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

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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 cordon blood. For this, $0.5x10^6$ 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 $1.31\pm 0.21x10^6$ 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

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. (Fig. 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. 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 (Api 2001; Kavlock et al. 2002; Latini 2005; Meeker et al. 2009; Staples et al. 1997). This explains why in humans, phthalates have been found in urine, blood, sweat, breast milk, saliva, amniotic fluid, and umbilical cord blood (Genuis et al. 2012; Hays et al. 2011; Latini et al. 2003; Main et al. 2006; Tranfo et al. 2014). 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 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, 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.

Material 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 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 cytocines (IMDMcit) 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 IMDMcit medium (400xg 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 (Louis and Siegel 2011; Phelan and May 2007).

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 CSF-GM 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 (UFC-E), erythroid burst forming units (UFB-E), granulocyte forming units (UFC-G), forming units of monocytes (UFC-M), granulocyte and monocyte forming units (UFC-GM) or multipotent forming units (UFC-GEMM) (Andrade-Zaldívar et al. 2014; De León et al. 1998).

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.

Results

Representative kinetics of cell expansion of DEHP treatments

Fig. 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 to 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. 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 to 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

Effect of phthalates on hematopoietic cells of umbilical cord blood

In the present study, the cytotoxic effect of phthalates DBP, BBP and DEHP on human hematopoietic cells was demonstrated. Andersen in 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 (Andersen et al. 1999). 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 are toxic at concentrations of 13.9 μg and 15.6 μg respectively for the B4G12 cell line which is derived from corneal endothelial cells (Manz et al. 2014) demonstrated that DEHP is toxic to promyelocytic leukemia cells at concentrations of 100 μg/mL, however, at 10 μg/mL no toxic effects were observed. 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 3a-c); where the DBP turned out to be the most toxic between these compounds tested; since as seen in figure 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.

Clonogenic assays of DBP, BBP, DEHP and DEP

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 figure 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; Kovats 2015; Liu et al. 2014). For example, Liu et al 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 (Liu et al. 2014). 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 (Figure 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. 2014).

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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Figure captions

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.

Fig. 2 Kinetics of diethylhexyl phthalate (DEHP)

The graph show 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 culture. 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.

Fig. 3 Maximum cell expansion of hematopoietic cells exposed to DBP, BBP, DEHP and DEP

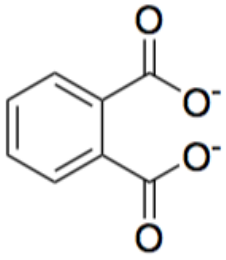
Different concentrations of DBP, BBP, DEHP and DEP (0.1-100 μg/mL) were tested for 14 days. The cultures were started with 500,000 cells/mL. A) Cultures exposed to DBP showed a significant decrement at lower concentrations of 0.1 μg/mL with respect to the controls ($p<0.05$) n=3. B) Cultures exposed 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 control ($p<0.05$; $p<0.01$). 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 Clonogenic assays of 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 DEP and DEHP in concentrations of 0.1 to 100 μg/mL. Cultures were started with 10,000 cells/ml. A)

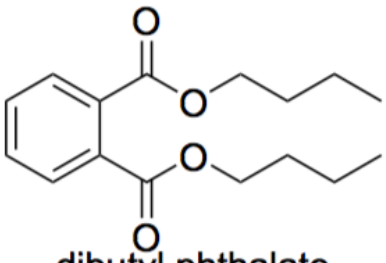
DBP treatments did show a significant decrement at 10 and 100 μg/mL. n=3 ($p<0.05$). B) Cultures exposed to 100 μg/mL BBP showed a significant decrement. n=3 ($p<0.05$). C) Treatments with DEHP showed a significant decrement in the amount of CFUs exposed to 100 μg/mL compared to controls ($p<0.05$) n=2. D) Treatments with DEP did not show any significant difference between treatments and controls.

a)



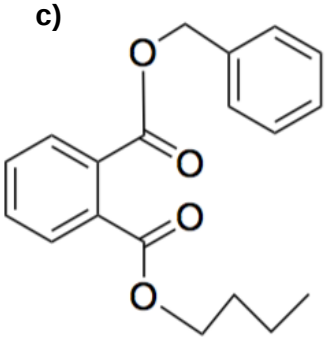
phthalate

b)



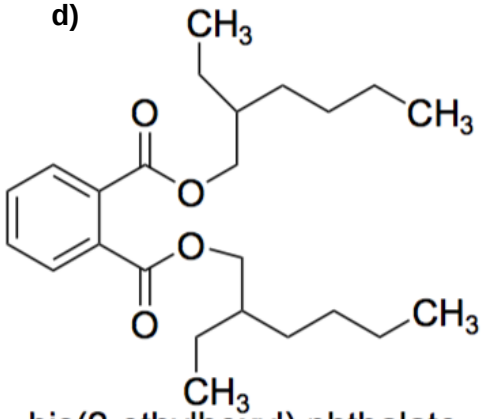
dibutyl phthalate

c)



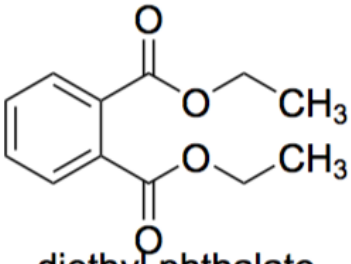
benzyl butyl phthalate

d)



bis(2-ethylhexyl) phthalate

e)



diethyl phthalate

Fig. 1

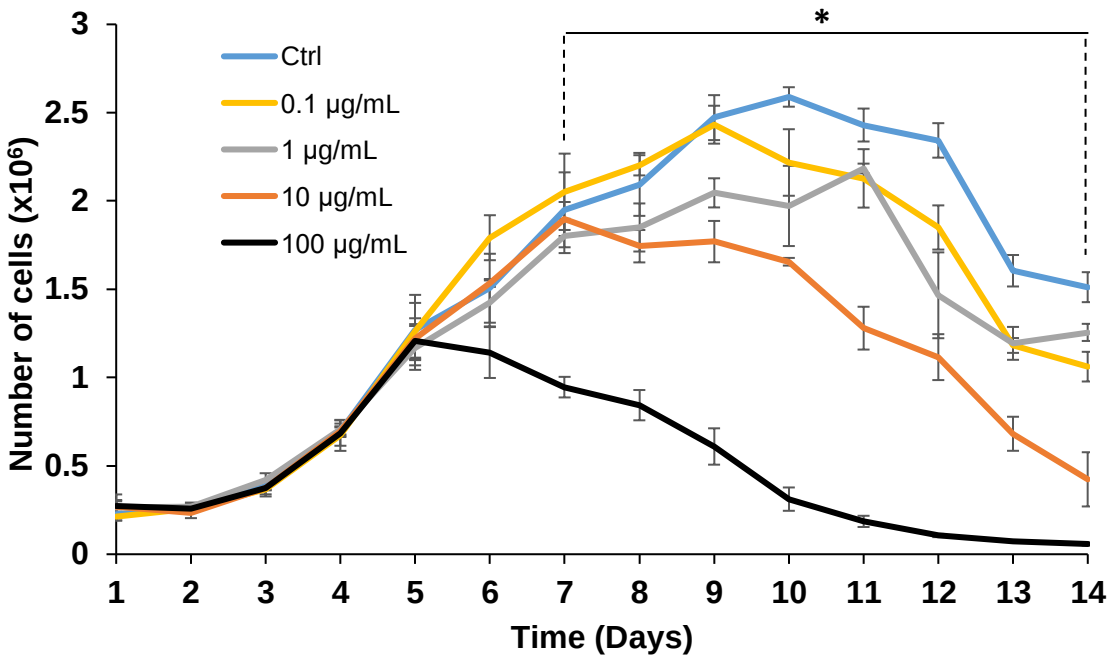

*
Ctrl
0.1 µg/mL
1 µg/mL
10 µg/mL
100 µg/mL
Number of cells (x10⁶)
Time (Days)
0
0.5
1
1.5
2
2.5
3
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Fig.2

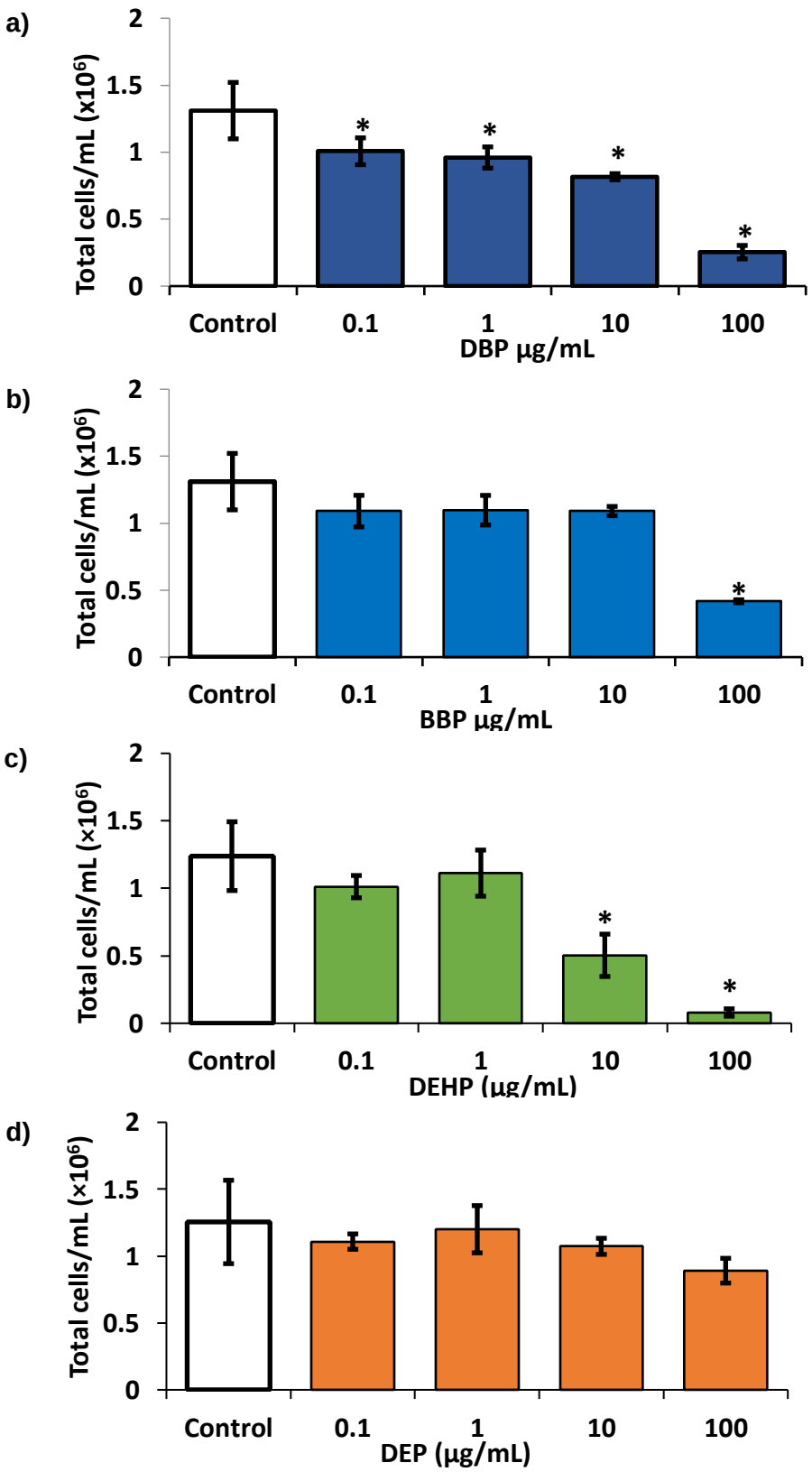

a)
Total cells/mL (x10⁶)
2
1.5
1
0.5
0
Control
0.1
1
10
100
DBP µg/mL
b)
Total cells/mL (x10⁶)
BBP µg/mL
c)
Total cells/mL (×10⁶)
DEHP (µg/mL)
d)
Total cells/mL (×10⁶)
DEP (µg/mL)

Fig. 3

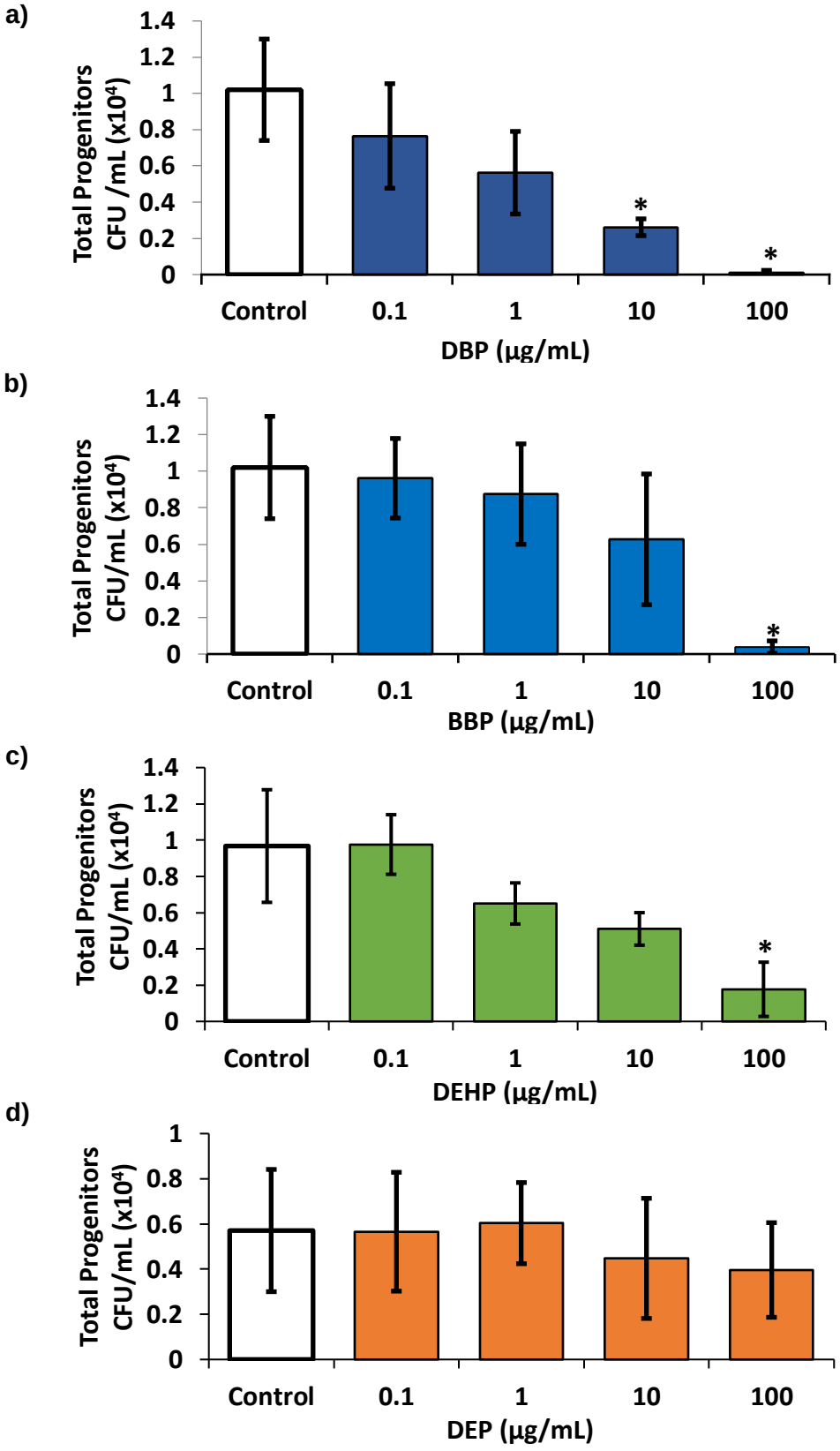

a)
Total Progenitors CFU /mL (x10⁴)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
0.1
1
10
100
DBP (µg/mL)
b)
Total Progenitors CFU/mL (x10⁴)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
0.1
1
10
100
BBP (µg/mL)
c)
Total Progenitors CFU/mL (x10⁴)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
0.1
1
10
100
DEHP (µg/mL)
d)
Total Progenitors CFU/mL (x10⁴)
1
0.8
0.6
0.4
0.2
0
Control
0.1
1
10
100
DEP (µg/mL)

Fig. 4