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ORIGINAL ARTICLE

Phthalates affect the in vitro expansion of human hematopoietic stem cell

Ana K. Gutiérrez-García · José M. Flores-Kelly · Tomás Ortiz-Rodríguez · Marco Antonio Kalixto-Sánchez · Antonio De León-Rodríguez

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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, 0.5×10^6 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 $1.31 \pm 0.21 \times 10^6$ 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.

Keywords Phthalates · Toxicity · Hematopoietic stem cells · Hematopoietic progenitors

Ana K. Gutiérrez-García and José M. Flores-Kelly have contributed equally to this work.

A. K. Gutiérrez-García · J. M. Flores-Kelly · T. Ortiz-Rodríguez · A. De León-Rodríguez (✉)
División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, A.C., Camino a la Presa San José 2055, Col. Lomas 4ª Sección, CP 78216 San Luis Potosí, SLP, Mexico
e-mail: aleonr@me.com; aleonr@ipicyt.edu.mx

M. A. Kalixto-Sánchez
Hospital General del ISSSTE, Carlos Diez Gutiérrez 915, Col. Julian Carrillo, CP 78340 San Luis Potosí, SLP, Mexico

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 (Thomas et al. 1984). Benzyl butyl phthalate (BBP) is most commonly found in vinyl products including flooring, paints, adhesives, children's toys, food packaging, etc. (Braun et al. 2013). 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 1) (Latini 2005; Shaz et al. 2011). It has been reported that in some cases phthalates can represent up to 40% of a finished product for direct use by the consumer (Singh et al. 1972). Specifically in the area of health, some medical materials have been analyzed and 20–40% of phthalates have been found in them (Kostić et al. 2016). 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

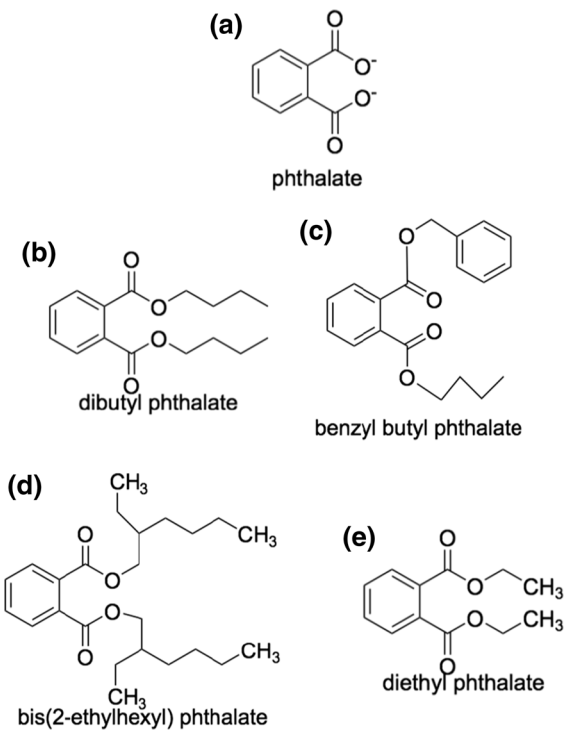

Fig. 1 Structure of common phthalates. The structures of the phthalates: **a** General structure of phthalates. **b** Dibutyl phthalate (DBP) structure, **c** benzyl butyl phthalate (BBP) structure, **d** bis (2-ethylhexyl) phthalate (DEHP) structure, **e** diethyl phthalate (DEP) structure

(Witorsch and Thomas 2010; Kusk et al. 2011; Mankidy et al. 2013; Ahmad et al. 2014). 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 (Staples et al. 1997; Api 2001; Kavlock et al. 2002; Latini 2005; Meeker et al. 2009). This explains why in humans, phthalates have been found in urine, blood, sweat, breast milk, saliva, amniotic fluid, and umbilical cord blood (Table 1) (Latini et al. 2003; Main et al. 2005; Hays et al. 2011; Genuis et al. 2012; Tranfo et al. 2014). 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. 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 (Calafat and McKee 2006), and DBP 1.0 μg/kg-bw/day for general population (Marsee et al. 2006). An important human exposition to DEHP occur during medical procedures using PVC-containing devices and blood stored in plastic bags (Lovekamp and Davis 2001). For example, patients undergoing hemodialysis can receive as much as 150 mg of DEHP in 5 h (Gibson et al. 1976). Lagerberg et al. (2015) 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. (2005) 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 (Cairo and Wagner 1997). 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

Table 1 Concentration 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]	Koch et al. (2003), Genuis et al. (2012)
Maternal blood	18.90 μg/mL	7.67 μg/mL	–	8.84 μg/mL	Lin et al. (2008)
Sweat	3.94–750 μg/g[a]	8–58.6 μg/g	–	8–576 μg/g	Genuis et al. (2012)
Breast milk	0.31 ng/g	0.62–1.2 ng/g	1.2 μg/L[c]	156–398 ng/g	Main et al. (2005), Mortensen et al. (2005), Zhu et al. (2006)
Saliva	91.4 ng/mL[a]	22.4 ± 9.8 nmol[b]	353.6 ng/mL[c]	1017 ± 147 μg/g	Steiner et al. (1998), Silva et al. (2005)
Amniotic fluid	0.70 μg/L[a]	3.53 μg/L[b]	–	1.47 μg/L[d]	Tranfo et al. (2014)
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	Lin et al. (2008), Huang et al. (2014)

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

because they exhibit a greater potential for proliferation and expansion (Andrade et al. 2015).

Since phthalates can cause cell damage and death and are present in blood storage bags (Phillips et al. 1986; Inoue et al. 2005; Benachour and Aris 2009; Lagerberg et al. 2015; Manz et al. 2015), 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.

Materials and methods

Reagents

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

Cell culture and exposure conditions

The umbilical cord blood was centrifuged at 450×g 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 550×g for 15 min at 25 °C. The white cell pack was collected and washed with PBS and centrifuged at 800×g 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 0.5×10^6 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 (400×g for 15 min at 25 °C) modified from (De León et al. 1998; Mayani et al. 1998). 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 (Phelan and May 2007; Louis and Siegel 2011).

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) (De León et al. 1998; Andrade-Zaldívar et al. 2014).

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.

Results

Representative kinetics of cell expansion of DEHP treatments

Figure 2 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.

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. 3a). 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. 3a). 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. 3b). 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. 3c). 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. 3d). 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%.

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

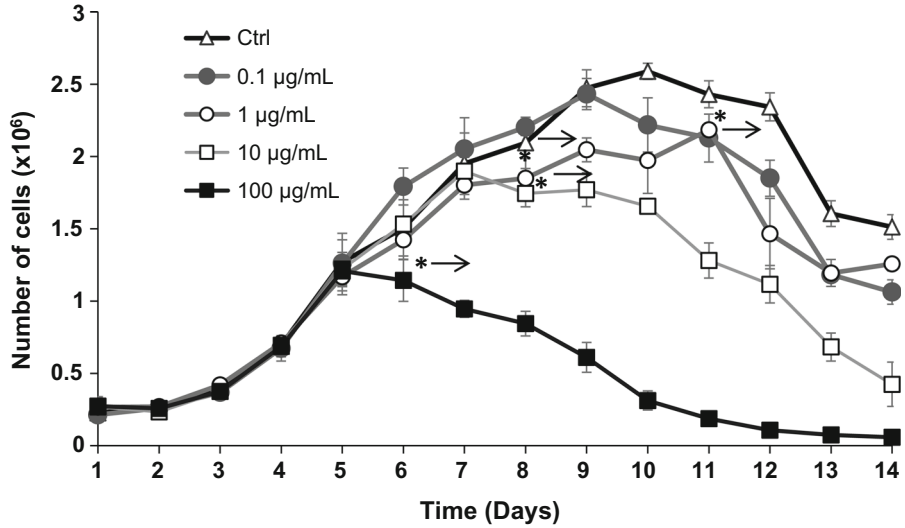

Fig. 2 Kinetic of hematopoietic cell expantion exposed to the treatments with DEHP. The graph shows the curve of total cellular expansion during the incubation period of the human mononuclear cells of umbilical cord blood exposed from 0.1 to 100 µg/mL of DEHP and the respective control cultures. The 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

($p < 0.05$) (Fig. 4a). 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. 4b). 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. 4c). 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. 4d). 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.

Discussion

In the present study, the cytotoxic effect of phthalates DBP, BBP and DEHP on human hematopoietic cells was demonstrated. Andersen et al. (1999) 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. (1975) 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. (2012) 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. (2014) 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. (1999) showed that DEHP was cytotoxic and produce DNA damage in human leucocytes and lymphocytes. Sicińska (2018) 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 (Melzak et al. 2018). 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 (Fig. 3a–c); where

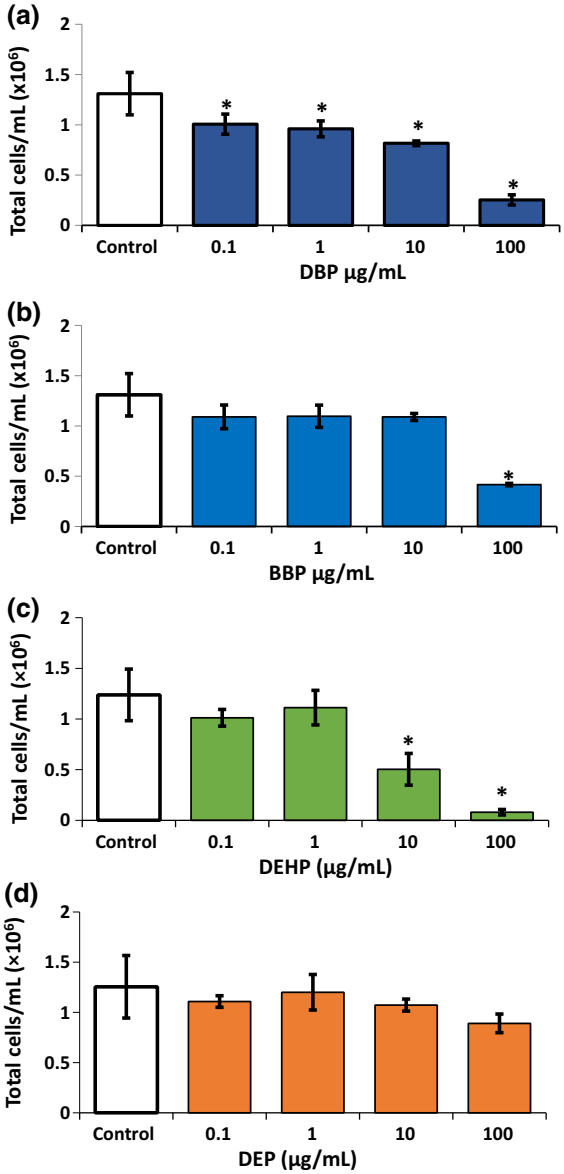

Fig. 3 Maximum cell expansion of hematopoietic cells exposed to DBP, BBP, DEHP and DEP. Different concentrations of DBP, BBP, DEHP and DEP (0.1–100 µg/mL) were tested for 14 days. The cultures were started with 500,000 cells/mL. **a** Cultures exposed to DBP showed a significant decrement at lower concentrations 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

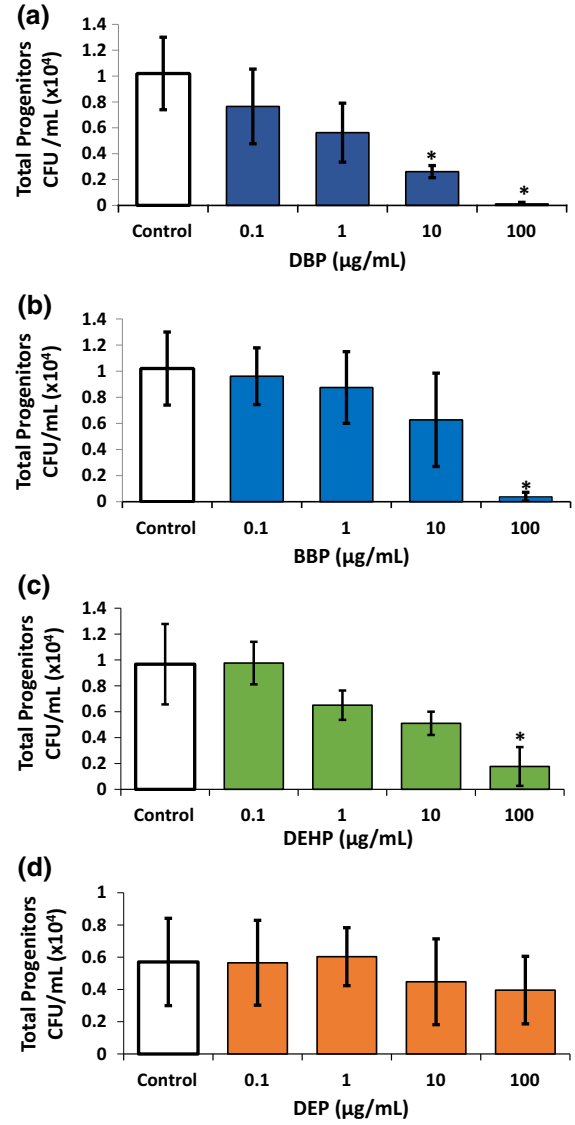

Fig. 4 Maximum hematopoietic progenitor expansion exposed to DBP, BBP, DEHP and DEP. The graphs show the number of colony forming units (CFU) of the cultures exposed to DBP, BBP, DEHP and DEP in range of 0.1–100 µg/mL. Cultures were started with 10,000 cells/mL. **a** DBP treatments did show a significant 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

the DBP turned out to be the most toxic between these compounds tested; since as seen in Fig. 3a, 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 (Mierzejewska et al. 2015). 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 (Kim et al. 2019). 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 (Singh and Li 2012).

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) (Pereira et al. 2007; Sarma et al. 2010; Wognum et al. 2013).

As observed in Fig. 4 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, (Chighizola and Meroni 2012; Liu et al. 2014; Kovats 2015). For example, Liu et al. (2014) 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 (Fig. 4a–c). This is important since the presence of phthalates in blood and umbilical cord serum has been demonstrated (Huang et al. 2014; Liu et al. 2014). 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 (Manz et al. 2015).

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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