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POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

Evaluation of phthalates exposition in sirtuin transcription in HepG2 cells and its toxicity in human hematopoietic stem cells

Tesis que presenta

Ana Karen Gutiérrez García

Para obtener el grado de

Doctora en Ciencias en Biología Molecular

Director de la Tesis:

Dr. Antonio De León Rodríguez

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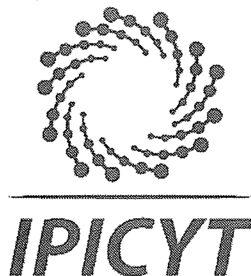

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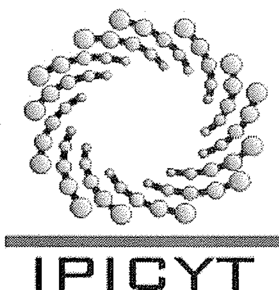

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Dedicatorias

Dedico este trabajo y el esfuerzo puesto en él, a las personas

que me han apoyado en todo momento a lo largo de mi carrera

profesional, mi familia.

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Resumen

"Evaluación de la exposición de ftalatos en la transcripción de sirtuinas en células HepG2 y su toxicidad en células madre hematopoyéticas humanas"

Los plastificantes son sustancias incorporadas en polímeros plásticos para aumentar su flexibilidad y maleabilidad. Los ésteres de ácido ftálico (ftalatos) se desarrollaron para su uso como plastificantes en la década de 1920 y cada año se venden más de 8 millones de toneladas de plastificantes en todo el mundo, representando el 70% de todos los plastificantes industriales actuales. Los ftalatos no están unidos covalentemente al polímero plástico y pueden migrar al medio ambiente. Por lo tanto, los humanos están expuestos a estos compuestos; su exposición se ha asociado con retrasos en la fertilidad, un mayor riesgo de alergias, asma, obesidad, diabetes y cáncer. Debido al peligro potencial para la salud humana y el medio ambiente, varias organizaciones internacionales de salud y medio ambiente han clasificado los ftalatos como contaminantes prioritarios. Por lo tanto, evaluamos la citotoxicidad del diisononil ftalato (DINP), su efecto sobre la expresión de sirtuinas en células HepG2 y su efecto sobre los niveles de especies reactivas de oxígeno (ROS). Los resultados mostraron que 1 µg/mL DINP disminuyó significativamente la expresión de los genes Sirt1, Sirt2, Sirt3 y Sirt5. Además, los niveles de proteínas de Sirt1 y Sirt3 fueron significativamente disminuidos por 1 µg/mL DINP. Por otro lado, 100 µg/mL DINP duplicaron los niveles de proteínas de acetilación en lisina, así como las especies reactivas de oxígeno. Además, evaluamos el efecto de cuatro ftalatos dibutil ftalato (DBP), bencil butil ftalato (BBP), dietil ftalato (DEP) y dietilhexil ftalato (DEHP) en la expansión *in vitro* de células hematopoyéticas humanas. Para esto, 0.5×10^6 células/mL se expusieron a concentraciones que oscilaban entre 0.1 y 100 µg/mL de ftalatos y se determinó la expansión celular total después de 14 días de cultivo en medio IMDM-citocinas. Los cultivos control alcanzaron $1.31 \pm 0.21 \times 10^6$ cell/mL mientras, que los cultivos expuestos a DBP, BBP y DEHP mostraron una reducción del 23 al 81%, del 17 al 69% y del 15 al 93.5%, respectivamente.

PALABRAS CLAVE: células HepG2, células madre hematopoyéticas, ftalatos, toxicidad

Abstract

“Evaluation of phthalates exposition in sirtuin transcription in HepG2 cells and its toxicity in human hematopoietic stem cells”

Plasticizers are substances incorporated into plastic polymers to increase their flexibility and workability. Phthalic acid esters (phthalates) were developed for use as plasticizers in the 1920´s and more than 8 million tons of plasticizers are sold globally every year, representing the 70% of all current industrial plasticizers. Phthalates are not covalently bound to the plastic polymer and can migrate to the environment. Therefore, humans are exposed to these compounds; its exposure has been associated with delays in fertility, increased risk of allergies, asthma, obesity, diabetes and cancer. Due to the potential hazard to human health and the environment, several international health and environment organizations have classified phthalates as priority pollutants. Therefore, the cytotoxicity of diisononyl phthalate (DINP) was evaluated on sirtuin expression in HepG2 cells and its effect on the levels of reactive oxygen species (ROS). Results showed that 1 μg/mL DINP significantly down-regulated Sirt1, Sirt2, Sirt3, and Sirt5 gene expression. Furthermore, protein levels of Sirt1 and Sirt3 were significantly down-accumulated by 1 μg/mL DINP. On the other hand, 100 μg/mL DINP doubled the levels of lysine acetylation proteins as well as reactive oxygen species (ROS). Also, the effect of four phthalates: dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), diethyl phthalate (DEP) and diethylhexyl phthalate (DEHP) was evaluated on the *in vitro* expansion of human hematopoietic cells. For this, 0.5 x 10^6 cells/mL were exposed to concentrations ranging from 0.1 to 100 μg/mL and the total cell expansion was determined after 14 days of culture in IMDM-cytokines medium. The control cultures attained 1.31 ± 0.21 x 10^6 cell/mL, whereas the cultures exposed to DBP, BBP and DEHP showed a reduction from 23 to 81%, 17 to 69% and 15 to 93.5%, respectively.

KEYWORDS: HepG2 cells, Hematopoietic stem cells, Phthalates, Toxicity

Chapter 1

Diisononyl Phthalate Differentially Affects Sirtuin Expression in the HepG2 Cell Line

Abstract

Human exposure to phthalates has received special attention due to their possible adverse human health effects. Diisononyl phthalate (DINP) is a plasticizer still widely used in many products, despite being considered an endocrine disruptor. In this study, we evaluated DINP's cytotoxicity, its effect on the levels of reactive oxygen species (ROS), and its effect on sirtuin expression in HepG2 cells. Results showed that 1 μg/mL DINP significantly down-regulated Sirt1, Sirt2, Sirt3, and Sirt5 gene expression ($p < 0.05$), while other sirtuins remained unaffected. Furthermore, protein levels of Sirt1 and Sirt3 were significantly down- regulated by 1 μg/mL DINP. On the other hand, 100 μg/mL DINP doubled the levels of lysine acetylation proteins (increased 2-fold) as well as reactive oxygen species (ROS) compared with the controls. In conclusion, our study suggests, for the first time, that DINP regulates the potential epigenetic disruptor sirtuin family and leads to induction of ROS via sirtuins.

Gutiérrez-García AK, Choudhury M, De Leon-Rodriguez A. Diisononyl Phthalate Differentially Affects Sirtuin Expression in the HepG2 Cell Line. Chemical Research in Toxicology. 2019, 32, 9, 1863-1870.

1. INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are defined as exogenous substances that have the ability to cause adverse health effects in an intact organism by altering functions of the endocrine system [1]. It was originally thought that EDCs act primarily through nuclear hormone receptors; however, it is now widely accepted that EDCs act through a variety of signaling mechanisms, which include nuclear steroid receptors, nonsteroid receptors, orphan receptors, epigenetic modifications, and enzymatic pathways ultimately responsible for maintaining endocrine homeostasis [2]. Phthalates are a group of endocrine disruptors used as plasticizers in materials such as polyvinyl chloride, along with being involved in the manufacturing processes of many other products. Diisononyl phthalate (DINP) is one of the primary phthalates most used in the industry. It is a mixture of compounds consisting of isononyl esters of phthalic acid (Figure 1). DINP is widely used in flooring, wire and cable, dip coating, coated fabrics, tubing, shoes, sealants, and artificial leather; humans may be exposed to DINP by oral, dermal, and inhalation routes [3]. The environmental ubiquity of DINP is known, and its presence has even been reported in river water, drinking water, outdoor air, and indoor air [4]. DINP and its metabolites are widely studied and have received considerable attention recently because of specific concerns about dietary or medical exposure in pediatric patients [5]. It has been proposed that population exposure to DINP would not exceed the levels of di(2-ethylhexyl) phthalate (DEHP) [3], which are estimated at 3−30 μg/kg body weight/day [6]. Phthalates, including DINP, are not covalently bound to plastics and can migrate into saliva, where they are swallowed [3,7]. Thus, children may be exposed to higher levels of DINP than adults, because infants and small children mouth toys and other articles containing DINP [3,8]. The chronic health effects of DINP, including organ toxicity, carcinogenicity, and reproductive toxicity, have been reviewed in dietary studies [3,8]. Early life exposure to phthalates has been associated with a variety of adverse effects, particularly those involving endocrine processes [3,9]. It has been noted that levels of phthalate metabolites in urine and serum are associated with

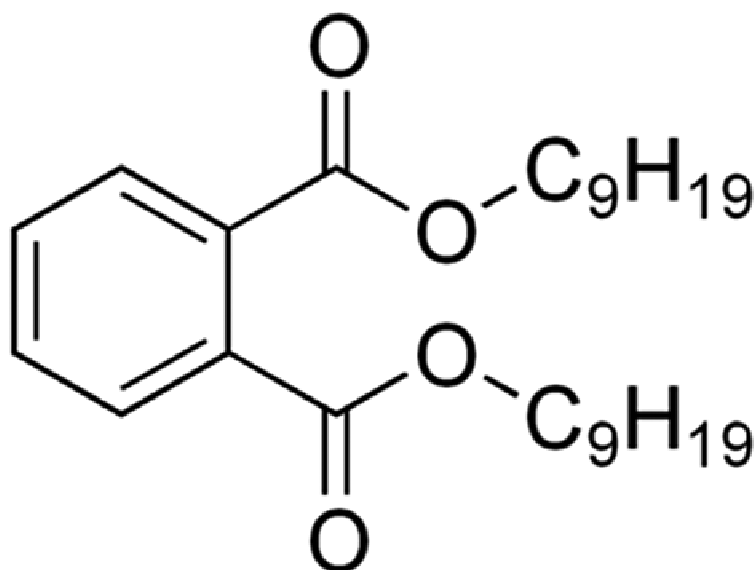


Figure 1 Chemical structure of diisononyl phthalate (DINP)

central obesity and insulin resistance in adults [10,11], suggesting that adult exposure to phthalates may link obesity with related metabolic disorders. This is in addition to a possible contributing role in the development of obesity, as shown by recent data reporting an association between urinary levels of phthalates and higher odds for obesity (body mass index) in children and adolescents [9]. Sirtuins (Sirts) are a group of mitochondrial NAD+-dependent histone deacetylases which have emerged as key epigenetic regulators that act as cellular sensors by detecting energy availability and modulating metabolic processes [12]. Sirtuins are involved in several cellular functions including chromosomal stability, DNA repair, the cell cycle, apoptosis, metabolism, and aging by deacetylating a variety of transcription factors, histones, and nonhistone proteins. Several studies identified Sirt3 as a potentially important factor in the pathogenesis of diabesity. For instance, Zhang et al. showed that butyl benzyl phthalate (BBP) decreases Sirt1 and Sirt3 gene expression and protein levels in HepG2 cells [13]. Additionally, our group also showed that several phthalates and persistent organic pollutants differentially modulate the sirtuin family in macrophage cells [14]. The aim of this study was to investigate sirtuin regulation, levels of lysine acetylation proteins, and the generation of reactive oxygen species under exposure to diisononyl phthalate (DINP) by HepG2 cells.

2. MATERIALS AND METHODS

2.1. Cell Culture and Exposure Conditions.

The human hepatocellular carcinoma cell line (HepG2) was cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin/streptomycin (Sigma) at 37 °C in 5% CO_2 in an incubator. For treatments with DINP (Sigma), the cells were cultured in 24-well plates (2×10^5 cells per well in 1 mL of medium) for 1 day before using them for exposition. A stock of DINP 100 mg/mL in dimethyl sulfoxide (DMSO, Sigma) was prepared, and logarithmic dilutions with DMSO were performed until to obtain solutions with DINP 10 000, 1000, and 100 μg/mL, and then the required volume was added to each well to obtain the desired concentration. The control used to compare was medium plus DMSO. HepG2 cells were cultured with various concentrations of DINP (0.1, 1, 10, and 100 μg/mL). After 48 h of treatment, mRNA was extracted and quantified for gene expression. To analyze protein expression of Sirt1, Sirt2, Sirt3, Sirt5, and acetylated proteins, HepG2 cells were cultured with the same DINP concentrations (0.1, 1, 10, and 100 μg/mL), and after 72 h of treatment, nuclear and mitochondrial proteins were extracted and analyzed by Western blot. Briefly, the cells were harvested in ice-cold buffer B (containing 20 mM HEPES, 1.5 mM $MgCl_2$, 25% glycerol, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA) and supplemented with protease inhibitor cocktail and 0.5 mM PMSF (Sigma).

2.2. Cell Viability Assay.

Cell viability was determined by the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using thiazolyl blue tetrazolium bromide powder (Sigma) as described previously.[13] Briefly, HepG2 cells were plated and incubated with different concentrations of DINP at 48 h. The cells were incubated with MTT solution (10 μL per well, 5 mg/mL in PBS) for 4 h at 37 °C. The supernatant was then removed, and formazan crystals were dissolved in 100 μL of DMSO with orbital shaking. Optical densities of the resultant solutions were determined colorimetrically at 490 nm using a microplate reader (BioRad). The percentage of viable cells was determined by comparing the optical densities of cells incubated with the varying concentrations of DINP and DMSO control.

2.3. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

The total RNA was extracted using TRI reagent (Sigma). The cDNA was made from 1 µg of total RNA by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The forward and reverse primers used in the present study are shown in Table 1. Real-time PCR was performed using SYBR select master mix (Applied Biosystems). The 18S cDNA level was used as a reference gene, the expression levels were normalized to 18S, and gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and expressed as fold change [15]. All assays were carried out in triplicate.

Table 1. Gene primers used in this study.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
h18S (F4)	CTCTAGATAACCTCGGGCCG	GTCGGGAGTGGGTAATTTGC
hSirt1 (F2)	TAGCCTTGTCAGATAAGGAAGGA	ACAGCTTCACAGTCAACTTTGT
hSirt2 (F1)	ATCCACCGGCCTCTATGACAA	CGCATGAAGTAGTGACAGATGG
hSirt3 (F1)	GACATTCGGGCTGACGTGAT	ACCACATGCAGCAAGAACCTC
hSirt4 (F2)	GAATCGGGGATACCAGACTACA	GCCAGCCTACGAAGTTTCTCG
hSirt5 (F1)	GCCATAGCCGAGTGTGAGAC	CAACTCCACAAGAGGTACATCG
hSirt6 (F1)	CCCGGATCAACGGCTCTATC	GCCTTCACCCTTTTGGGGG
hSirt7 (F1)	CGTCCGGAACGCCAAATAC	GACGCTGCCGTGCTGATT

2.4. Western Blotting.

Once the nuclear and mitochondrial proteins were extracted, the concentration was estimated with BSA reagents from Thermo Scientific. Equal amounts of protein were separated using SDS-PAGE and transferred to nitrocellulose membranes (Thermo Scientific). Membranes were blocked with 5% milk in TBS containing 0.1% Tween 20 (Sigma) for 1 h, and they were incubated with the respective primary antibody 1:1000: Sirt1 (Cell Signaling, MA, #2496), Sirt2 (Cell Signaling, #12672), Sirt3 (Cell Signaling, #2627), Sirt5 (Santa Cruz, CA, #271635), acetylated-lysine (Cell signaling, #9441), or β-actin (Cell Signaling, #8457). AP conjugated secondary antibody (Invitrogen #T2191) was used for detection and quantification of immunoblots. Membranes were developed using enzymatic substrate. Band

densities were analyzed by ImageStudio Lite software (LI-COR). All assays were made in triplicate.

2.5. Measurement of Reactive Oxygen Species.

Reactive oxygen species levels (ROS) were measured using 2′ ,7′ -dichloro-fluorescein diacetate (DCFDA, Sigma). Briefly, DINP-treated cells were seeded in a 96-well plate for 48 h. Cells were then incubated with 25 mM DCFDA for 45 min at 37 °C, and the fluorescence was measured using a plate reader (Fluoroskan Ascent FL, Thermo Scientific).

2.6. Statistical Analysis.

All data are presented as the mean ± standard error (SE). Statistical significance was determined by One- way ANOVA ($p < 0.05$). Pairwise comparisons were made using Tukey's test ($p < 0.05$).

3. RESULTS

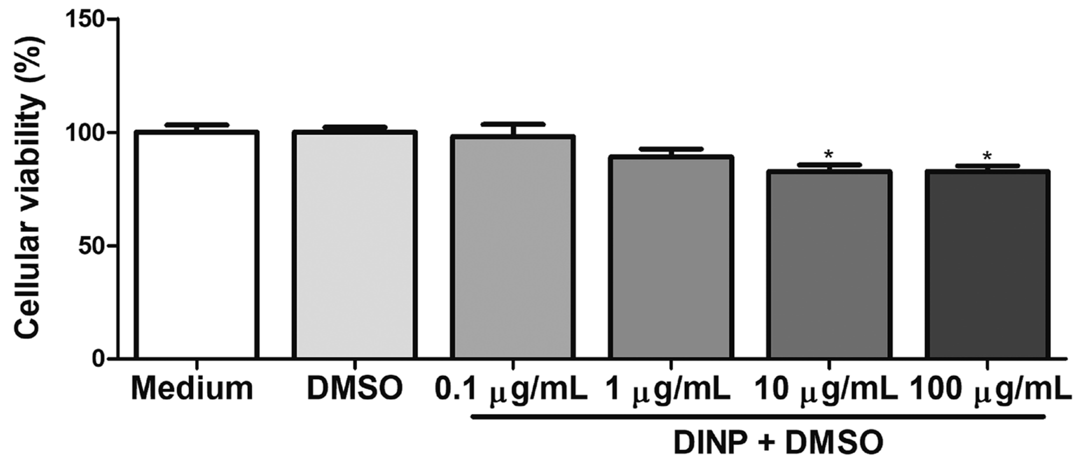
3.1. DINP Affects Cell Viability in the HepG2 Cell Line.

To determine the effect of DINP on cell viability, HepG2 cells were treated with different doses of DINP (0.1, 1, 10, and 100 μg/mL) for 48 h. The results showed that DINP has a dose- dependent effect on cell viability in HepG2 cell lines (Figure 2). Low concentrations of DINP (0.1 and 1 μg/mL) showed insignificant levels of cell death. Treatments with 10 and 100 μg/mL were significantly affected by DINP as compared to the control ($p < 0.05$); however, 85% of cells were viable for both concentrations. To evaluate if the DINP diluent could affect the cellular viability, two diluents, DMSO (Figure 2A) and ethanol (Figure 2B), were tested. In both cases no effect of the diluents on the cellular viability was observed.

3.2. DINP Differentially Regulates Sirtuin Expression.

The effect of DINP treatment on the gene expression of sirtuins was determined by treating HepG2 cells with different doses of DINP (0.1, 1, 10, and 100 μg/mL) for 48 h. Sirt1, Sirt2, and Sirt5 gene expression was significantly decreased at a low concentration (1 μg/mL) of DINP when compared to control ($p < 0.05$). The gene expression levels of Sirt1, Sirt2, and Sirt5 decreased to 50% at 1 and 10 μg/mL DINP, while gene expression decreased by 75% with DINP 100 μg/mL (Figure 3A, B, E). For Sirt3, the gene expression levels did not reach below 50% for all concentrations tested. Interestingly, gene expression of Sirt3 showed a significant decrease at 0.1 μg/mL DINP ($p < 0.001$) (Figure 3C), displaying a dose-dependent effect. Others sirtuins (Sirt 4, 6, 7) evaluated remain unaffected (Figure 3D, F, G).

A)



B)

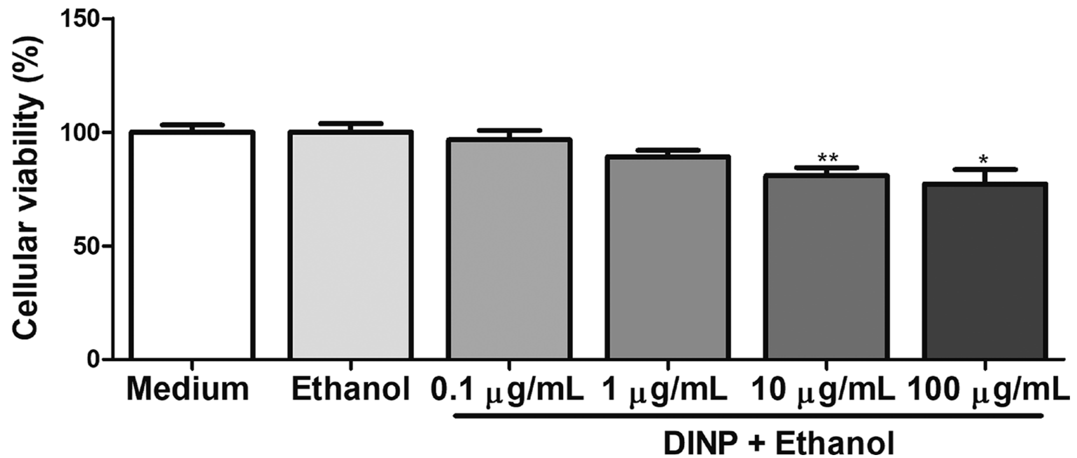


Figure 2. DINP induced cytotoxicity in HepG2 cells. HepG2 cells were incubated with different concentrations of DINP for 48 h. Cell viability was determined by MTT assay. Two different (A) DMSO and (B) EtOH diluents were tested, demonstrating that neither causes an adjuvant or cytotoxic effect. The labels medium, DMSO, and ethanol represents DMEM medium, medium+DMSO, and medium+ethanol, respectively. Data represent the means ± SE (n = 6), *$p < 0.05$, **$p < 0.01$, versus untreated control.

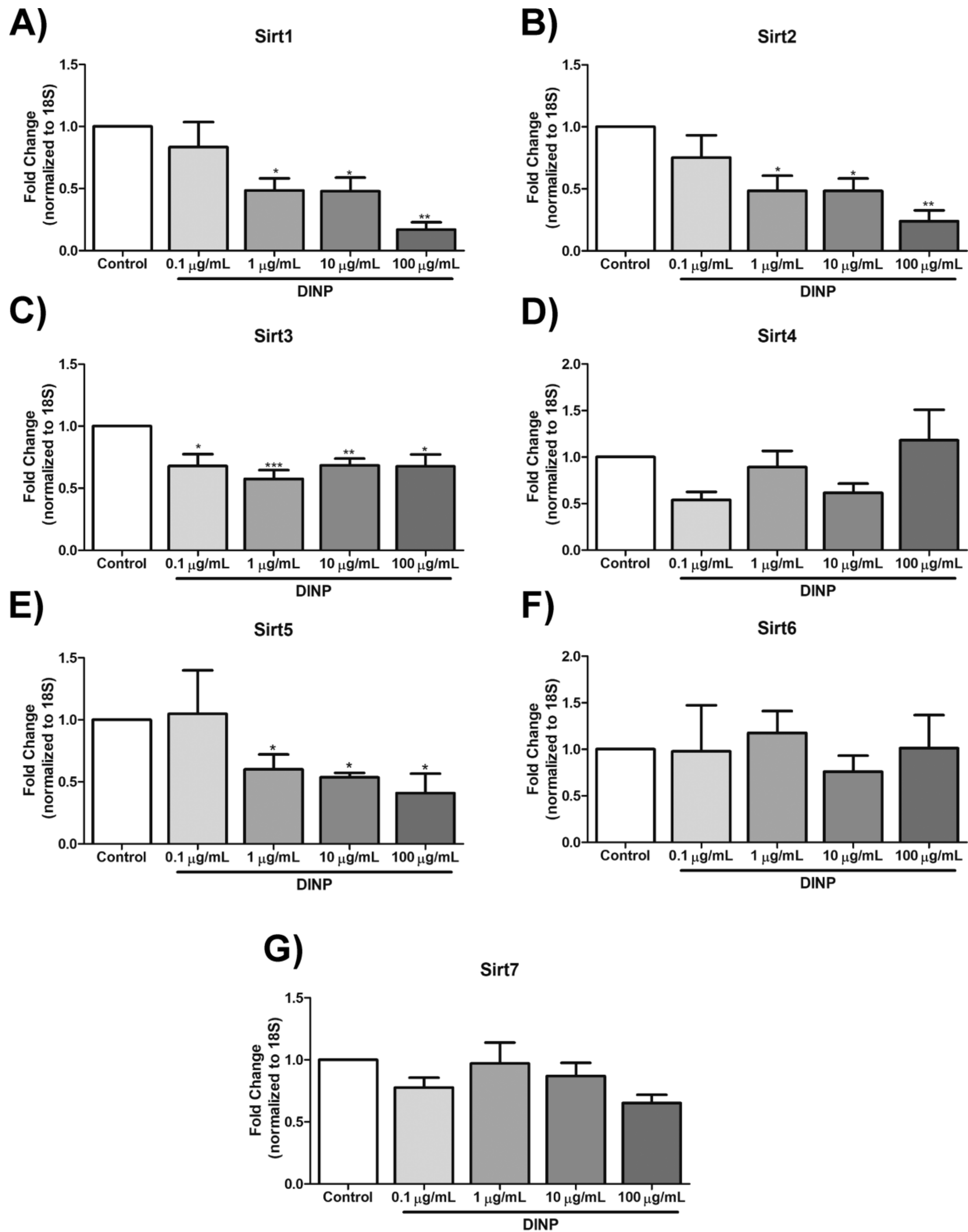


Figure 3. DINP decreased the expression of Sirt1, Sirt2, and Sirt3 in HepG2 cells. HepG2 cells were exposed for 48 h at different concentrations (0.1, 1, 10, and 100 μg/mL) of DINP. (A−G) The mRNA levels of Sirts 1 to 7 were determined by qPCR. The 18S gene served as an endogenous control. All data are mean ± SE, *p < 0.05, **p < 0.01, ***p < 0.001 compared to an untreated control; n = 3.

3.3. DINP Decreases Sirt1 and Sirt3 Protein Levels and Increases Acetylated Protein in HepG2 Cells.

To determine the effect of DINP on Sirt1, Sirt2, Sirt3, Sirt5, and acetylated protein levels, HepG2 cells were treated with different doses of DINP (0.1, 1, 10, and 100 μg/mL) for 72 h. Treatments of 1 to 100 μg/mL DINP significantly decreased Sirt1 and Sirt3 protein levels ($p < 0.05$) (Figure 4A,B). The protein expression levels of Sirt1 at 1 μg/mL DINP decreased 25% and at 100 μg/mL decreased 50% compared to the control. Similarly, protein levels of Sirt3 treated with 1 to 100 μg/mL decreased to 50% compared to the control (Figure 4B). On the other hand, 100 μg/mL doubled the protein acetylation levels ($p < 0.05$) (Figure 4C, D). These results are consistent with the gene expression results. Interestingly, Sirt2 and Sirt5 did not show significant changes (Figure 4B).

3.4. DINP Treatment Increases Reactive Oxygen Species (ROS) Levels.

To investigate if DINP induced ROS production, HepG2 cells were treated with doses of DINP (0.1 to 100 μg/mL) for 48 h. The results showed that DINP increased the ROS levels in a dose-dependent manner and ROS levels were significantly increased ($p < 0.05$) in the DINP 100 μg/mL treatments (Figure 5).

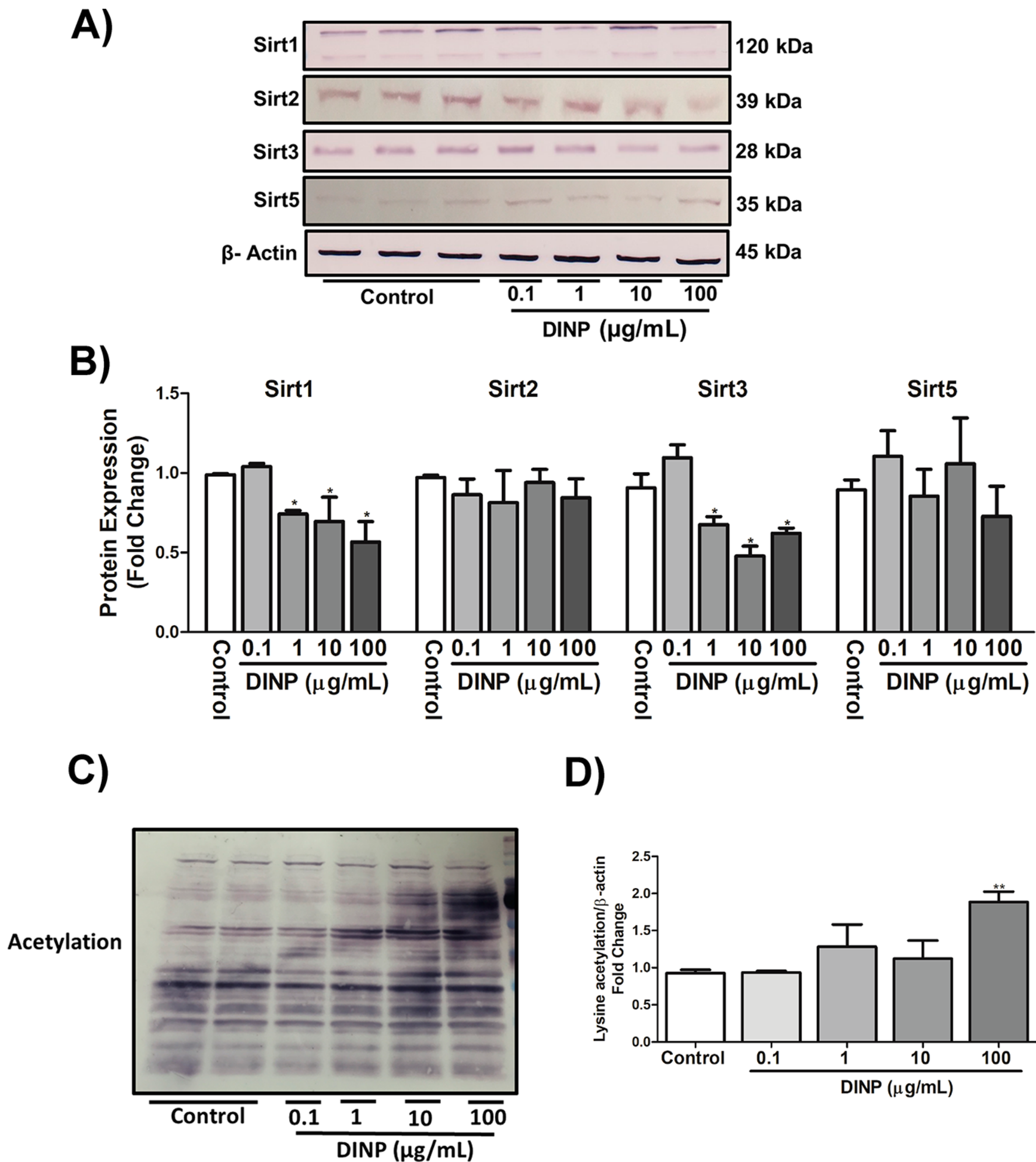


Figure 4. Protein levels of Sirt1 and Sirt3 were affected at 1 μg/mL of DINP, and acetylation protein levels were increased. HepG2 cells were exposed for 72 h at different concentrations (0.1, 1, 10, and 100 μg/mL) of DINP. (A, B) Protein levels of Sirt1, Sirt3. (C, D) Lysine acetylation was determined by Western blot analysis. Quantification of expression was described as the ratio of protein level to β-actin level. One representative blot is shown. All data are mean ± SE, *p < 0.05, **p < 0.01 compared to an untreated control; n = 3.

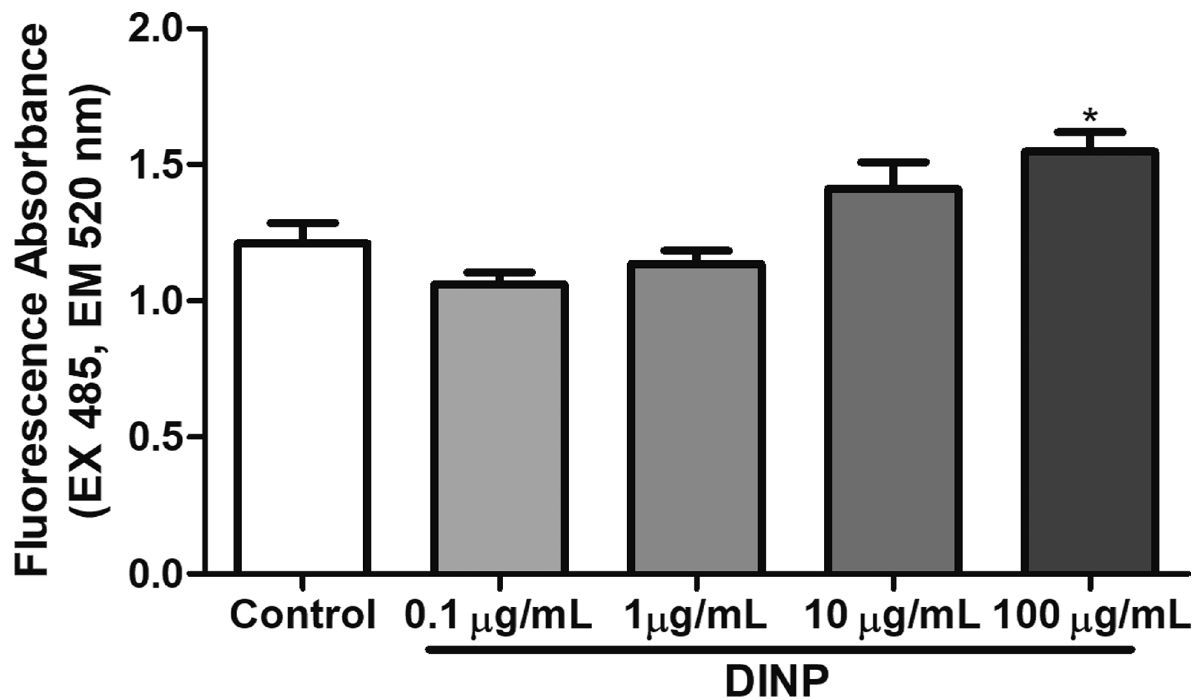


Figure 5. Highest concentrations of DINP increased ROS levels in HepG2 cells. Reactive oxygen species (ROS) levels were determined by measuring oxidized dichlorofluorescein (DCF) levels using 2,7-dichlorofluorescein diacetate (DCFDA). Data represent the mean ± standard error versus untreated control. $^*p < 0.05$, $n = 4$.

4. DISCUSSION

Several studies have shown that phthalates have toxic effects at levels similar to those to which average populations are currently exposed [16-18]. DINP has been identified as a food contaminant, and it has been banned and restricted by regulatory agencies in the EU [19,20]. Children may be particularly susceptible to the effects of DINP because they have higher relative exposures compared with adults (due to greater dietary intake per kilogram), their metabolic (i.e., detoxification) systems are still developing, and key organ systems are undergoing substantial changes and maturations that are vulnerable to disruptions. In 2009−2010 NHANES data, it was reported that urinary metabolites of DINP were detected in 98% of the population. Additionally, cross-sectional data from NHANES from 2009 to 2012 show positive associations of DINP metabolite concentrations with insulin resistance and systolic blood pressure z scores in children and adolescents [21]. Therefore, in the present study, we investigated the possible effects of exposure to DINP phthalate on sirtuin gene and protein expression levels. Because phthalate exposure in humans is widespread, it is important to know if phthalates can interact with epigenetic regulators such as sirtuins, which can promote long-term changes in metabolic homeostasis, potentially leading to deleterious physiological consequences. Our results showed, for the first time, that DINP decreased Sirt1, Sirt2, and Sirt3 gene expression (Figure 3) and Sirt1 and Sirt3 protein levels were downregulated in a dose-dependent manner (Figure 4). The study by Zhang et al. [13] discovered that when HepG2 cells were treated with benzyl butyl phthalate (BBP), Sirt1 and Sirt3 gene and protein expression were decreased, which agrees with our results. Reduced levels of Sirt1 and Sirt3 expression have been shown to cause impaired metabolic function or age-related complications [22,23]. Therefore, DINP-induced down-regulation of Sirt1 and Sirt3 may have a plausible correlation to recent metabolic-related health concerns.

Oxidative stress is due to the continuous production of ROS, which imbalances the production of free radicals and the antioxidant system and can usually induce oxidative damage such as DNA oxidation, protein oxidation, and lipid peroxidation [24,25]. Thus, oxidative stress can be considered an overwhelming generation of reactive species or a general disruption of redox cellular homeostasis. It has been

reported to play an important role in the pathogenesis of diabetes, asthma, and other diseases [26-28]. ROS may function as regulators of cell signaling, which may impact the development of a metabolic disorder. The production of ROS is negatively associated with cell viability, energy metabolism, and metabolic diseases, and it is already known that during conditions of metabolic stress such as obesity and metabolic syndrome, an oxidative stress environment is created [25]. Oxidative damage by release of ROS has been attributed to some phthalates, including DEHP. Recently, several studies have reported an association between exposure to phthalates including DINP and oxidative stress. For example, Liu et al. [29] observed that ROS levels were increased significantly by mono(2-ethylhexyl) phthalate (MEHP). Aly et al. [30] showed that dibutyl phthalate (DBP) induced testicular toxicity by oxidative stress. MEHP induces apoptosis through ROS-mediated mitochondrial-dependent pathway in HUVEC cells [31]. Franken et al. [32] showed a highly significant association of phthalate exposure with oxidative stress via DEHP and its main metabolite MEHP. Kang et al. [33] showed that DINP contributes to the development of allergic asthma by promoting the elevation of oxidative stress and activating the NF-Kβ signaling pathway. The toxic effects of DINP such as acute toxicity, mutagenicity, carcinogenicity, and reproductive and developmental toxicity [34-36] have been associated with ROS accumulation. For instance, Ma et al. [37] demonstrated that the hepatic injury in mice was caused by the toxic effect of ROS accumulation by DINP. Similarly, our results showed that ROS production increased when cells are exposed to DINP (Figure 5). The toxic effects associated with altered levels of subcellular ROS are largely prevented by various antioxidants, many of which are regulated by sirtuins and appear to be an integral part of an important cellular defense mechanism against oxidative stress and ROS formation. Several studies support the idea that sirtuins play very important roles in maintaining proper cellular redox balance and seem to be protecting the body from the adverse effects of oxidative stress and associated diseases. Therefore, sirtuins have emerged as key players in regulating the antioxidative capacity of cells. For instance, several reports support the idea that Sirt1 can mediate an oxidative stress response, directly deacetylating several transcription factors that regulate antioxidant genes [38-41].

For example, sirtuins can regulate oxidative stress mainly through forkhead transcription factor (FOXO), which controls a variety of cellular processes including ROS production, DNA repair, and apoptosis [39]. Results reported by Brunet et al. [38] demonstrated that Sirt1 deacetylates FOXO1 and FOXO3a and increases cellular resistance to oxidative stress in HEK 293 cells, where FOXO deacetylation confers cell resistance to oxidative stress. During oxidative stress, Sirt1-FOXO3a interaction increased the transcription of stress-resistant genes and decreased the expression of FOXO3a-dependent proapoptotic genes [38]. In our case, DINP indeed acetylated several proteins in HepG2 cells (Figure 4C, D). Sirt2 has a critical role in the modulation of the oxidative stress response. Sirt2 is a central regulator of the defense mechanism against ROS and has been shown to deacetylate and activate Forkhead box O3 (FOXO3a), a transcriptional activator of superoxide dismutase 2 (SOD2) which in turn reduces the ROS level [42]. On the other hand, Sirt3 is known to mediate the flow of mitochondrial oxidative pathways, plays an important role in the detoxification of ROS, and therefore regulates the production of ROS [43]. Sirt3 has been shown to mediate the deacetylation of enzymes that are responsible for the reduction of ROS, leading to protection against oxidative stress. For instance, Sirt3 has been shown to deacetylate and activate isocitrate dehydrogenase 2 (IDH2), SOD2 (by direct deacetylation and activation of the enzymatic function) [44-46], and catalase, all key enzymes in reducing the cellular levels of ROS [25, 47, 48]. Sirt3 is shown to activate antioxidant machinery in the mouse heart by inducing the expression of SOD2 and catalase through deacetylation of the transcription factor FOXO3a [47]. In addition, decreased Sirt3 levels have been found in human epidermal keratinocytes after ozone exposure, which was correlated with increased DNA damage, higher levels of cellular $H2O2$, and reduced SOD2 protein levels [49], and loss of Sirt3 has been shown to increase the production of ROS [50]. It has been reported that Sirt5 also desuccinylates and activates the ROS-detoxifying enzyme superoxide dismutase 1 (SOD1) and, at least in the brain, regulates the SOD2 expression [51, 52]. These findings strengthen evidence that phthalates can activate oxidative stress via sirtuins (Figure 6). In this work, decrease of sirtuins 1 and 3 was observed at 1 μg/mL of DINP; however, cellular toxicity and

protein deacetylation started at 100 µg/mL of DINP. Therefore, it might be that the residual sirtuin content and activity was sufficient to maintain cellular vitality. The use of sirtuin inhibitors and activators could help to clarify this issue.

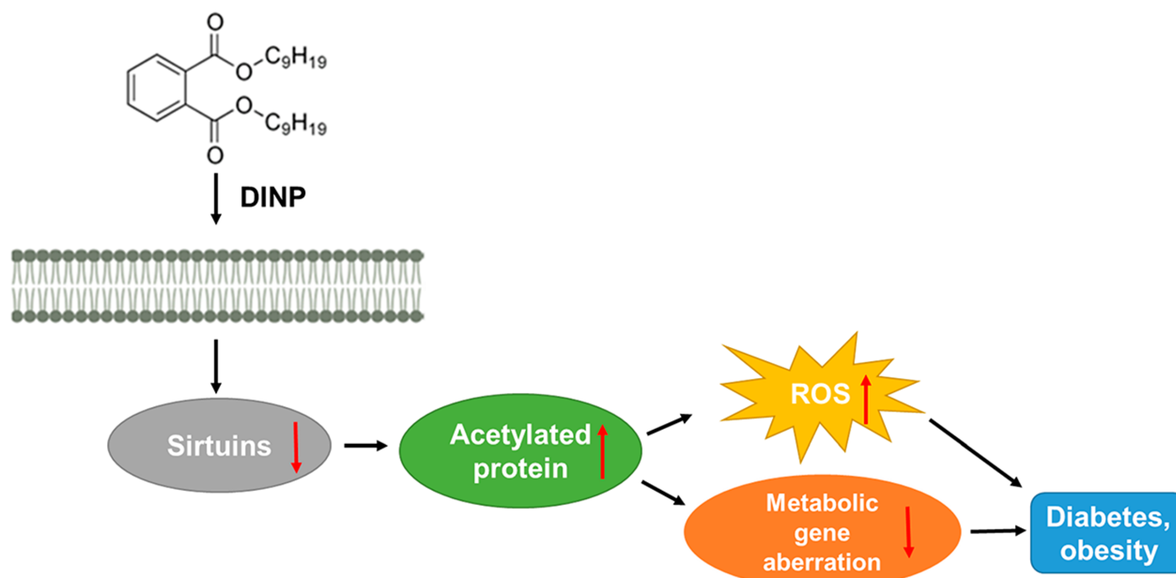


Figure 6. Proposed model for the effect of DINP in sirtuin regulation. DINP treatment induces downregulation of sirtuins, which leads to increased levels of ROS production by acetylation of several proteins involved in the oxidative stress pathway and modulation of metabolic genes.

5. CONCLUSION

Our study suggests that DINP can alter the potential epigenetic disruptor sirtuin family and thus lead to the induction of ROS via sirtuins. We observed a decrease in gene expression and protein levels of Sirt1 and Sirt3 proteins after exposure to DINP at concentrations that do not affect HepG2 cell viability. This can be correlated with an increase in acetylated proteins, which as a result leads to an increase in ROS levels. However, considering the widespread exposure to DINP in the population, in vivo studies will be required to understand the effect of DINP on the regulation of sirtuins. Identifying the mechanism whereby phthalate exposure is associated with a metabolic syndrome remains an important area of research.

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Chapter 2

Phthalates affect the *in vitro* expansion of human hematopoietic stem cell

Abstract

Phthalates are esters of phthalic acid used industrially as plastic additives; however, these are not covalently bound to the polymer matrix and therefore can be released to the environment. The aim of this study was to evaluate the effect of four phthalates: dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), diethyl phthalate (DEP) and diethylhexyl phthalate (DEHP) on the *in vitro* expansion of human hematopoietic cells from umbilical cord blood. For this, 0.5 x 10^6 cells/mL were exposure to concentrations ranging from 0.1 to 100 µg/mL and the total cell expansion was determined after 14 days of culture in IMDM-cytokines medium. The control cultures attained 1.31 ± 0.21 x 10^6 cell/mL, whereas the cultures exposed to DBP, BBP and DEHP showed a reduction from 23 to 81%, 17 to 69% and 15 to 93.5%, respectively. DEP did not affect the total cell expansion. The most significant decrease on total cell expansion was observed at 0.1 µg/mL DBP, 100 µg/mL BBP and 10 µg/mL DEHP ($p < 0.05$). Additionally, the effect of these compounds on the expansion of hematopoietic progenitors was analyzed by clonogenic assays as colony forming units (CFU). The CFU decreased considerably compared with respect to the control cultures. The reduction was 74.6 and 99.1% at 10 and 100 µg/mL DBP respectively, whereas 100 µg/mL BBP and 100 µg/mL DEHP reduced the CFU expansion in 97.1% and 81%, respectively. Cultures exposed to DEP did not show significant differences. The results demonstrate the toxicity of DBP, BBP and DEHP on the human hematopoietic stem cells.

Gutiérrez-García AK, Flores-Kelly JM, Ortiz-Rodriguez T, Kalixto-Sanchez MA, De Leon-Rodriguez A. Phthalates affect the *in vitro* expansion of human hematopoietic stem cell. Cytotechnology. 2019, 71, 2, 553-561.

1. INTRODUCTION

Phthalate esters, also called phthalates, are a group of synthetic, liquid, colorless, viscous and lipophilic chemical compounds. These compounds are used as plasticizer additives to provide flexibility to the finished plastic product or as a vehicle for coloring, gloss or fragrance. Dibutyl phthalate (DBP) is used as a component of latex adhesives. It is also used in cosmetics and other personal care products as a plasticizer in cellulose plastics, and as a solvent for dyes [1]. Benzyl butyl phthalate (BBP) is most commonly found in vinyl products including flooring, paints, adhesives, children's toys, food packaging, etc. [2]. Phthalates, such as diethylhexyl phthalate (DEHP) and diethyl phthalate (DEP), are some of the most widely used and can be found in a wide variety of products such as tablecloths, curtains shower, etc. (Figure 7) [3, 4]. It has been reported that in some cases phthalates can represent up to 40% of a finished product for direct use by the consumer [5]. Specifically, in the area of health, some medical materials have been analyzed and 20–40% of phthalates have been found in them [6]. When used as plasticizers these additives do not chemically bond to the polymers of the plastic and therefore can be released, migrate and evaporate to the environment around them. These contaminants have been found in food, air, soil, water and sediments [7-10]. Therefore, humans are in contact with them through different exposure routes. For example, oral, medical, dermal and inhalation exposure is very common for high molecular weight phthalates such as BBP or DEHP. For the DEP, because it is of low molecular weight, the main routes of exposure are dermal and inhalation since it is used mainly as a solvent and vehicle for fragrances and ingredients for cosmetics, instead of as a plasticizer [3, 11-14]. This explains why in humans, phthalates have been found in urine, blood, sweat, breast milk, saliva, amniotic fluid, and umbilical cord blood (Table 2) [15-19]. Phthalates are used extensively in our daily life, the effect of phthalates exposure has become an important issue, due to their persistence in the environment, resistance to chemical or enzymatic degradation and sequestration and storage in adipose tissue.

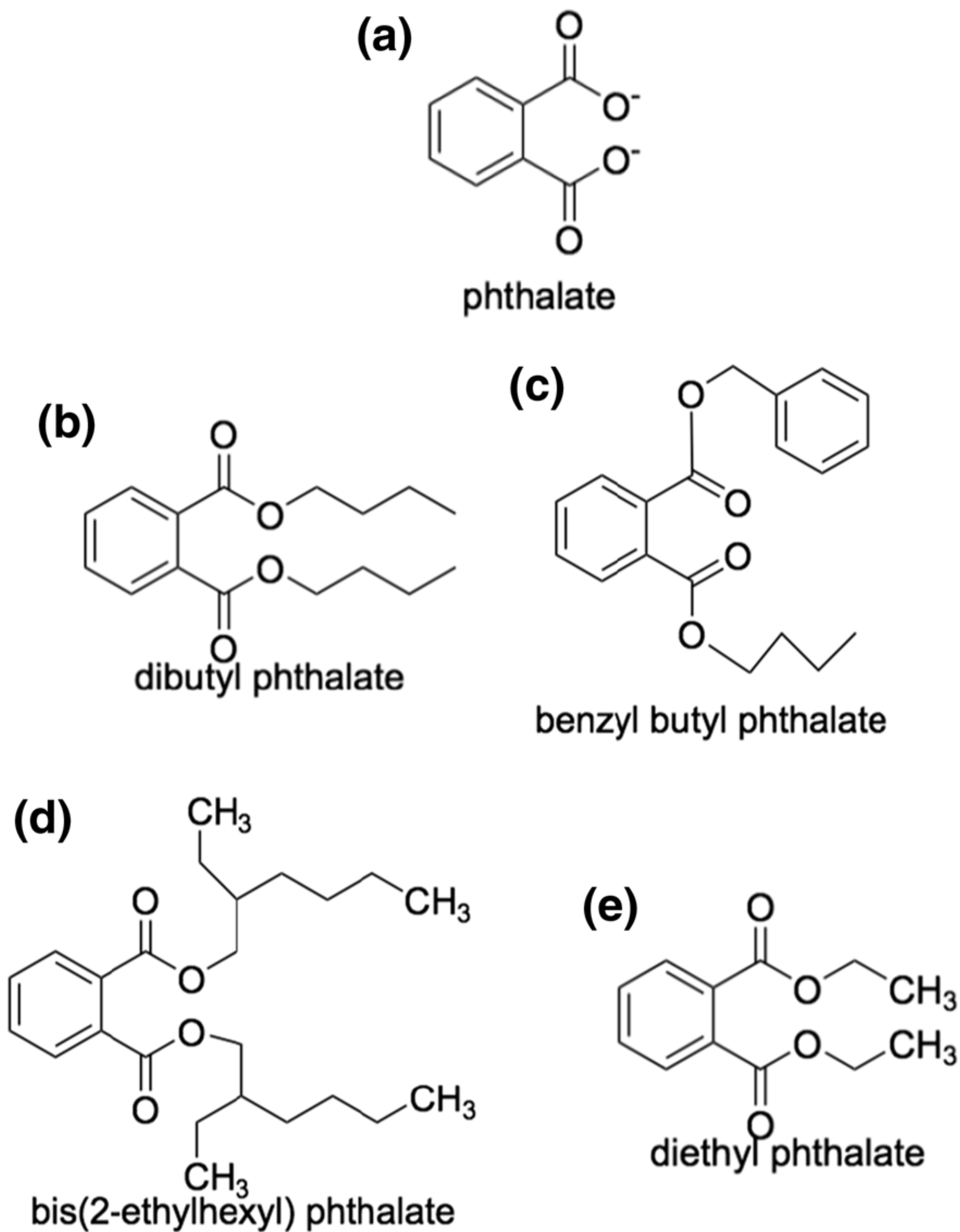

Figure 7. Structure of common phthalates.The structures of the phthalates: a General structure of phthalates. b Dibutyl phthalate (DBP) structure, c benzyl butyl phthalate (BBP) structure, d bis (2-ethylhexyl) phthalate (DEHP) structure, e diethyl phthalate (DEP) structure.

Table 2. Concentrations range of phthalates or the main metabolite found in body fluids

Phthalate	DEP	DBP	BBP	DEHP	Reference
Urine	6.76-6978 µg/g [a]	20.7-342 µg/g [b]	37.9 µg/L [c]	1.11-108 µg/g [d]	[15, 53]
Maternal Blood	18.90 µg/mL	7.67 µg/mL	-	8.84 µg/mL	[54]
Sweat	3.94-750 µg/g [a]	8-58.6 µg/g	-	8-576 µg/g	[15]
Breast Milk	0.31 ng/g	0.62-1.2 ng/g	1.2 µg/L [c]	156-398 ng/g	[18, 55, 56]
Saliva	91.4 ng/mL [a]	22.4±9.8 nmol [b]	353.6 ng/mL [c]	1017±147 µg/g	[57, 58]
Amniotic Fluid	0.70 µg/L [a]	3.53 µg/L [b]	-	1.47 µg/L [d]	[19]
Umbilical Cord Blood	11.92 µg/mL 8.99 µg/L	5.71 µg/mL 68.14 µg/L	22.5 µg/L	5.20 µg/mL 187.16 µg/L	[52, 54]

Main metabolites: [a]MEP (Mono-ethyl phthtalate) [b]MnBP (Mono-n-butyl phthalate) [c]MBzP (Monobenzyl phthalate) [d]MEHP (Mono-(2-ethylhexyl) phthalate)

The estimate daily exposure (EDI) of DEHP in United State are: 2.2–7.4 and 2.6–3.8 µg/kg-bw/day in adults and children, respectively. DEP are 5.5–11.4 and 1.7–6.3 µg/kg-bw/day [20], and DBP 1.0 µg/kg-bw/day for general population [21]. An important human exposition to DEHP occur during medical procedures using PVC-containing devices and blood stored in plastic bags [22]. For example, patients undergoing hemodialysis can receive as much as 150 mg of DEHP in 5 h [23]. Lagerberg et al. [24] reported that plasma storage bags release 5720 µg/mL of DEHP after 24 h of storage, while in stored erythrocytes the concentration of DEHP increased from 4.1 ± 0.9 µg/mL at 33 ± 11 µg/mL at day 42 of storage. Inoue et al. [25] found in total blood stored for 21 days up to 83.2 µg/mL of DEHP. In cord blood up to 17.8 ± 2.7 µg/mL of DEHP was found in the first 24 h of storage. It is of particular

interest to know what effect phthalates exert on cell viability when interacting with umbilical cord blood that is used as a source of stem cells [26]. Hematopoietic stem cells extracted from umbilical cord blood have shown advantages over hematopoietic stem cells from bone marrow or mobilized peripheral blood, because invasive techniques are not used to obtain them and because they exhibit a greater potential for proliferation and expansion [27]. Since phthalates can cause cell damage and death and are present in blood storage bags [24, 25, 28-30], it is of clinical importance to study the effect of them on the total cell population of umbilical cord blood as it is used for stem cell transplants as a treatment for recurrent malignant hematological tumors, bone marrow failure syndromes, severe congenital immunodeficiency states and some metabolic alterations.

2. MATERIALS AND METHODS

2.1. Reagents

DBP, BBP, DEHP and DEP were purchased from Sigma. MethoCult GF (H4434) was obtained from StemCell Technologies, Inc.

2.2. Cell culture and exposure conditions

The umbilical cord blood was centrifuged at 450xg for 15 min at 25 °C. Subsequently, the white globular bundle was recovered and diluted with PBS pH 7.2. This cell suspension was placed with 7 mL of Ficoll- Paque Plus reagent (Pharmacia) and centrifuged at 550xg for 15 min at 25 °C. The white cell pack was collected and washed with PBS and centrifuged at 800xg for 20 min at 25 °C. Isolated mononuclear cells were resuspended in Iscove modified Dulbecco culture medium (IMDM, Sigma, St. Louis,MO, USA) and 10% Bovine Fetal Serum (SFB, Gibco Grand Island, NY, USA). Cells were grown in 24-well plates, inoculating 0.5 x 10^6 cells/mL in culture medium (IMDM, Sigma) with 10% FBS, 0.1 mg/mL streptomycin, 100 U/mL penicillin and 0.25 µg/mL of amphotericin B (Sigma). The following cytokines (IMDMcyt) were added to the base medium: 5 ng/mL Interleukin-3 (IL-3), 12.5 ng/mL Interleukin-6 (IL-6), 5 ng/mL Seminal cell factor (SCF), 5 ng/mL of FLt-3 receptor ligand (Flt-3-L) (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL of Granulocyte Colony Stimulating Factor (G-CSF) (FILATIL®), 10 ng/mL of Granulocyte and Macrophage Colony Stimulation Factor (GM-CSF) (GRAMAL®) and 3 U/mL of Erythropoietin (Epo) (BIOYETIN®) (Probiomed, Mexico City, Mexico). The plates were placed in an incubator at 37 °C with a 5% CO_2 atmosphere. Later on, day 5, half of the cell suspension was replaced by new IMDMcyt medium (400xg for 15 min at 25 °C) modified from [31, 32]. The cultures were exposed to different concentrations of phthalates (DBP, BBP, DEHP and DEP) for 14 days and a condition without compound (control) was placed. The number of total cells was determined by the trypan blue exclusion method using a hematocytometer [33, 34].

2.3. Determination of hematopoietic progenitors

10,000 mononuclear cells were inoculated in 1 mL of medium (MethoCult® GF H4434 classic), (StemCell Technologies, Inc. Vancouver British Columbia, Canada)

this medium contains the following cytokines: 50 ng of SCF, 10 ng of IL-3, 10 ng of GM-CSF and 3 ng of Epo. The cell suspension was transferred to a 35 mm Petri dish. The plates were incubated for 14 days at 37 °C with a 5% CO_2 atmosphere. The colonies identified and quantified by means of the clonogenic assay were named as: erythroid colony forming units (CFU-E), erythroid burst forming units (BFU-E), granulocyte forming units (CFU-G), forming units of monocytes (CFU-M), granulocyte and monocyte forming units (CFU-GM) or multipotent forming units (CFU-GEMM) [31, 35].

2.4. Statistical analysis

All data are presented as the mean ± standard error (S.E.). Statistical significance was determined by One-way ANOVA ($p < 0.05$) and post hoc analysis by Dunnett's. The statistical analysis was performed using Microsoft Excel v 14.0.

3. RESULTS

3.1. Representative kinetics of cell expansion of DEHP treatments

Figure 8 shows a representative kinetics of cell expansion of human mononuclear cells from cultures exposed from 0.1 to 100 µg/mL of DEHP and the control culture during 14 days of incubation. It was found that for the 4 treatments the lag phase was 4 days. The control presented the exponential phase on day 5, the stationary phase on day 9 and on day 12 the decay phase. In cultures exposed to 0.1 µg/mL DEHP the exponential phase was presented on day 5, starting on day 10 the slope began to decrease, observing a significant difference on cell viability at day 11. The cultures treated with 1 and 10 µg/mL DEHP presented a similar behavior; the exponential phase was on day 5 and the decay phase on day 11; a significant difference was observed on cell viability with respect to control from days 8 and 9, respectively. In cultures exposed to 100 µg/mL DEHP, a significant decrease in cell viability was observed after day 6.

3.2. Effect of phthalates on the *in vitro* expansion of hematopoietic cells

Hematopoietic cells isolated from umbilical cord blood were exposed to different concentrations of phthalates: DBP, BBP, DEHP and DEP ranging from 0.1 to 100 µg/mL. The samples were compared with respect to the control, which obtained a maximum cell expansion of $1.31 \times 10^6 \pm 2.1 \times 10^5$ cell/mL. All DBP concentration used were statistically significant and showed a reduction in cell expansion ($p < 0.05$) (Fig. 9a). For the cultures exposed to concentrations of 0.1, 1, 10 and 100 µg/mL of DBP, a maximum cell expansion of 76.8, 73.2, 62.3 and 19.3% respectively, was obtained (Fig. 9a). In the case of BBP concentrations of 0.1, 1 and 10 µg/mL, did not show a significant reduction with respect to the control. A maximum cell expansion of 83.2, 83.7 and 83.2% respectively was obtained. Nevertheless, at 100 µg/mL BBP a significant reduction was observed ($p < 0.05$), obtaining only 31.7% of cell expansion (Fig. 9b). On the other hand, cultures exposed to 0.1 and 1 µg/mL DEHP did not showed a significant reduction, but at 10 and 100 µg/mL DEHP a significant reduction was observed ($p < 0.05$), showing a maximum cell expansion of 59.3 and 6.5% respectively (Fig. 9c).

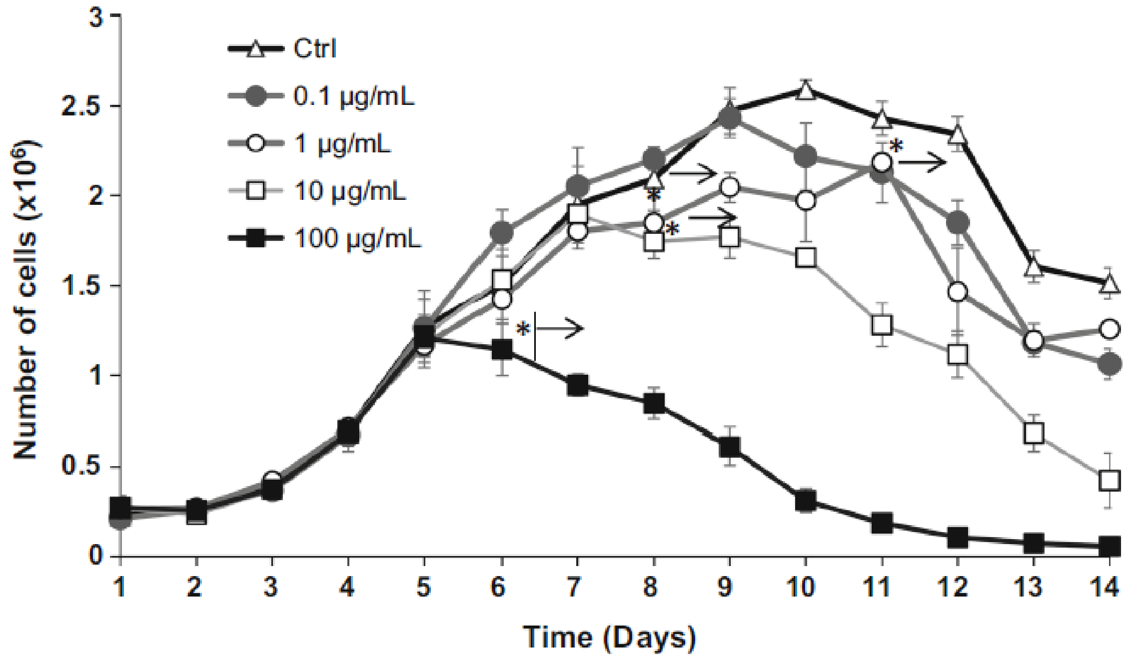

Figure 8. Kinetic of hematopoietic cell expansion exposed to the treatments with DEHP.The graph shows the curve of total cellular expansion during the incubation period of the human mononuclear cells of umbilical cord blood exposed from 0.1 to 100 μg/mL of DEHP and the respective control cultures. The assays were started with 500,000 cells/mL. The assay was carried out in triplicate for the cultures exposed to the different concentrations to be analyzed of phthalate and for the control culture. The statistical analysis showed that, with respect to the control culture, on day 6 the cultures exposed to 100 μg/mL showed significant differences, on the other hand, on day 8 the cultures exposed to 1 and 10 μg/mL showed a significant difference and cells exposed to 0.1 μg/mL DEHP showed a significant difference at day 11

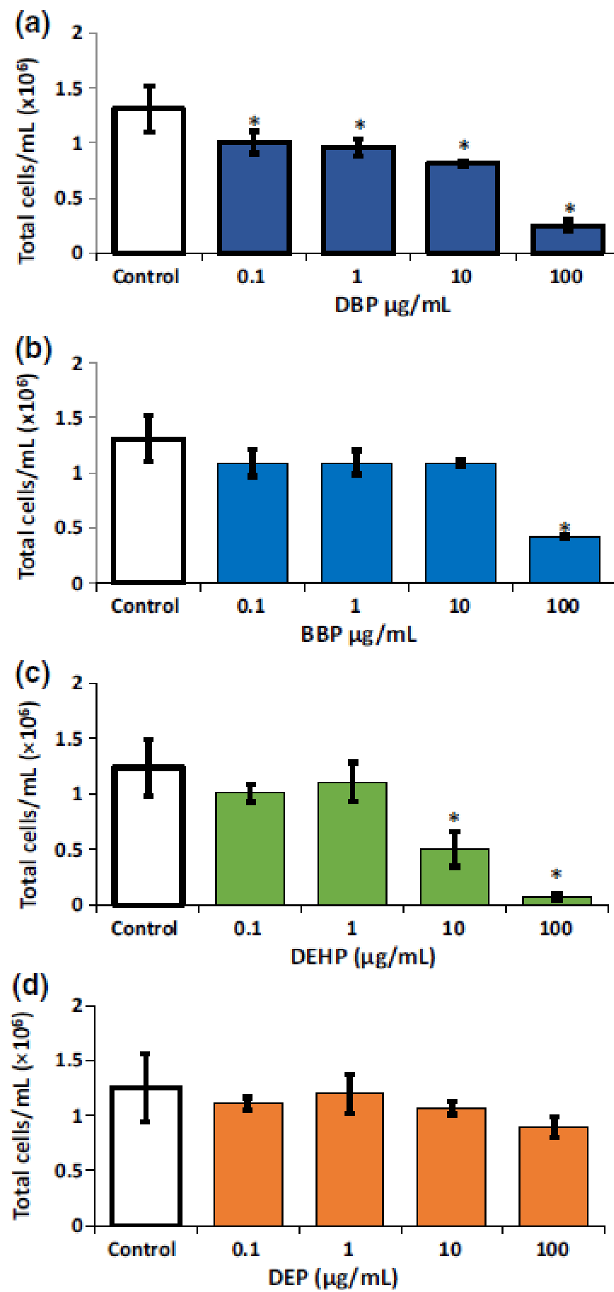

Figure 9. Maximum cell expansion of hematopoietic cells exposed to DBP, BBP, DEHP and DEP. Different concentrations of DBP, BBP, DEHP and DEP (0.1–100 µg/mL) were tested for 14 days. The cultures were started with 500,000 cells/mL. (a) Cultures exposed to DBP showed a significant decrement at lower concentrations of 0.1 µg/mL with respect to the controls ($p < 0.05$) n = 3. (b) Cultures exposed to BBP showed a significant decrement at 100 µg/mL with respect to the controls ($p < 0.05$) n = 3. (c) Treatments of DEHP at 10 and 100 µg/mL showed a significant decrement with respect to the controls ($p < 0.05$; $p < 0.01$). n = 2. (d) Cultures exposed to DEP from 0.1 to 100 µg/mL did not show significant differences with respect to the controls

Cultures exposed to 0.1, 1, 10 and 100 μg/mL of DEP did not show a significant difference in maximum cell expansion with respect to the control (Fig. 9d). These data demonstrate that DBP is the most toxic phthalate due to at lower concentration a significant reduction is observed. But at higher concentration (100 μg/mL) DEHP presents the highest toxicity, since it showed a reduction of 93.5%.

3.3. Effect of phthalates on hematopoietic progenitor cells of umbilical cord blood

The hematopoietic progenitor cells present in umbilical cord blood MNCs exposed to different concentrations of DBP, BBP, DEHP and DEP (0.01–100 μg/mL) were evaluated by means of the Colony Forming Unit (CFU) assay. Cells exposed to 10 and 100 μg/mL DBP showed a significant reduction of the amount of CFU, where 25.4 and 0.9% of CFU were obtained ($p < 0.05$) (Fig. 10a). In the case of BBP, only cells exposed to 100 μg/mL BBP showed a significant reduction ($p < 0.05$), the amount of CFU obtained was 2.9% (Fig. 10b). On the other hand, treatments with 100 μg/mL of DEHP showed a significant decrease of hematopoietic progenitors of 81% compared to the controls ($p < 0.05$) (Fig. 10c). This indicates that DEHP also negatively affected the expansion of hematopoietic progenitors. Interestingly, the CFUs present in treatments of 0.1–100 μg/mL of DEP did not show a significant decrease with respect to the control (Fig. 10d). According to these results it is deduced that DBP presents greater toxicity than the rest of the phthalates (BBP, DEHP and DEP) since at the concentration of 10 μg/mL the number of progenitors was significantly reduced.

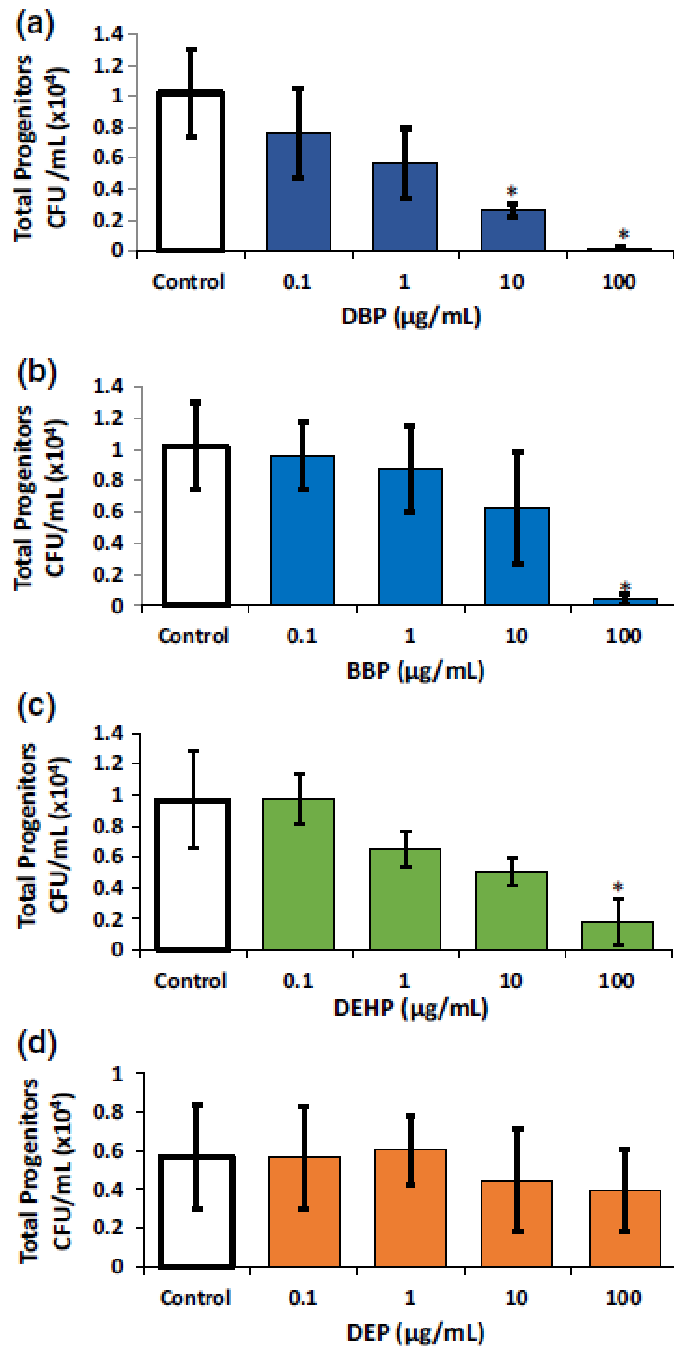

Figure 10. Maximum hematopoietic progenitor expansion exposed to DBP, BBP, DEHP and DEP. The graphs show the number of colony forming units (CFU) of the cultures exposed to DBP, BBP, DEHP and DEP in range of 0.1–100 µg/mL. Cultures were started with 10,000 cells/mL. (a) DBP treatments did show a significant decrement at 10 and 100 µg/mL and n = 3 ($p < 0.05$). (b) Cultures exposed to 100 µg/mL BBP showed a significant decrement and n = 3 ($p < 0.05$). (c) Treatments with DEHP showed a significant decrement in the amount of CFUs exposed to 100 µg/mL compared to controls ($p < 0.05$) n = 2. (d) Treatments with DEP did not show any significant difference between treatments and controls

4. DISCUSSION

In the present study, the cytotoxic effect of phthalates DBP, BBP and DEHP on human hematopoietic cells was demonstrated. Andersen et al. [36] reported the disruptive effect of DBP and BBP, since they found that these compounds have affinity to the estrogen receptor, in addition to inducing the proliferation of MCF-1 cells. Jones et al. [37] found that different phthalates (including DBP) have toxicity against the WI-38 cell line, which is derived from fibroblasts of human lung tissue. Krüger et al. [38] showed that both DBP and BBP were toxic at concentrations of 13.9 µg/mL and 15.6 µg/mL respectively for the B4G12 cell line, which is derived from corneal endothelial cells. Manz et al. [39] demonstrated that DEHP was toxic to promyelocytic leukemia cells (HL-60) at concentrations of 100 µg/mL, however, at 10 µg/mL no toxic effects were observed but shown an alteration in the migration process. Anderson et al. [40] showed that DEHP was cytotoxic and produce DNA damage in human leucocytes and lymphocytes. Sicińska [41] demonstrated that DBP and BBP induce haemolysis in human erythrocytes at concentrations of 10 and 5 µg/mL, respectively and eryptosis at concentrations of 1 µg/mL DBP and 2.5 µg/mL BBP. On the other hand, DEHP induce the formation of stomatocytes in red blood cells (RBCs) at concentrations as low as ng/mL [42]. These agree with the results obtained in the present work, given that at concentrations of 100 µg/mL DBP, BBP and DEHP had the highest toxicity (Figure 9a–c); where the DBP turned out to be the most toxic between these compounds tested; since as seen in Figure 9a, all DBP concentrations evaluated showed a significant decrease in the number of cells with respect to the control, however, in the case of BBP, DEHP and DEP at lower concentrations, most of them did not present toxicity for the MNC's. Nevertheless, the possibility that these cells undergo some type of modification is not ruled out since the endocrine disruptors can produce a range of effects similar to those that estrogen would produce naturally on different hematopoietic strains. A previous study reported that hematopoietic stem progenitor cells contain the receptors for follicle-stimulating hormone, luteinizing hormone, prolactin, androgens, β-estrogen and progesterone capable of stimulating hematopoiesis [43]. Therefore, phthalates may be exerting their action through these nuclear receptors. On the other hand, it has been reported that phthalates can cause DNA damage [44]. For instance, DBP,

BBP and DEHP may affect DNA methylation, histone modifications (acetylation, methylation, phosphorylation) and expression of non-coding RNAs, including miRNAs in utero and neonatal exposure [45]. In this study we used the colony forming unit (CFU) method, due to, is a method frequently used in clinical therapy laboratories to measure the content of progenitor cells in bone marrow, peripherally blood and umbilical cord blood sample and allows to evaluate the functional integrity of the cells after handling (volume reduction, erythrocyte removal, cryopreservation and thawing) [46-48]. As observed in Figure 10 the concentration of 0.1 μg/mL for all tested compounds did not show adverse effect. However, many studies confirm that the variation in the levels of estrogen or endocrine disruptors can induce effects such as activation in the expression of different cytokines in the different hematopoietic lineages [49-51]. For example, Liu et al. [51] demonstrated that macrophages exposed to concentrations of 22 μg/mL of the endocrine disruptor bisphenol-A induce the expression of IL-6 and TNF-α. On the contrary, at concentrations of 1, 10 and 100 μg/ mL, DBP, BBP and DEHP a decrease in the number of hematopoietic progenitors was observed (Figure10 4a–c). This is important since the presence of phthalates in blood and umbilical cord serum has been demonstrated [51-52]. The negative effect of phthalates on hematopoietic progenitors has been reported in cultures initiated with $CD34^+$ cells in the presence of DEHP for 72 h, where a significant reduction of 57.4% was reported in the number of colonies formed compared to the control [30].

5. CONCLUSIONS

We demonstrated that the presence of DBP, BBP and DEHP phthalates affects the *in vitro* expansion of human hematopoietic stem cells isolated from the umbilical cord blood, the DBP being the most cytotoxic of the phthalates tested. Considering the widespread exposure to phthalates in the population, future molecular studies will be necessary to understand the effect of phthalates on hematopoietic stem cell transplantation. Our findings are expected to open new research horizons to investigate the effects of the endocrine disruptors on the transplantation of hematopoietic stem cells.

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CONCLUSIONS

After the exposure of DINP on HepG2 cells, a decrease in gene expression and protein levels of Sirt1 and Sirt3 proteins was observed. As well as an increase in acetylated proteins and ROS levels. On the other hand, the presence of DBP, BBP and DEHP affects the *in vitro* expansion of human hematopoietic stem cells isolated from the umbilical cord blood.

Considering the widespread exposure to phthalates in the population, future molecular and *in vivo* studies will be necessary to understand the effect of phthalates on human cells. Our findings are expected to open new research horizons to investigate the effects of phthalates on the transplantation of hematopoietic stem cells as well as identifying the mechanism whereby phthalate exposure is associated with a metabolic syndrome.

ANNEXES

RNA Extraction

Monolayer cells:

NOTE: TRI Reagent is **not** compatible with plastic culture plates.

1. Wash the cells 2-3 times with PBS.
2. Resuspend the cells with PBS and place in an Eppendorf tube.
3. Centrifuge for 10 min at 1000 rpm.
4. Discard the supernatant and resuspend the cells with TRI Reagent (1 mL of TRI Reagent per 10 cm^2 of glass culture plate surface area). After addition of the reagent, the cell lysate should be passed several times through a pipette to form a homogeneous lysate.
5. Allow samples to stand for 5-10 min at room temperature. Add 0.2 mL of chloroform per mL of TRI Reagent used. Shake vigorously for 15 seconds and allow standing for 2-15 minutes at room temperature. Centrifuge the resulting mixture at 13000 rpm for 15 min at 4°C.
6. Transfer the aqueous phase to a fresh tube and add 0.5 mL of 2-propanol per mL of TRI Reagent used in sample preparation and mix. Allow the sample to stand for 5-10 min at room temperature. Centrifuge at 13000 rpm for 10 min at 4°C. The RNA precipitate will form a pellet on the side and bottom of the tube.
7. Remove the supernatant and wash the RNA pellet by adding a minimum of 1 mL of 75% ethanol per 1 mL of TRI Reagent used in sample preparation. Vortex the sample and then centrifuge at 13000 rpm for 5 min at 4°C.
8. Briefly dry the RNA pellet for 5-10 min by air-drying. Do not let the RNA pellet dry completely, as this will greatly decrease its solubility.
9. Add an appropriate volume of DEPC-water to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55-60°C for 10-15 min.

NOTE: final preparation of RNA is free of DNA and proteins. It should have a A_{260}/A_{280} ratio of >1.7.

AB High Capacity cDNA Synthesis (2X mixture)

	Each (μL)
10X RT Buffer	2
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2
MultiScribe Reverse Transcriptase	1
RNase Inhibitor	1
Total	6.8

Thermal Cycler

Lid heat	105°C
1st cycle	25°C 10 min
2nd cycle	37°C 120 min
3rd cycle	85°C 5 min
	4°C forever
Total volume	20 μL

Add 13.2 μL RNA + 6.8 μL 2X RT Master Mix

qPCR reaction mix protocol

	Volume
cDNA	1 μL
Forward primer (10 μM)	0.4 μL
Reverse primer (10 μM)	0.4 μL
H_2O	3.2 μL
Total volume	10 μL

Protein Extraction Procedure

Buffer A/B method (Cytoplasmic, Nuclear and Mitochondrial Extraction)

1. Add PMSF and Protease Inhibitor to Working Solutions (Buffer A and Buffer B). Keep everything on ice.
2. Aspirate media and wash with PBS (3 times).
3. Add Buffer A (as less as possible)- 500 μL for 35 cm^2 dish.
4. Scrape the cells using cell scrapper into the Eppendorf (same scraper may be used for all dishes as long as it is washed with PBS every time).
5. Incubate for 10 min in 4°C vortex.
6. Centrifuge for 10 min (high speed in 4°C).
   a. Supernatant= cytoplasmic proteins
   b. Pellet= nucleus, mitchondria
7. Pipet supernatant into an Eppendorf tube labeled as CYTOPLASMIC PROTEINS.
8. Resuspend pellet in 25-30 μL (or less if pellet is small) of Buffer B.
9. Incubate for 45 min in 4°C vortex.
10. Centrifuge for 10 min (high speed in 4°C)
    a. Supernatant= desired protein
    b. Pellet= cell debris
11. Pipet supernatant into an Eppendorf tube labeled as NUCLEAR PROTEINS and discard pellet.
12. Store the samples at -70°C.

Protein quantification (Bradford method)

1. Dilute the extracted protein to $1/20^{th}$ of its concentration in a new tube (keep protein and diluted lysate on ice during entire process).
2. Add 5 μL of diluted protein to at least three Wells of a 96-well transparent plate.
3. After adding all simples to be quantified in serial order in your plate, begin to add your standards in similar way.

4. Standards are to be made in diluted lysis buffer (1/20th buffer A/B or RIPA depending on extraction and fraction) using BSA. Make standards of 0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1 mg/mL.
5. Dilute 5 mL of Bio-rad proteins assay reagent in 20 mL H_2O.
6. Add 250 µL of diluted reagent from reservoir to 96-well plate. Work quickly but carefully.
7. Hold plate away from light for 20 min.
8. Read the absorbance at 595 nm, mixing for 10 s before read.
9. After obtaining concentrations make your samples ready for electrophoresis by adding Laemmli buffer.
10. For 100 µL working solution of sample loading buffer (Laemmli buffer) add 5 µL β-mercaptoethanol to 95 µL of Laemmli.
11. Boil at 95°C for 5 min.
12. Store samples at -20°C.

Electrophoresis SDS-PAGE

1. Make resolving/separating gels of desired percentage

SEPARATING GEL

	gel %age -->	6%	7%	8%	10%	12%
Use one depending on Acrilamide %	30% Acrylamide solution (mL)	2	2.32	2.66	3.34	4
	40% Acrylamide solution (mL)	1.5	1.75	2	2.5	3
	4X Separation buffer (mL)	2.5	2.5	2.5	2.5	2.5
Depending on Acrilamide %	dH_2O (30% Ac)	5.5	5.18	4.84	4.16	3.5
	dH_2O (40% Ac)	6	5.75	5.5	5	4.5

Add 100 µL of 10% APS

Add 10 µL of TEMED

2. Make stacking gel

STACKING GEL

0.6 mL 30% Acrylamide (or 0.45 mL 40%)
1.25 mL 4X Stacking buffer
3.15 mL dH_2O (3.3 mL when using 40%)
50 µL 10% APS
5 µL TEMED

3. Centrifuge simples and Ladder (protein marker) for 30s.
4. Load simples and Ladder on gel and run at 200V till marker runs completely (usually 45-60 min).

Western Blotting

1. After transfer, block the membrane with 5% milk solution for 1 h at RT on rocking platform.
2. Wash 3 times with TBST 5-15 min each.
3. Incubate with primary antibody overnight at 4°C on rocking platform.
4. Wash 3 times with TBST 5-15 min each.
5. Incubate with secondary antibody for 1.5-2 h at RT.
6. Wash 3 times with TBST 5-15 min each.
7. Equilibrate the membrane with AP buffer (with $MgCl_2$) for 15 min.
8. Development solution: 10 mL AP buffer (with $MgCl_2$), 33 µL BCIP and 66 µL NBT, add to the membrane until bands came up. Stop the reaction with dH_2O.

MTT Assay

1. Seed your cells in 96 well plates and treat your cells according to your study.
2. After exposure, the cells were incubated in the dark with 10 µL MTT solution (stock=5 mg/mL PBS 1X) for 4 h in CO_2 incubator.
3. Aspirate media.
4. Add 100 µL of DMSO in each well, and the plates were agitated to dissolve the formazan crystal product.

5. Absorbance was then measured at 490 nm using a multi well plate reader.
6. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%.

Measured of ROS levels by DCFDA assay

1. Seed your cells in 96 well or 24 well plates and treat your cells according to your study.
2. After exposure, cells were treated with 25 μM DCFDA for 45 min at 37 °C.
3. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.
4. The fluorescence intensity corresponds to the ROS generation in the treated cells.

Recipes:

Protein Lysis Buffers

Chemical	Buffer A (Cytoplasmic)	Buffer B (Mitochondrial and nuclear)
HEPES	10 mM	20 mM
KCl	0.5 mM	-
$MgCl_2$	10 mM	1.5 mM
Glycerol	-	25%
NaCl	-	420 mM
DTT	0.5 mM	0.5 mM
IGEPAL CA-630	0.1%	-
EDTA	-	0.2 mM
dH_2O	Up to final volume	
PMSF	0.5 mM	0.5 mM
Protease Inhibitor	10 μL/mL	10 μL/mL

4X Separation buffer	4X Stacking buffer	5X Running buffer (1 L)	6X Sample buffer
90 g Tris base 2 g SDS*	30.25 g Tris base 2 g SDS*	15.14 g Tris base 72.05 g Glycine 25 mL 20% SDS*	7 mL 0.5 M Tris pH 6.8 3 mL Glycerol 1 g SDS 1.2 mg bromophenol blue
Adjust pH to 8.8 with HCl dH_2O to 500 mL, store at RT	Adjust pH to 6.8 with HCl dH_2O to 500 mL, store at RT	dH_2O to 1 L (pH 8.3- do not adjust), store at 4°C	REMEMBER: working solution contains 5% β-mercaptoethanol

*Add after dissolving other components

TBST	Blocking and secondary antibody solution	Primary antibody solution	Towbin 10X[1]	AP buffer[2]
2.428 g Tris 29.22 g NaCl	5% milk in TBST	5% BSA in TBST	3. 03 g Tris base 14.4 g Glycine	1.21 g Tris base 0.58 g NaCl 1.51 g $MgCl_2$-$6H_2O$
Adjust pH to 7.5 with HCl dH_2O to 1 L 1 mL Tween-20		0.02% NaN_3 to preserve antibody for long term use	Adjust pH to 8.3 MilliQ H_2O to 100 mL	Adjust pH to 9.5 dH_2O to 100 mL

[1]Use at 1X with 20% of methanol

[2]Add $MgCl_2$ before use