

DEHP exposure impairs human skeletal muscle cell proliferation in primary culture conditions

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Abstract

The plasticizer di (2-ethylhexyl) phthalate (DEHP) inhibits differentiation, impairs glucose metabolism, and decreases mitochondrial function in murine muscle satellite cells; however, if these effects are translated to human cells is unknown. The goal of this study was to evaluate changes in morphology and proliferation of primary human skeletal muscle cells exposed to DEHP. *Rectus abdominis* muscle samples were obtained from healthy women undergoing programmed cesarean surgery. Skeletal muscle cells were isolated and grown under standard primary culture conditions, generating two independent sample groups of 25 subcultures each. Cells from the first group were exposed to 1 mM DEHP for 13 days and monitored for changes in cell morphology, satellite cell frequency and total cell abundance, while the second group remained untreated (control). Differences between treated and untreated groups were compared using generalized linear mixed models (GLMM). Cell membrane and nuclear envelope boundary alterations, loss of cell volume and presence of stress bodies were observed in DEHP-treated cultures. DEHP-treated cultures also showed a significant reduction in satellite cell frequency compared to controls. Exposure to DEHP reduced human skeletal muscle cell abundance. Statistical differences were found between the GLMM slopes, suggesting that exposure to DEHP reduced growth rate. These results suggest that exposure to DEHP inhibits human skeletal muscle cell proliferation, as evidenced by reduced cell abundance, potentially compromising long-term culture viability. Therefore, DEHP induces human skeletal muscle cell deterioration potentially inducing an inhibitory effect of myogenesis by depleting satellite cells.

Introduction

Di (2-ethylhexyl) phthalates (DEHP) are widely used as additives in plastic products to increase flexibility, endurance and transparency (ATSDR 2019). However, these chemical compounds are toxic and teratogenic (Park et al. 2020). According to the U.S. Environmental Protection Agency (EPA), DEHP is the most widespread plasticizer and one of the six phthalates considered priority pollutants worldwide (EPA 2012). Because DEHP is not covalently bound to the plastic polymer, this phthalate can be released and transported by air, water and soil (Halden 2010). DEHP concentrations in nature depend on the environment where DEHP is deposited. Net et al. (2015) reported that DEHP dust concentration varies between $600 \mu\text{g g}^{-1} \text{d}^{-1}$ in the U.S. and $3,000 \mu\text{g g}^{-1} \text{d}^{-1}$ in Denmark, estimating a general absorption per person of $70 \mu\text{g kg}^{-1} \text{d}^{-1}$. Despite a maximum exposure concentration of DEHP per day established at $25 \mu\text{g kg}^{-1}$ for a human adult (60 kg) (World Health Organization 2017), concentrations as low as $100 \mu\text{M}$ could be harmful for different tissues and systems (Brassea-Pérez et al. 2022).

Human exposure to DEHP mostly occurs by consuming food and beverages packaged in plastic (Kim et al. 2014; Gurdemir et al. 2019), but also through inhalation (Franken et al. 2017), across the skin (Wu et al. 2015) and via parenteral administration (Fromme 2011; Kelley et al. 2012). Steiner et al. (1998) found that DEHP concentration in human saliva after sucking on a PVC film, common in dental vacuum forming sheet, vinyl toys, and snorkel mouthpieces, is $1017 \mu\text{g g}^{-1}$ equivalent to 2.6 mM ($2,604 \mu\text{M}$).

DEHP is a lipophilic compound that can cross biological membranes; it is absorbed and metabolized in the intestine, and distributed through the vascular system reaching the liver, before excretion takes place (Rael et al. 2009; Choi et al. 2013). Within cells, esterase and lipase-mediated metabolism hydrolyze DEHP into its primary metabolite mono-(2-ethylhexyl) phthalate (MEHP) (Koch and Calafat 2009; Choi et al. 2013). This monoester is lighter (lower molar mass) than DEHP and, therefore, preferentially transported into the vascular system. Thus, MEHP is a more reactive and potentially hazardous compound to human health than DEHP (Choi et al. 2018). Chronic exposure to phthalates in humans and other animals is associated with endocrine dysfunction (Cho et al. 2015), developmental alterations (Agarwal et al. 1986; Zuo et al. 2014), cancer (Wang et al. 2012; Yavasoglu et al. 2014; Crobeddu et al. 2019), and loss of cell proliferation and viability in different tissues (Ma et al. 2018; Molino et al. 2019; Chen et al. 2020). Despite the available information on the hazardous effects of DEHP in mammals, the potential impact of this compound in human skeletal muscle is still unclear (ATSDR 2019).

Skeletal muscle is composed of cells with multiple nuclei that form long fibers; these cells are involved in voluntary movements and represent 30% and 38% of body mass in adult women and men, respectively (Janssen et al. 2000; Hill and Olson 2012). As a contractile apparatus, skeletal muscle fibers need to be continuously repaired. The proliferation or population growth capacity of skeletal muscle cells and fibers depends on self-renewal of myogenic satellite cells (Snijders et al. 2015). Skeletal muscle satellite cells maintain cell populations which would spread or proliferate, differentiate into myoblasts, fuse forming multinuclear myotubes, and lead to myofiber formation (Etienne et al. 2020). This regeneration process compensates for tissue loss due to attrition, exposure to xenobiotics or injury (Snijders et al. 2015; Chen et al. 2020; Etienne et al. 2020).

Chen et al. (2020) suggested that DEHP/MEHP induces mitochondrial dysfunction and inhibits myogenesis in murine skeletal muscle cells. Moreover, exposure to phthalates promotes fragmentation of the mitochondrial reticulum, compromising mitochondrial efficiency (Hoppins 2014; Lackner 2014). By decreasing mitochondrial energy production, DEHP/MEHP could compromise muscle satellite cell viability and myogenic regeneration. Furthermore, skeletal muscle dysfunction may lead to metabolic disorders such as insulin resistance, obesity (Rabinowitz and Zierler 1962) and even sarcopenia (Yang et al. 2022). As plasticizers are found in many daily products, exposure to DEHP has increased in the last decades (Ferguson et al. 2011; Kim et al. 2014; Gurdemir et al. 2019). The aim of this study was to analyze potential changes in skeletal muscle cell proliferation, abundance and morphology in primary human skeletal muscle cells exposed to DEHP.

Materials And Methods

Sample collection

Rectus abdominis muscle biopsies (~ 3 g) were collected from five healthy adult (18–35 years old) females undergoing programmed cesarean surgery and antenatal care at Instituto Mexicano del Seguro Social (IMSS). Prior to sampling, informed consent was obtained from all volunteers. The research

protocol and informed consent forms were registered and approved by Comité de Ética en Investigación and Comité Hospitalario de Bioética (F-CNIC 2019 – 174 and R 2000-785-008), Comisión Nacional de Investigación Científica del Instituto Mexicano del Seguro Social (IMSS; 2018-785-010), as well as by Comisión Nacional de Bioética (CONBIOÉTICA-09-CEI-009-20160601). Sample collection was carried out in accordance with the guidelines of CONBIOÉTICA and the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Primary cell isolation and culture

Human skeletal muscle cells were grown from tissue explants in culture medium consisting of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, 1X; Corning®), calf serum (FBS, 12.4% v/v; Gibco™), penicillin/streptomycin (10 U mL⁻¹; Gibco™), L-alanine-L-glutamine (GlutaMAX, 100X; Gibco™), sodium pyruvate (100 mM; Gibco™) and sulfonic acid (HEPES, 1 M; Gibco™). Skeletal muscle cells were incubated at 35 ± 1°C in a humidified 5% CO₂ incubator. Medium was changed every 3 days. Cells were allowed to reach ~ 98–100% confluence before being sub-cultured until enough biomass for the bioassays was obtained.

Cell viability at different di (2-ethylhexyl) phthalate (DEHP) doses

DEHP toxicity in skeletal muscle cells was tested prior to bioassays to establish the *in vitro* DEHP theoretical concentration at which cell viability declines. The theoretical concentration was estimated according to the ratio (v/v) of dissolved DEHP in FBS and culture medium (Jones et al. 1975; Li et al. 2015). DEHP partially dissolves in cell culture medium; thus, initial concentration of DEHP in a stock solution is not maintained and the actual DEHP content (approximately 15% of the initial concentration) can be estimated as follows:

$$[DEHP]_{dissolved} = 0.1528x + 1.5263, R^2 = 0.96, p = 0.021[1]$$

Where x represents the DEHP concentration in the stock solution.

The proportion of live skeletal muscle cells was determined by trypan blue exclusion using a hemacytometer (Ehrlich 1904; Louis and Siegel 2011). By estimating cell viability, it was found that, at a theoretical concentration of 925 µM DEHP, 40% of the cells were dead between 10 and 13 days of exposure (Fig. 1). Considering the apparent self-diffusion coefficient (D_{app}) of DEHP in the medium to be 4.04 x 10⁻⁷ cm min⁻¹ (Hara 1993; Bernard et al. 2021), the concentration of dissolved DEHP in culture medium was estimated to be approximately 1000 µM (1 mM). Where dissolved DEHP is the portion of the phthalate in contact with the cells in culture and can be absorbed by them. Therefore, further bioassays were carried out for 13 days using a 1 mM DEHP concentration. Prior to each assay, DEHP was diluted in FBS (12.4% v/v; Gibco™) for 24 hours and added to the culture medium.

Di (2-ethylhexyl) phthalate (DEHP) bioassay

Skeletal muscle cells were trypsinized (Freshney 2016) and seeded in 25 cm² T flasks (n = 50 subcultures). Cells were then assigned to two groups, one group was treated with a theoretical concentration of DEHP (1 mM, n = 25) (Jones et al. 1975) for 13 days; the second group was kept as an untreated control (n = 25). At the beginning of each bioassay, each T flask started at ~ 40–50% confluence.

Cell morphology, proliferation, and regeneration capacity

Human skeletal muscle cell cultures were monitored using an inverted microscope and ZEN 2.0 software© (Carl Zeiss Microscopy GmbH 2011). Photographs were taken under a 20X objective from random quadrants of each flask and saved in JPEG format (5 mp). Photograph quality parameters, including brightness (normal 0%), contrast ratio (20%), resolution (96 ppp), and noise (0%) were considered to select representative images.

The images that met the required quality parameters were used for further processing, including determination of cell morphology, cell abundance, and satellite cell frequency. Changes in physical cell characteristics, including cell volume loss (plasmolysis), presence of bodies in the cytosol (Ravel-Chapuis et al. 2016), and changes in the nuclear envelope (Ye et al. 2017), were registered. Cell confluence measured as the percentage of the surface culture area that is covered with cells was also considered. The frequency of satellite cells, which are a major component of the regenerative capacity of muscles (Charifi et al. 2003), was used as a proxy of the regeneration capacity of skeletal muscle. Satellite cells were identified as those showing a characteristic round shape and approximate size of $25 \pm 15 \mu\text{m}$ (Allbrook 1981; Gregory 2004). Cell proliferation was quantified based on changes in cell abundance and used as an indicator of cell population growth. Cell abundance was estimated using ImageJ (Schneider et al. 2012). A reference scale was set for each image using ImageJ software (Schneider et al. 2012), considering that 574 pixels in each 20X objective image is equivalent to 0.02 cm. Total cell abundance was then calculated by extrapolating the number of cells counted in each photograph (0.0061178 cm^2) to the total surface of each flask (25 cm^2). Total cell count was reported as total cell number per 25 cm^2 per day. Satellite cell absolute frequency was divided into two sets to calculate the average daily frequency of satellite cells in the first (1–7 days) and second (8–13 days) weeks of the bioassays, respectively.

Statistical analyses

Shapiro–Wilks (W) and Levene's tests were used to evaluate statistical assumptions of normality and homoscedasticity, respectively, before statistical analyses were performed (Hector 2015). Non-parametric Wilcoxon test was applied to estimate statistical differences in satellite cell frequency between control and treatment groups through the first (1–7 days) and second week (8–13 days) of bioassays.

To avoid observation bias during cell counts, the full data set was subjected to bootstrapping, using 1000 iterations; no statistical differences were observed between sample and resample distributions ($X^2 = 2400$, $p = 0.2405$). Hence, raw data were used to compute further analyses. Cell abundance quantified in each flask was treated as an independent subsample of each human skeletal muscle cell culture (n = 5).

For each culture condition (control, DEHP exposure), generalized linear mixed-effects models (GLMMs) (Bates et al. 2015; Handayani et al. 2017) were adjusted to analyze skeletal muscle cells response, in terms of cell proliferation (response variable). Random slopes, intercepts and non-random effect from independent cultures were considered for model building (Table I).

Table I. Factors used to build generalized linear mixed-effects models (GLMMs) to analyze human skeletal muscle cell population growth in untreated (control) and di (2-ethylhexyl) phthalate (DEHP, 1 mM) treated cell cultures for 13 days.

| Treatment | Variable | Type | Description | Formula |
|-----------|----------------|-------------|--------------------------------|---|
| Control | Cell abundance | Continuous | Response variable | $y \sim \beta_0 + \beta_1x + (x \text{human})$ |
| | Culture time | Continuous | x: Explanatory variable | |
| | Human | Categorical | Random effect | |
| DEHP | Cell abundance | Continuous | Response variable | $y \sim \beta_0 + \beta_1x + (x \text{human})$ |
| | Culture time | Continuous | x: Explanatory variable | |
| | Human | Categorical | Random effect random effect | |

(II) Indicates non-random effect from independent samples.

GLMM were designed following a gamma distribution with an identity link function ($f(x) = x$) as it yielded the best fit for model building and data distribution showed no statistical differences with respect to a reference gamma distribution ($X^2 = 32,680$, $p = 0.275$). Goodness of fit and model selection for correlated and uncorrelated intercepts and slopes were based on Akaike's information criterion (AIC). Nagelkerke R-squared, an alternative test for fitted models, was estimated as well as the significant relationship described by the model (p value), by using the likelihood ratio test (Chi-square test) (Kabacoff 2015). For this estimation, each GLMM is compared against a null model which is nested in the fitted models. GLMM data are presented as means and standard deviations. The slope coefficient and constants were statistically tested to evaluate differences in skeletal muscle cells between DEHP-treated and control cells using Student's t-test (Ferson and Burgman 2000). All analyses were performed using RStudio 4.0.3® (RStudio Team 2020) and all reported p values lower than 0.05 ($\alpha = 0.05$) were considered statistically significant.

Results

A DEHP theoretical concentration of 1 mM (cell viability < 60%) was used for further analysis as other tested concentrations (10, 100 and 200 μM) did not affect skeletal muscle cell viability ($\geq 90\%$).

Di (2-ethylhexyl) phthalate (DEHP) induces morphological changes in human skeletal muscle cells in primary culture

From the 526 total photographs obtained, 413 (78.5%) images met the required quality parameters; total cell abundance and satellite cell frequency were calculated throughout the bioassay. Figure 2 shows representative images of the morphological changes observed in primary human skeletal muscle cells exposed to DEHP (1 mM). From day 3 of the bioassay, phthalate micelles were observed in the cytosol. Satellite cell membranes shrunk and contracted, and some skeletal cells lost volume; these changes were observed especially in cells without adjacent or neighboring cells. On day 6, DEHP particles were observed inside the nucleus and the nuclear envelope appeared dissociated. At this time, some satellite and skeletal muscle cells were seen floating in the media. Plasmolysis and stress bodies formation in the cytosol increased with DEHP exposure time. On day 9, plasmolyzed cells were prevalent and the intercellular spaces became more evident with time in cells exposed to DEHP. During the second week of DEHP exposure, the cytoplasmic material was almost covered by stress bodies surrounding the nuclei. Control cultures started to show cells growing on top of other cells, forming layers, when 85–90% confluence was reached. By day 13, more DEHP micelles were observed in the cytosol and nuclei, leading to cell shape change, loss of cell adherence to the culture flask, loss of cell layering, and cell death (Fig. 2). In contrast, in the control samples skeletal muscle cell layers were piled one on top of the other (~ 3 layers) and large multinucleated myotubes were observed.

Figure 3 shows the frequency of satellite cells within primary human skeletal muscle cells exposed to DEHP (1 mM) for 13 days. The absolute frequency of satellite cells was significantly lower in cells exposed to DEHP compared to controls (without DEHP); this was observed from day 1, and by day 7 (first week, Fig. 3A), as well as by day 8 to 13 (second week, Fig. 3B). DEHP-treated cells showed a decrease in satellite cell frequency despite available spaces in the culture flask. In contrast, skeletal muscle cells continued proliferating during the entire bioassay and satellite cells were frequently observed in the control group. Higher frequency of satellite cells in controls could be related to a higher potential of these cells to be resilient and cope with DEHP effects after 13 days of exposure.

Di (2-ethylhexyl) phthalate (DEHP) reduces human skeletal muscle cell proliferation under primary culture conditions

Significance contribution of the explanatory variable culture time was found for both, control (t-test = 121.5, $p < 0.001$) and DEHP-treated GLMMs (t-test = 94.18, $p < 0.001$), meaning that for in models, culture time is a predictor variable for cell proliferation, regardless of culture condition. The GLMMs suggest that the time of culture contributes to explain 91% and 90% of deviance of the total number of skeletal muscle cells in control and DEHP treatment conditions, respectively (Fig. 4). Residuals from GLMM for both control ($W = 12$, $p = 0.055$) and DEHP-treated ($W = 15$, $p = 0.34$) cells were normal, achieving linearity in both cases. The variable human, independent samples, was tested as random effects in the models, but its contribution does not improve model goodness of fit test, so no random effects from human independent samples were assumed for GLMM building.

According to the GLMM for control cells, the average cell abundance after the average culture time (6.5 days) was 223,489 cells in 25 cm² plus the starting cell quantity for the bioassay (~ 200,000 cells), considering a standard error of 231 cells. For DEHP-treated cells, the expected average cell abundance at 6.5 days was 203,660.3 cells in 25 cm² plus the starting cell number (~ 200,000 cells), considering a standard error of 260 cells (Table II).

Table II. Summary of the effects of culture time in both control and di (2-ethylhexyl) phthalate (DEHP, 1 mM) treated cells on human skeletal muscle cell proliferation capacity during 13 days of bioassay.

| Treatment | Variable | Estimate | Std. Error | t-test | p | AIC |
|-----------|-----------|-----------|------------|--------|-----------|-------|
| Control | Intercept | 223,488.6 | 231.1 | 967.0 | < 0.001** | 615.5 |
| | Time | 33,236.7 | 273.6 | 121.5 | < 0.001** | |
| DEHP | Intercept | 203,660.3 | 259.8 | 783.91 | < 0.001** | 602.3 |
| | Time | 17,866.9 | 519.6 | 34.39 | < 0.001** | |

Std. Error, standard error; t-test, test for non-parametric Student's t-distribution; p, significance level; AIC, Akaike information criterion; ** statistical differences.

The prediction equations for the GLMMs are represented as follows:

$$\text{Control}\hat{y}_i = 223,488.6 + 33,236.7x_i \quad [2]$$

$$\text{DEHP}\hat{y}_i = 203,660.3 + 17,866.9x_i \quad [3]$$

where \hat{y} is indexed i for i -th human samples, x represents culture time.

For control cells, the expected increase in cell abundance per day was $33,237 \pm 273.6$ cells, which is higher than and statistically different from (t-test = -56.18, $p < 0.001$) the expected value for DEHP-treated cells ($17,867 \pm 520$ cells) (Fig. 4). These results suggest that human skeletal muscle cells proliferated at a lower rate when exposed to DEHP compared to control cells. Moreover, positive slopes were observed in both GLMMs, indicating that cell proliferation was maintained throughout the bioassay; however, cell abundance significantly decreased with time in DEHP-treated but not in control cells (Fig. 4).

Discussion

We found that exposure to DEHP concentration lower than 1 mM did not significantly reduce skeletal muscle cell viability, as reported in previous studies (Chen et al. 2020). High DEHP concentration, such as 1 mM, was used in prior studies with grass carp (*Ctenopharyngodon idella*) hepatocytes (cultured in 96-well plates) (Cui et al. 2020, 2021), but not in studies with other vertebrates' skeletal muscle cells. Negative effects of DEHP exposure have been observed in other cells, such as human endometrial cells

(Cho et al. 2015), erythrocytes (Melzak et al. 2018), placental cells (Tetz et al. 2013), and gametes (Al-Saleh et al. 2019). Some authors describe cell alterations, including decreased cell size or plasmolysis, nucleus fragmentation (Alberts 2013), DNA damage (Al-Saleh et al. 2019), vacuolization (Sung et al. 2003), and lower cell density (Patel et al. 2015). These changes have been related to programmed cell death and cellular senescence (Alberts 2013; Baar et al. 2018). In the freshwater prawn (*Macrobrachium rosenbergii*), exposure to phthalates (including DEHP) produced alterations in nuclear morphology of hemocytes and promoted cell vacuolization leading to cell death via apoptosis and necrosis (Sung et al. 2003). The authors observed that prawn hemocytes treated with 100 mg mL^{-1} of DEHP primarily die via necrosis on the first 10 minutes of exposure; then, at 40 minutes the main cell death pathway was apoptosis (Sung et al. 2003).

In the present study, the observed changes in skeletal muscle cell morphology, such as plasmolysis, could be associated to cell death, which could explain cell deterioration and population loss. The origin of cell stress bodies observed during the bioassays was not confirmed; but, according with the literature, they could be vacuoles (Sung et al. 2003) or apoptotic bodies produced as a consequence of cell disfunction (Alberts 2013). Alternatively, these bodies could be lipid droplets, which have cytoprotective functions against lipotoxic agents and lipid peroxidation promoters (Jarc and Petan 2019), or peroxisomes, which are produced massively as cytoprotective factors during stress processes (Elcombe and Mitchell 1986; Lapinskas et al. 2005). Stress granules, cytoplasmic aggregates of protein and RNA that contribute to cellular protection, have been observed in arsenite-treated (0.5 mM) C_2C_{12} mouse myoblasts; these granules are more evident after 45 minutes of exposure (Ravel-Chapuis et al. 2016). Chen et al. (2020) observed stress granule formation in C_2C_{12} mouse myoblasts at different stages of differentiation (proliferating, quiescent and differentiated) upon exposure to DEHP and its primary metabolite, MEHP.

Satellite cells, which are muscle stem cells, differentiate into myoblasts and have a crucial role in muscle maintenance and repair (Snijders et al. 2015). The presence of DEHP/MEHP in skeletal muscle cells promotes alterations in mitochondrial morphology, such as changes from its filamentary reticular network form into vesicles, which are less efficient at producing ATP (Chen et al. 2020). Likewise, phthalates block insulin-induced glucose cell uptake (Chen et al. 2020). Without glucose, and with less efficient mitochondria, satellite cells are not able to differentiate into myoblasts (Chen et al. 2020). This process leads to loss of cell abundance, decreased satellite cell recruitment and differentiation, and concomitant inhibition of muscle regeneration (Chen et al. 2020).

Human skeletal muscle cell proliferation was maintained, but satellite cell frequency decreased significantly after 13 days of DEHP exposure ($1 \text{ mM} = 390.564 \text{ } \mu\text{g mL}^{-1}$). Gutiérrez-García et al. (2019) found that human hematopoietic stem cells from umbilical cord blood lost 82% of the cell population after 14 days of *in vitro* DEHP exposure ($100 \text{ } \mu\text{g mL}^{-1}$). Differentiated skeletal muscle cells were more resilient than satellite cells. This could be explained due to satellite cells being more sensitive to epigenetic alterations that impair cell function (Pérez et al. 2019), which reduce their capacity to deal with exogenous agents, including phthalates.

Differentiated skeletal muscle cells have high energy requirements due to their contractile function (Kanatous et al. 1999; Ravussin and Smith 2006). This activity is matched with high blood flow demand which makes skeletal muscle more vulnerable to circulating xenobiotics (Molina-Ortiz et al. 2013). It could be expected that metabolically active tissues that naturally deal with other kind of stressors, such as the contractile effort in muscle cells, could deal with xenobiotic effects (Rodrigues-Lima et al. 2003). Skeletal muscle, as a major organ in the human body, could have a role in degradation of xenobiotic compounds (Cooper and Plum 1987; Chen et al. 2020), including phthalates (ATSDR 2019). Further information is needed to assess the pathways in skeletal muscles that derive into DEHP/MEHP metabolization.

Based on the results from this study, it can be speculated that exposure to DEHP could aggravate muscular pathologies or syndromes such as sarcopenia, which is characterized by loss of muscle mass and function (Huang et al. 2021). Loss of satellite cells reduce myoblast recruitment, promoting cell culture deterioration and loss of cellular integrity. These processes are similar to those associated with cellular senescence (Serrano et al. 2008), which involves cellular aging and related diseases. *In vivo* skeletal muscle cell deterioration could involve other factors including xenobiotic exposure (Chen et al. 2020), chronic diseases (Morley 2001), malnutrition, vitamin D deficit (Malafarina et al. 2012), thermal, mechanical, oxidative, or pharmacological stresses (Mcardle et al. 2002), among others.

Conclusion

Di (2-ethylhexyl) phthalate (DEHP) exposure for 13 days induces alterations in cell membrane and nuclear envelope boundaries, cell volume loss, presence of stress bodies, and reduced frequency of satellite cells in primary human skeletal muscle cell cultures. Based on the slope comparisons, we suggest that cells exposed to DEHP show lower proliferation rates than control cells. The results from this study suggest a potential link between DEHP exposure and functionality in human skeletal cells in primary culture. The lower frequency of satellite cells in DEHP-treated cells as compared to controls could overwhelm the repair function leading to cell biomass loss over time. Exposure to DEHP reduced human skeletal muscle cell proliferation capacity as evidenced by reduced cellular abundance, which is more evident at 13 days of xenobiotic exposure.

In summary, changes in both morphology and proliferation capacity were observed in human skeletal muscle cells under primary cell culture following exposure to DEHP (1 mM) for 13 days. These results contribute to understand the toxic effects of phthalates in human skeletal muscle and support previous research on the myogenic inhibitory effect of DEHP in skeletal muscle cells in other mammalian species and could suggest a potential link between DEHP exposure and muscle cell functionality.

Declarations

All samples were collected in accordance with the guidelines of Mexico's Comisión Nacional de Bioética (CONBIOÉTICA) and the Code of Ethics of the World Medical Association (Declaration of Helsinki). The

research protocol and informed consent forms were registered and approved by Comité de Ética en Investigación and Comité Hospitalario de Bioética (F-CNIC 2019-174 and R 2000-785-008), Comisión Nacional de Investigación Científica del Instituto Mexicano del Seguro Social (IMSS; 2018-785-010), as well as by CONBIOÉTICA (09-CEI-009-20160601).

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Data availability - The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing Interests and Funding

The authors whose names are listed above certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Figures

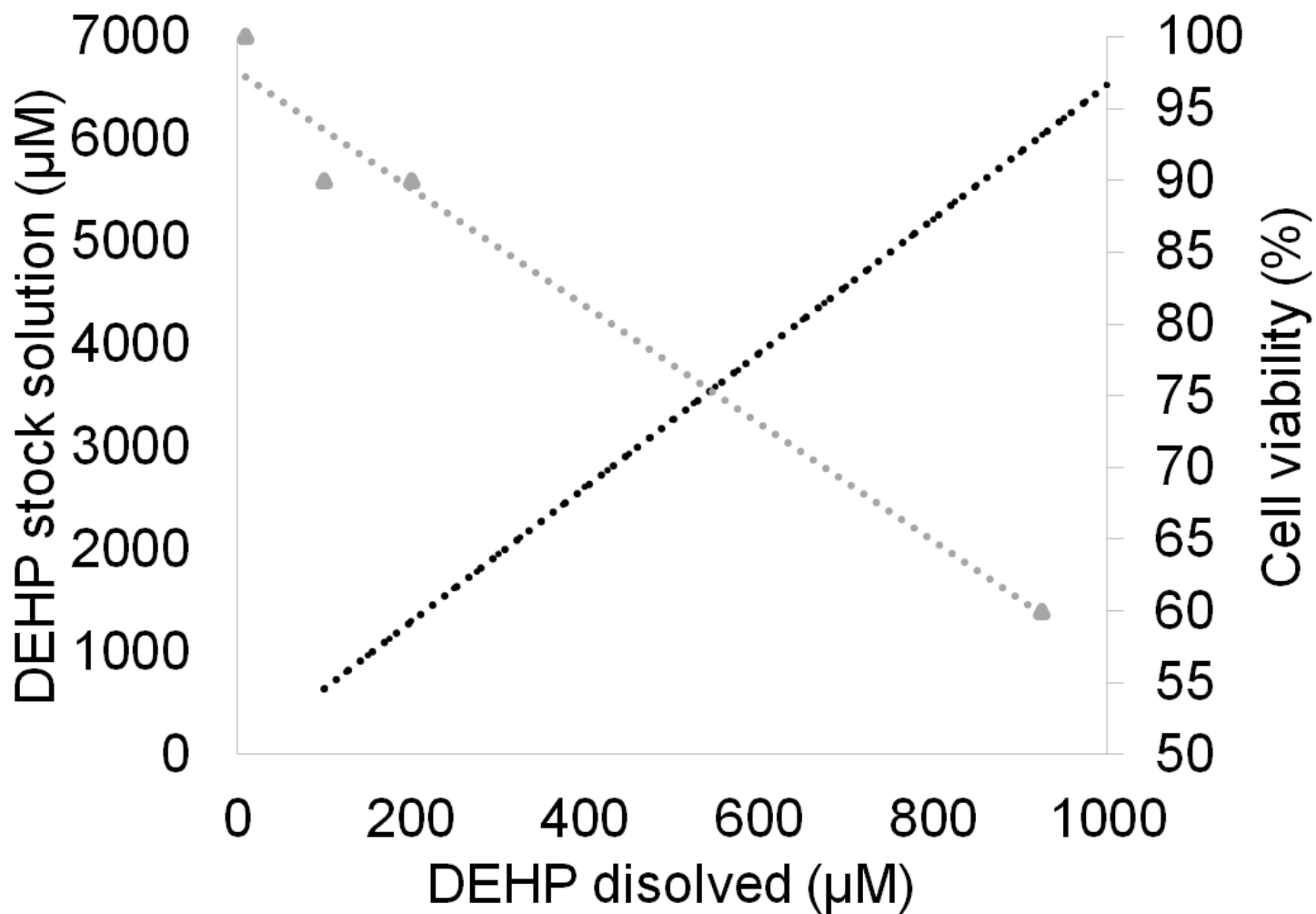


Figure 1

Relationship between the theoretical concentration (Jones et al. 1975; Li et al. 2015) of dissolved di (2-ethylhexyl) phthalate (DEHP) and DEHP stock solution (black circles) and cell viability (gray triangles) in human skeletal muscle cells in primary culture.

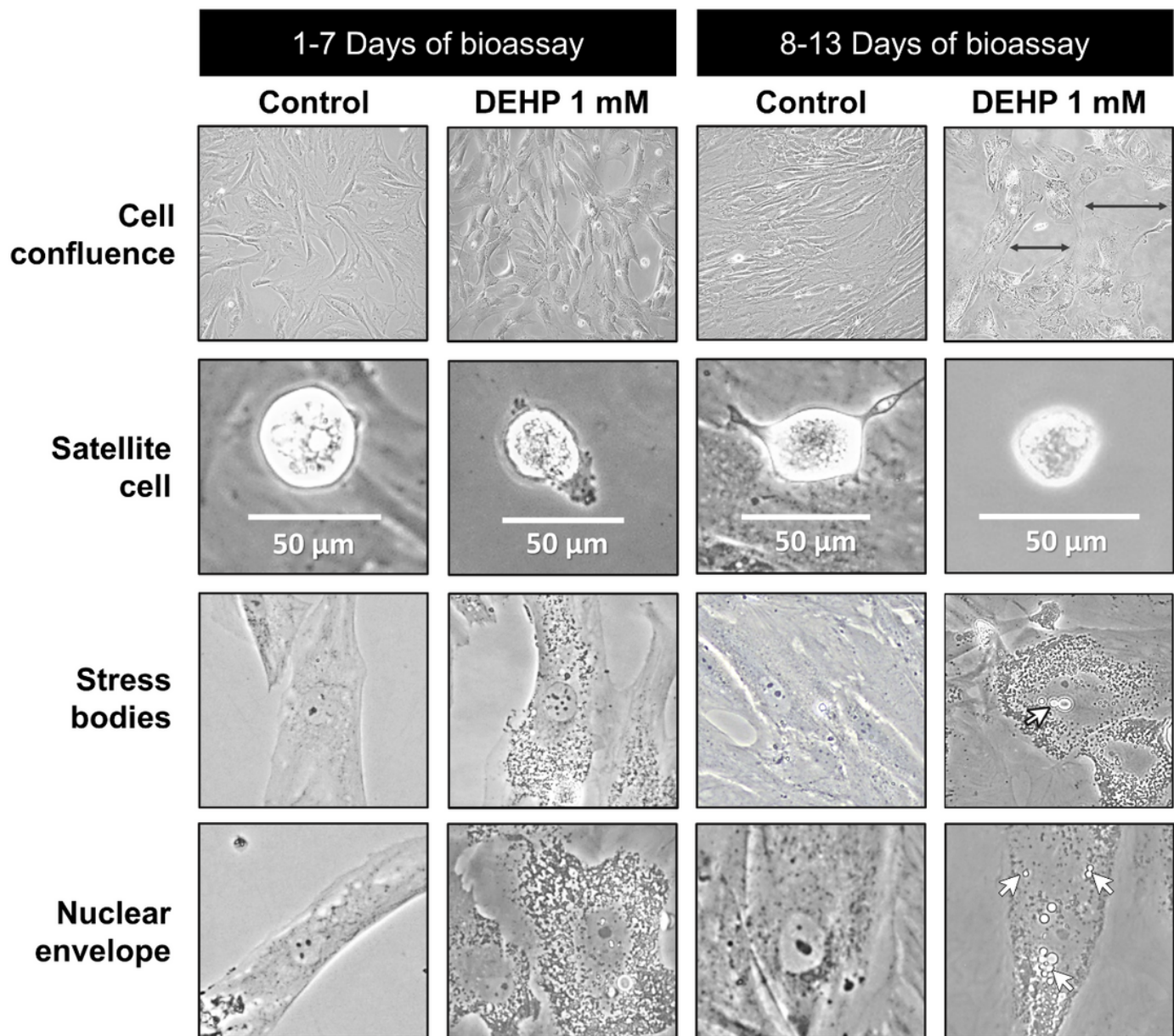


Figure 2. Morphological changes in primary human skeletal muscle cells exposed to 1 mM of di (2-ethylhexyl) phthalate (DEHP) for 13 days. All the images were taken under 20X objective and zoomed in for appreciation. Black arrows (\leftrightarrow) point at intercellular spaces and white arrows (\rightarrow) indicate phthalate micelles.

Figure 2

See image above for figure legend.

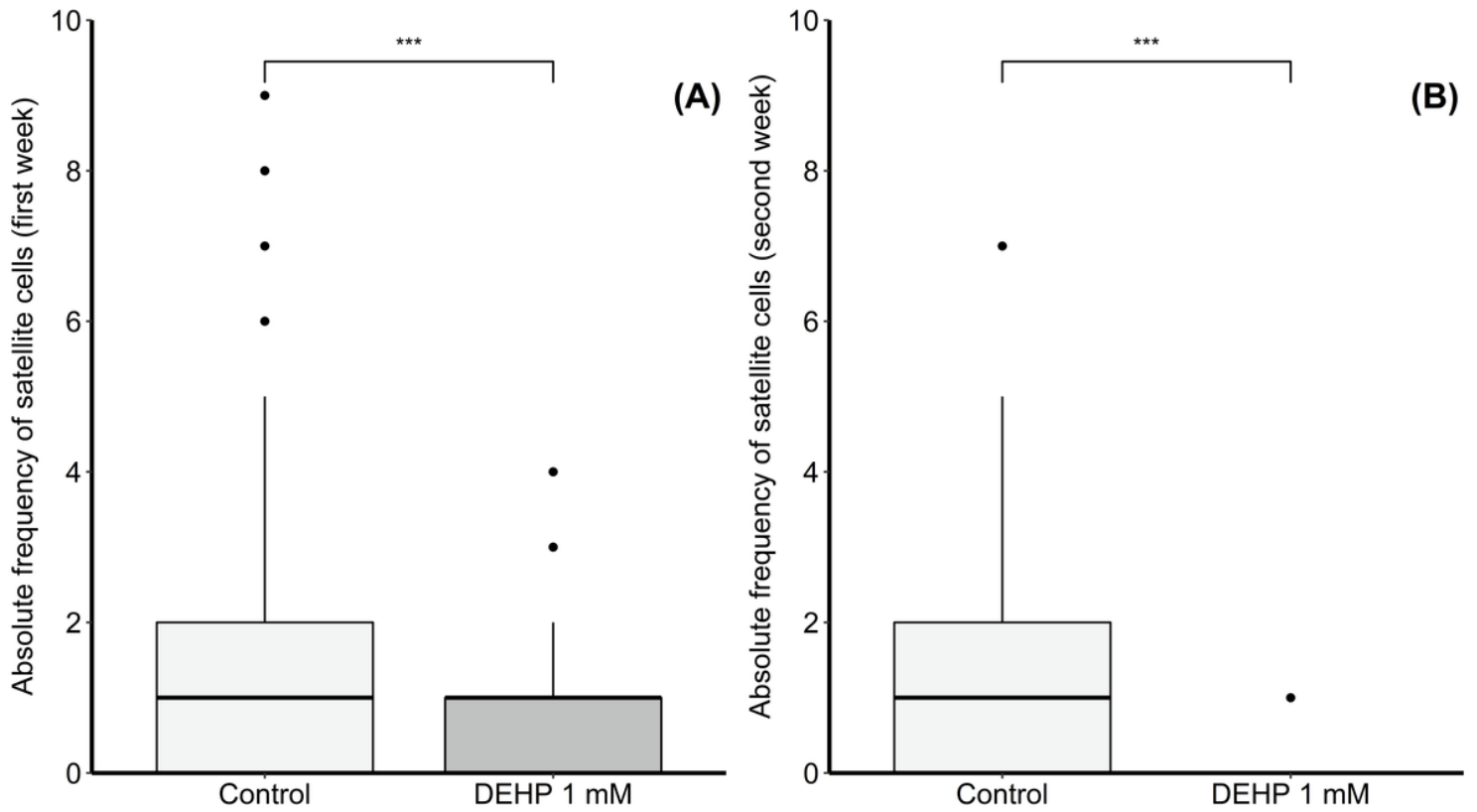


Figure 3

Absolute frequency of satellite cells within primary human skeletal muscle cells exposed 1 mM of di (2-ethylhexyl) phthalate (DEHP) for 13 days. (A) Data from day 1 to 7 of bioassay; (B) data from day 8 to 13 of bioassay. Statistical differences were estimated using Wilcoxon tests. *** significant differences ($p < 0.001$) between treatments; black circles denote outliers.

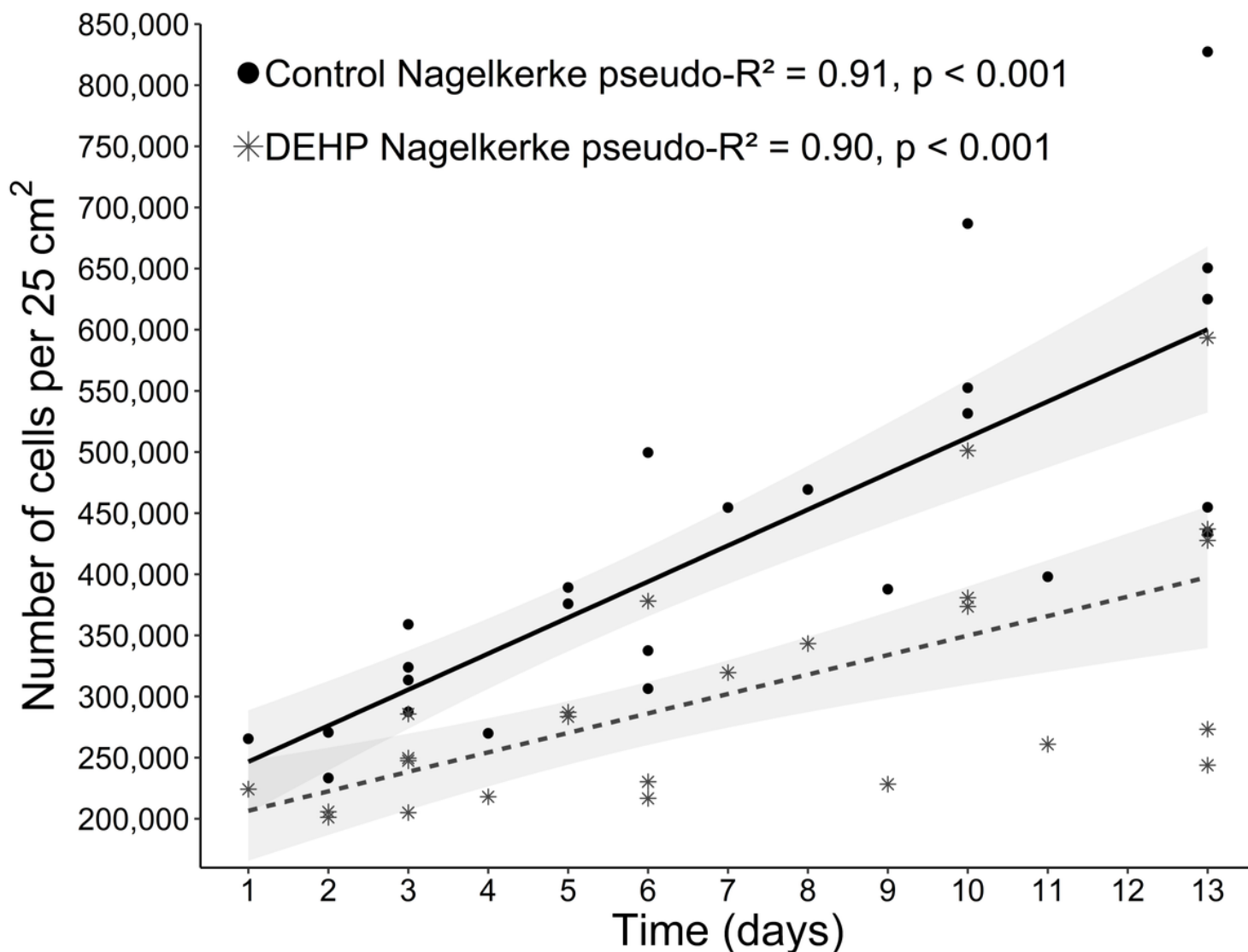


Figure 4

Generalized linear mixed models (GLMM) showing the total number of primary human skeletal muscle cells estimated in a 25 cm² T throughout 13 days. Lines represent the calculated linear regression models describing the association between number of cells and time of culture for cells exposed to di (2-ethylhexyl) phthalate (DEHP, 1 mM) (—) and the corresponding controls (-). Plotted values are those predicted by the GLMMs (n=25, per treatment). The gray area represents the standard error for each GLMM.

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