.5	20.6	3.5
Nucleolus diameter ( $\mu\text{m}$ )	80	3.1	9.0	4.7	6.2	5.3	5.5	1.2
Number of nuclei	80	1.0	2.0	1.0	1.0	1.0	1.1	0.3
Number of nucleoli	80	1.0	4.0	2.0	3.0	2.0	2.5	0.9
DEHP-exposed Cells								
Length ( $\mu\text{m}$ )	82	127.4	494.8	172.9	228.3	198.2	205.6	55.1
Width ( $\mu\text{m}$ )	82	13.4	167.2	28.0	77.8	43.6	58.6	37.4
Area ( $\mu\text{m}^2$ )	82	2,136	36,820	4,822	10,439	7,722	9,091	6,463
Projections length ( $\mu\text{m}$ )	82	14.6	236.7	41.4	71.6	50.6	58.4	29.9
Nucleus diameter ( $\mu\text{m}$ )	82	12.9	38.0	20.2	27.2	23.2	23.7	4.9
Nucleolus diameter ( $\mu\text{m}$ )	82	3.8	10.7	4.9	6.8	5.9	6.1	1.5
Number of nuclei	82	1.0	3.0	1.0	1.0	1.0	1.0	0.2
Number of nucleoli	82	1.0	4.0	1.0	2.0	2.0	2.0	0.8

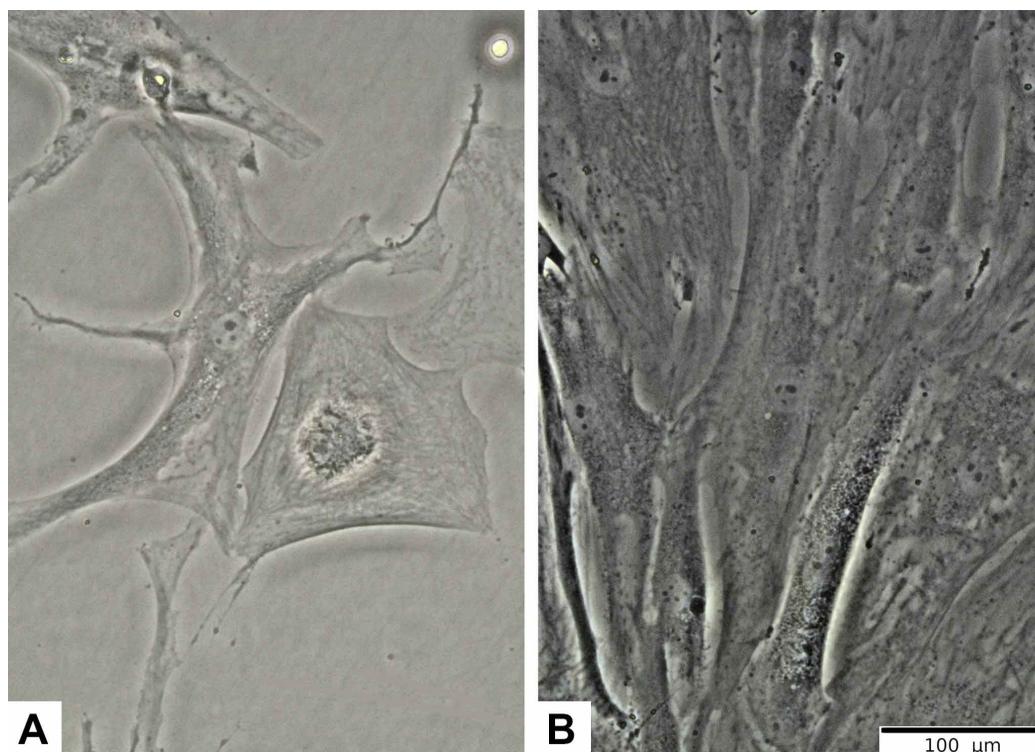


Fig. 1. Human skeletal muscle cells in control culture. A. Appearance at day 2 of the bioassay. In the center of the field, cells with irregular shape and projections of different sizes are observed. In the upper right corner, a spherical cell is observed floating in the culture medium. B. Appearance at day 13 of the bioassay. The cells cover most of the culture area, are mainly spindle-shaped and have short projections. On both days 2 and 13 of the bioassay, cytoplasm, nucleus, and nucleoli are clearly distinguishable. Photomicrographs taken *in vitro*, without dyes, but with phase contrast light.

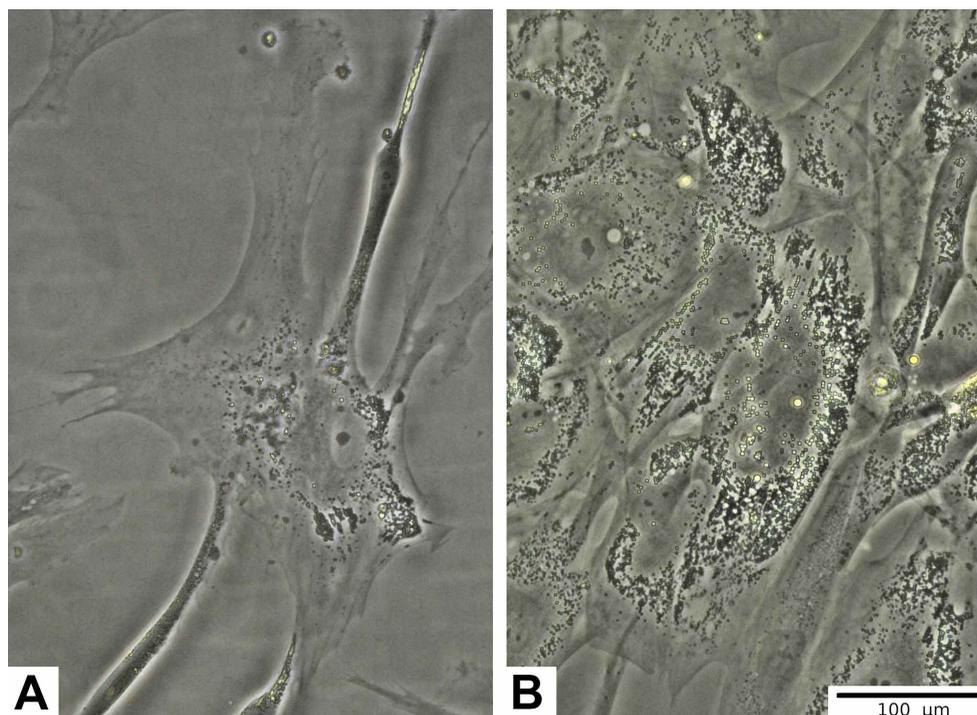


Fig. 2. Human skeletal muscle cells exposed to di-(2-ethylhexyl) phthalate (DEHP, 1 mM).

A. Appearance at day 2 of exposure to DEHP. In the center of the field, cells with irregular shape and projections of different sizes are observed. In the upper center a spherical cell is observed floating in the culture medium.

B. Appearance at day 13 of exposure to the phthalate. The cells are mainly irregular-shaped, have short projections and cover most of the culture area. On both day 2 and 13 of the bioassay, cytoplasm, nucleus, and nucleoli are clearly distinguishable. Photomicrographs taken *in vitro*, without dyes, but with phase contrast light.

the cytoplasm and by the increase in diameter (reaching up to 6 mm), was observed in DEHP exposed cells (Figs. 3B-C). Floating dead cells were present in DEHP exposed cell cultures (Fig. 3D) and were confirmed by trypan blue staining.

On day 13 of the bioassays, floating spherical cells, dividing cells, clustered cells, and a noticeable predominance

of spindle cells were observed in control cultures. Cell viability, growth area, and total protein concentration were optimal in control cultures (Table II). In contrast, reduced cellular activity and a perceptible predominance of irregular cells in DEHP exposed cells were observed. Compared to cells cultured under control conditions, cell viability was close to 50 %, the growth area was 12 % smaller, and the total protein concentration was 28 % lower in DEHP

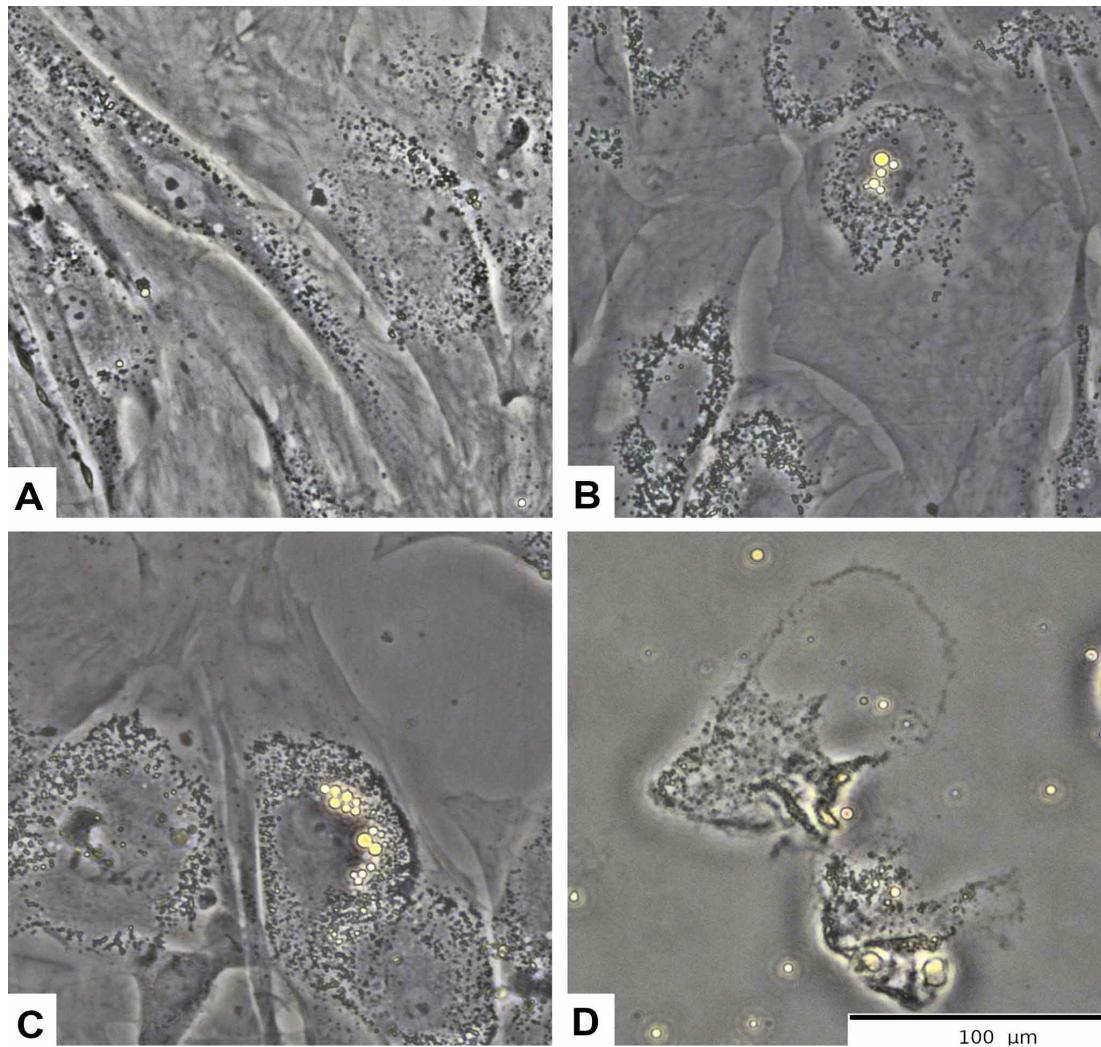


Fig. 3. Human skeletal muscle cells exposed to di-(2-ethylhexyl) phthalate (DEHP, 1 mM). A. Numerous droplets scattered in the cytoplasm were observed from the second day of exposure to the phthalate. B. During the bioassay, bright droplets grouped in clusters were also observed inside some of the cells. C. By day 13 of the bioassay, the bright droplets were more numerous and more frequently observed in cells. D. Dead desquamated cells were frequently observed floating in the culture medium; remains of the cell membrane and cytoplasmic content floating in the medium were distinguished. Photomicrographs taken *in vitro*, without dyes, but with phase contrast light.

Table II. Human skeletal muscle cell culture parameters at 13 days of exposure to di-(2-ethylhexyl) phthalate (DEHP, 1 mM) as compared to non-exposed cells (control). Mean  $\pm$  SD.

Treatment	n	Growth area, coverage (%)	Cell viability (%)	Total cell protein (mg mL <sup>-1</sup> )
Control	9	100 ( $\pm$ 0.0)	90.0 ( $\pm$ 3.7)	0.40 ( $\pm$ 0.2)
DEHP, 1mM	9	88 ( $\pm$ 3.5)	46.7 ( $\pm$ 12.8)	0.29 ( $\pm$ 0.1)

exposed cells ( $p < 0.05$ , Student's t-test). Differences in appearance (Figs. 1B, 2B) and potential differences in four morphometric characteristics (Fig. 4;  $p < 0.05$ , Student's t-test) were observed between human skeletal muscle cells grown under control conditions and those exposed to DEHP.

With respect to control, cells exposed to DEHP were wider ( $47.7 \pm 23.2 \mu\text{m}$  vs  $72.8 \pm 41.2 \mu\text{m}$ ), with greater cell area ( $7,727.4 \pm 3,681.4 \mu\text{m}^2$  vs  $11,178.5 \pm 7,490.8 \mu\text{m}^2$ ), and larger diameter of the nuclei ( $21.2 \pm 3.7 \mu\text{m}$  vs  $25.4 \pm 4.7 \mu\text{m}$ ) and nucleoli ( $5.4 \pm 1.1 \mu\text{m}$  vs  $6.2 \pm 1.5 \mu\text{m}$ ).

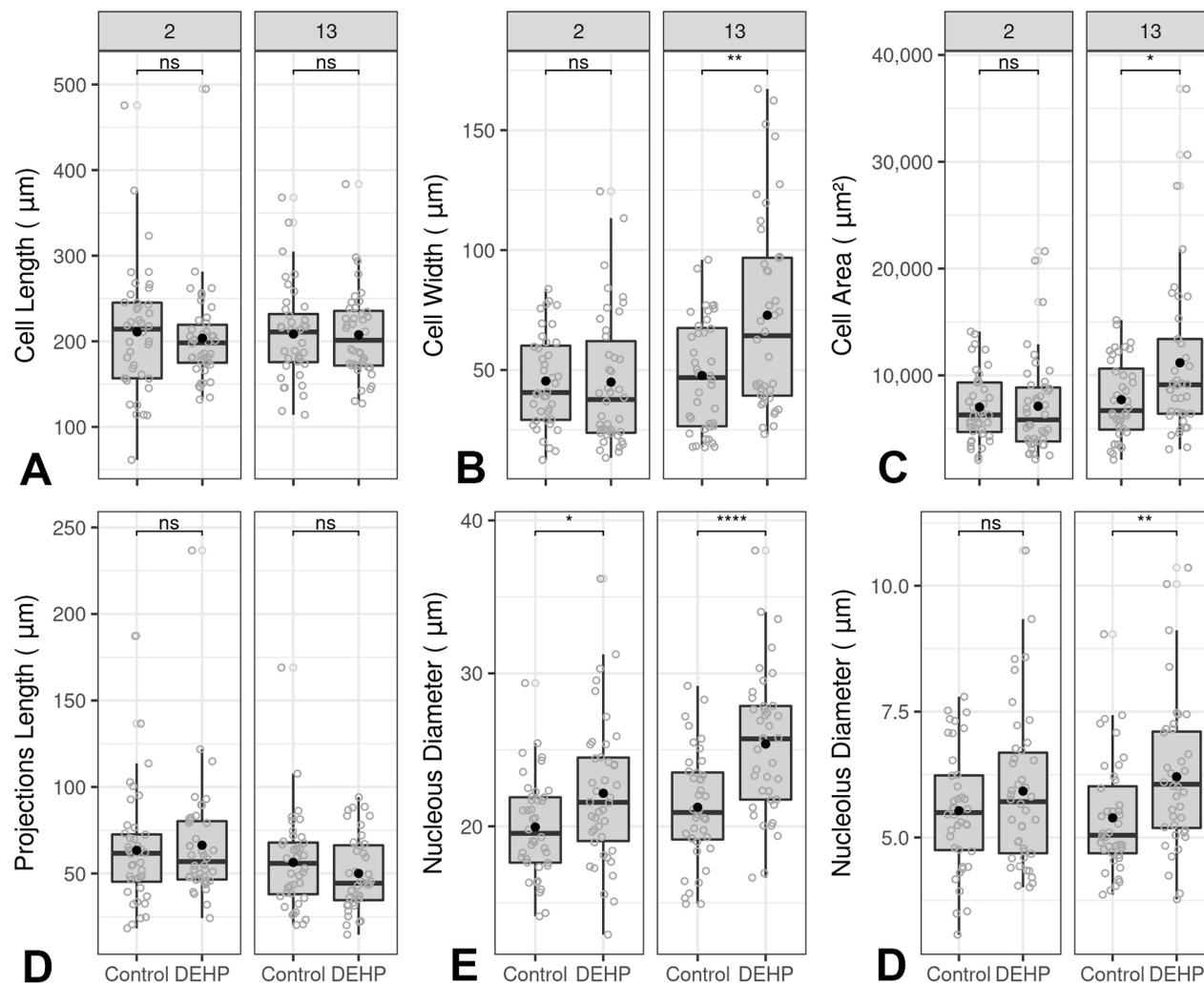


Fig. 4. Box-and-whisker plots comparing morphological characters of control cells and those exposed to di-(2-ethylhexyl) phthalate (DEHP) 1 mM, for bioassay days 2 and 13. A. Length, B. width, C. area, D. projections length, E. nuclei diameter, F. nucleoli diameter. Student's t-test, ns, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

## DISCUSSION

The toxicity of both DEHP and its metabolites has been studied in various cell types, but little is known about the effects on skeletal muscle cells (Lee *et al.*, 2020). The data obtained in the present study suggest that human skeletal muscle cells decreased their development, suffered morphological changes, and even died when exposed for 13 days to DEHP 1 mM under primary culture conditions.

Although the growth area in the DEHP exposed cells ( $88 \pm 3.5\%$ ) was apparently high ( $\geq 80\%$ ; Freshney, 2015), the presence of dead cells floating in the medium (Fig. 3D), the percentage of cell viability at the end of the experiment ( $46.7 \pm 12.8\%$ ), and the low concentration of total proteins ( $0.29 \pm 0.1 \text{ mg mL}^{-1}$ ), were related to the inhibition of cell development. These adverse effects correspond to those

described by Jones *et al.* (1975), in human diploid cell strain WI-38 exposed to DEHP for nine days, in which reduced growth areas and low protein concentrations were recorded. Also, the concentration (1 mM) and exposure time (13 days) used in the present study appear to approach a dose of 50 % of development inhibition ( $ID_{50}$ ) for human skeletal muscle cells under cell culture conditions. However, the solubility of DEHP in culture media is a technical challenge (Pérez-Albaladejo *et al.*, 2017) and one of the variables that can interfere in the calculation of specific cytotoxicity ( $ID_{50}$  or  $IC_{50}$ ), both for DEHP and its metabolites (Erkekoglu *et al.*, 2010). Despite the above, the lack of precision does not affect our results, nor the objective of exploring the morphological effects induced by DEHP.

Reports suggest that toxic concentration of DEHP is variable, and among other factors, depends on species, route of exposure, age of the donor (Wang *et al.*, 2019), cell type, and transformation to MEHP as its most toxic form (Erkekoglu *et al.*, 2010). Adverse effects of DEHP have been investigated in different cell types and concentrations ranging from nM ( $10^{-9}$ ) to mM ( $10^{-6}$ ). For example, effects have been reported in human breast tissue fibroblasts exposed to 100 nM DEHP for five days (Chen *et al.*, 2018) and in human placental JEG-3 cell line exposed to 400  $\mu$ M DEHP for two days (Martínez-Razo *et al.*, 2021). Effects of DEHP have also been tested in neonatal rat cardiomyocytes exposed to 128–256  $\mu$ M DEHP for three days (Posnack *et al.*, 2012), and chicken cardiomyocytes exposed to 500  $\mu$ M DEHP for two days (Cai *et al.*, 2019). Higher concentrations were used by Erkekoglu *et al.* (2010) who exposed MA-10 mouse interstitial cells (Leydig cells) to 110 mM DEHP for one day, calculating 3 mM as the  $LC_{50}$  for this cell type. Concentrations as high as the one used in this study, 1 mM (= 396  $\mu$ g DEHP  $mL^{-1}$ ), are not far from realistic values. Posnack *et al.* (2012) suggest that their experimental parameters (128–256 mM DEHP) were comparable to clinical exposures registered in newborns undergoing clinical procedures, that is, 50–100  $\mu$ g DEHP  $mL^{-1}$ . Concentrations of 180–380  $\mu$ g DEHP  $mL^{-1}$  have been reported in platelets stored in plastic containers (Rubin & Schiffer, 1976).

Morphological alterations related to DEHP exposure have been previously recorded in other cell types. Jones *et al.* (1975) described retraction of cytoplasmic processes and cell thinning of WI-38 strain. Cai *et al.* (2019) described hypertrophy of chicken cardiomyocytes (length, 235  $\mu$ m; width, 49  $\mu$ m; area, 3,682  $\mu$ m<sup>2</sup>) compared to control cells (length, 167  $\mu$ m; width, 35  $\mu$ m; area, 3,086  $\mu$ m<sup>2</sup>; maximum values measured from Figure 1 published by Cai *et al.* (2019). Komitowski *et al.* (1986) recorded morphological changes in the nuclear area and arrangement of chromatin within the nucleus of golden hamster hepatocytes. These alterations

are similar to those recorded in skeletal muscle cells (wider and larger in area, with larger nuclei and nucleoli) in the present study. The elevated percentage of coverage observed in skeletal muscle cells exposed to DEHP in the present study could be due to hypertrophy and not cell propagation. In all cases, the morphological alterations suggest cellular metabolism disruption in response to DEHP exposure; however, the mechanisms that lead to such morphological changes remain to be studied. Hypertrophy in cardiomyocytes is related to impairments in mitochondrial activity and glucose metabolism (Cai *et al.*, 2019). Structural abnormalities in sperm cells are related to alterations that DEHP causes in zinc metabolism, DNA, mitochondria, and increased production of reactive oxygen species (Rowdhwal & Chen, 2018). In the absence of cellular observations, changes related to oxidative stress were detected in rat skeletal muscle. Certain substances related to the integrity of the cell membrane were increased, and, therefore, metabolic dysfunctions in the cell structure occurred (Srinivasan *et al.*, 2011).

Interestingly, the number of bright droplets ( $3.2 \pm 1.2 \mu$ m) dispersed in the cytoplasm from the second day of exposure to DEHP and their increase in number and size (up to 6  $\mu$ m) towards the end of the bioassay remains to be explained. DEHP is known to induce proliferation of peroxisomes in hepatocytes and the transient accumulation of tiny lipid droplets that disappear when peroxisomes proliferate (Komitowski *et al.*, 1986; Lock *et al.*, 1989; Rowdhwal & Chen, 2018). While peroxisomes are vesicular organelles of  $\leq 1 \mu$ m, involved in the catabolism of fatty acids and reduction of reactive oxygen species (Lock *et al.*, 1989), lipid droplets are dynamic vesicles of 0.3–1.5  $\mu$ m in normal and healthy skeletal muscle (up to 3  $\mu$ m in modified cells for fat storage), involved in energetic processes (Bosma, 2016). Particularly, lipid droplets have been reported to be affected in interstitial tumor cells (Leydig tumor cells) exposed to MEHP (Dees *et al.*, 2001), and mouse adipocytes exposed to Bisphenol-A (Ariemma *et al.*, 2016). However, the morphological observations made in the present study do not allow a relationship with these or other subcellular structures.

It should be noted that oxidative stress triggered by exposure to DEHP is related to pathologies, such as insulin resistance (Kim *et al.*, 2013), development of obesity (Stojanoska *et al.*, 2017), and male infertility (Li *et al.*, 2014), as well as the proliferation of peroxisomes associated with carcinogenic processes in the liver (Lock *et al.*, 1989). Furthermore, the metabolites resulting from DEHP biotransformation (MEHP) promote changes in cell morphology and loss of substrate adhesion in rat testicular cells (Gray & Beamand, 1984), in addition to inducing

oxidative stress (oxidative damage to lipids, proteins, DNA) and apoptosis in human placental cells (Tetz *et al.*, 2013; Martínez-Razo *et al.*, 2021). The evidence leads to establishing that all types of cells are susceptible to being affected by DEHP. Therefore, knowing the effects of DEHP and other phthalates is essential in risk assessment and decision-making (*v.g.*, Agency for Toxic Substances and Disease Registry, 2022), particularly since our contact with these substances is imminent and daily.

## CONCLUSIONS

Exposure to DEHP (1 mM for 13 days) causes human skeletal muscle cells maintained in primary culture to decrease growth area coverage, total protein concentration, and cell viability. Lethal effects of DEHP are observed both in the first stages of propagation and in mature cells. Results suggest the entry of phthalate into cells and the disruption of metabolic processes that lead to morphological alterations, which include hypertrophy and the emergence of numerous tiny vesicles.

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**HERNÁNDEZ-ALMARAZ, P.; LUGO-LUGO, O.; GAXIOLA-ROBLES, R.; BITZER-QUINTERO, O. K.; RAMÍREZ-JIRANO, L. J.; BRASSEA-PÉREZ, E. & ZENTENO-SAVÍN, T.** Alteraciones morfológicas en células musculares esqueléticas humanas expuestas a ftalato de di-(2-etilhexilo) (dehp) en condiciones de cultivo celular primario. *Int. J. Morphol.*, 40(5):1209-1218, 2022.

**RESUMEN:** Diariamente los seres humanos tenemos contacto con aditivos plastificantes, el di-(2-etilhexil) ftalato (DEHP) se encuentra entre los más comunes. El DEHP puede liberarse del plástico e ingresar al cuerpo humano, donde es metabolizado y transformando en moléculas hidrofílicas oxidativas. Seguimientos en pacientes expuestos a este ftalato e investigaciones en cultivos de varios tipos celulares han aportado información sobre sus efectos. El DEHP es asociado con la inhibición del desarrollo de células humanas diploides y cambios morfológicos en células germinales en cultivo. Sin embargo, aún es poco lo que se sabe sobre los efectos en el músculo esquelético, a pesar de que este tejido representa alrededor

del 50 % de la masa corporal del humano. Para explorar y evaluar algunos efectos morfológicos en células de músculo esquelético, cultivos primarios fueron expuestos a DEHP (1 mM) durante 13 días. Se dio seguimiento a tres parámetros de desarrollo del cultivo y nueve características celulares. Al término de 13 días de exposición, los valores del área de crecimiento, viabilidad celular y concentración de proteínas totales fueron inferiores con respecto a los cultivos control. Se observaron cambios morfométricos en las células expuestas. Particularmente, el ancho y área celular, así como los diámetros del núcleo y nucleolos, fueron mayores a los registros en las células control. Estos resultados se interpretan como signos de citotoxicidad y sugieren efectos potencialmente adversos en el desarrollo de las células del músculo esquelético ante una exposición al DEHP, como se ha registrado para otros tipos celulares.

**PALABRAS CLAVE: Contaminantes emergentes; Ftalatos; Morfometría, plastificantes; Toxicidad.**

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