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

2

3

4   Ana K. Gutiérrez-García[a][1], José M. Flores-Kelly[a][1], Tomás Ortiz-Rodríguez[a], Marco Antonio Kalixto Sánchez[b]

5   , Antonio De León-Rodríguez[a][*]

6

7

8   [a]División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, A.C., Camino

9   a la Presa San José 2055, Col. Lomas 4ª Sección, C.P. 78216 San Luis Potosí, SLP, México.

10   [b]Hospital General del ISSSTE, Carlos Diez Gutiérrez 915, Col. Julian Carrillo CP 78340. San Luis Potosi,

11   S.L.P., México

12

13

14

16

17

18   [*]Corresponding author: Tel: +52 444 834 2044. Email address: aleonr@me.com, aleonr@ipicyt.edu.mx

19   [1]These authors contributed equally.

20

21

22

23

24

25

26 **Abstract**

27 Phthalates are esters of phthalic acid used industrially as plastic additives, however, these are not covalently

28 bound to the polymer matrix and therefore can be released to the environment. The aim of this study was to

29 evaluate the effect of four phthalates: dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), diethyl phthalate

30 (DEP) and diethylhexyl phthalate (DEHP) on the *in vitro* expansion of human hematopoietic cells from

31 umbilical cordon blood. For this, 0.5×10^6 cells/mL were exposure to concentrations ranging from 0.1 to 100

32 μg/mL and the total cell expansion was determined after 14 days of culture in IMDM-cytokines medium. The

33 control cultures attained $1.31 \pm 0.21 \times 10^6$ cell/mL, whereas the cultures exposed to DBP, BBP and DEHP

34 showed a reduction from 23 to 81%, 17 to 69% and 15 to 93.5%, respectively. DEP did not affect the total

35 cell expansion. The most significant decrease on total cell expansion was observed at 0.1 μg/mL DBP, 100

36 μg/mL BBP and 10 μg/mL DEHP ($p<0.05$). Additionally, the effect of these compounds on the expansion of

37 hematopoietic progenitors was analyzed by clonogenic assays as colony forming units (CFU). The CFU

38 decreased considerably compared with respect to the control cultures. The reduction was 74.6 and 99.1% at

39 10 and 100 μg/mL DBP respectively, whereas 100 μg/mL BBP and 100 μg/mL DEHP reduced the CFU

40 expansion in 97.1% and 81%, respectively. Cultures exposed to DEP did not show significant differences.

41 The results demonstrate the toxicity of DBP, BBP and DEHP on the human hematopoietic stem cells.

42 KEYWORDS: phthalates; toxicity; hematopoietic stem cells; hematopoietic progenitors

43

44

45

46

47

48

49

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).

77 Since phthalates can cause cell damage and death and are present in blood storage bags, it is of clinical

78 importance to study the effect of them on the total cell population of umbilical cord blood as it is used for

79 stem cell transplants as a treatment for recurrent malignant hematological tumors, bone marrow failure

80 syndromes, severe congenital immunodeficiency states and some metabolic alterations.

81

82 **Material and methods**

83

84 **Reagents**

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

86 StemCell Technologies, Inc.

87

88 **Cell culture and exposure conditions**

89 The umbilical cord blood was centrifuged at 450xg for 15 min at 25°C. Subsequently, the white globular

90 bundle was recovered and diluted with PBS pH 7.2. This cell suspension was placed with 7 mL of Ficoll-

91 Paque Plus reagent (Pharmacia) and centrifuged at 550xg for 15 min at 25°C. The white cell pack was

92 collected and washed with PBS and centrifuged at 800xg for 20 min at 25°C. Isolated mononuclear cells were

93 resuspended in Iscove modified Dulbecco culture medium (IMDM, Sigma, St. Louis, MO, USA) and 10%

94 Bovine Fetal Serum (SFB, Gibco Grand Island, NY, USA). Cells were grown in 24-well plates, inoculating

95 0.5×10^6 cells/mL in culture medium (IMDM, Sigma) with 10% FBS, 0.1 mg/mL streptomycin, 100 U/mL

96 penicillin and 0.25 μg/mL. of amphotericin B (Sigma). The following cytocines (IMDMcit) were added to the

97 base medium: 5 ng/mL Interleukin-3 (IL-3), 12.5 ng/mL Interleukin-6 (IL-6), 5 ng/mL Seminal cell factor

98 (SCF), 5 ng/mL of FLt-3 receptor ligand (Flt-3-L) (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL of

99 Granulocyte Colony Stimulating Factor (G-CSF) (FILATIL®), 10 ng/mL of Granulocyte and Macrophage

100 Colony Stimulation Factor (GM-CSF) (GRAMAL®) and 3 U/mL of Erythropoietin (Epo) (BIOYETIN®)

101 (Probiomed, Mexico City, Mexico). The plates were placed in an incubator at 37 °C with a 5% CO_2

102 atmosphere. Later on day 5, half of the cell suspension was replaced by new IMDMcit medium (400xg for 15

103 min at 25 °C) modified from (De León et al. 1998; Mayani et al. 1998). The cultures were exposed to

104 different concentrations of phthalates (DBP, BBP, DEHP and DEP) for 14 days and a condition without

105 compound (control) was placed. The number of total cells was determined by the trypan blue exclusion

106 method using a hematocytometer (Louis and Siegel 2011; Phelan and May 2007).

107 **Determination of hematopoietic progenitors**

108 10,000 mononuclear cells were inoculated in 1 mL of medium (MethoCult® GF H4434 classic), (StemCell

109 Technologies, Inc. Vancouver British Columbia, Canada) this medium contains the following cytokines: 50

110 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

111 Petri dish. The plates were incubated for 14 days at 37 °C with a 5% CO_2 atmosphere. The colonies identified

112 and quantified by means of the clonogenic assay were named as: erythroid colony forming units (UFC-E),

113 erythroid burst forming units (UFB-E), granulocyte forming units (UFC-G), forming units of monocytes

114 (UFC-M), granulocyte and monocyte forming units (UFC-GM) or multipotent forming units (UFC-GEMM)

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

116

117 **Statistical analysis**

118 All data are presented as the mean ± standard error (S.E.). Statistical significance was determined by One-way

119 ANOVA (p < 0.05) and post hoc analysis by Dunnett`s.

120

121 **Results**

122 **Representative kinetics of cell expansion of DEHP treatments**

123 Fig. 2 shows a representative kinetics of cell expansion of human mononuclear cells from cultures exposed

124 from 0.1 to 100 μg/mL of DEHP and the control culture during 14 days of incubation. It was found that for

125 the 4 treatments the lag phase was 4 days. The control presented the exponential phase on day 5, the

126 stationary phase on day 9 and on day 12 the decay phase. In cultures exposed to 0.1 μg/mL DEHP the

127 exponential phase was presented on day 5, starting on day 10 the slope began to decrease, observing a

128 significant difference on cell viability at day 11. The cultures treated with 1 and 10 μg/mL DEHP presented a

129 similar behavior; the exponential phase was on day 5 and the decay phase on day 11; a significant difference

130 was observed on cell viability with respect to control from days 8 and 9, respectively. In cultures exposed to

131 100 μg/mL DEHP, a significant decrease in cell viability was observed after day 6.

132

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.

161

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

7

188   reduction, erythrocyte removal, cryopreservation and thawing) (Pereira et al. 2007; Sarma et al. 2010;

189   Wognum et al. 2013).

190   As observed in figure 4 the concentration of 0.1 μg/mL for all tested compounds did not show adverse effect.

191   However, many studies confirm that the variation in the levels of estrogen or endocrine disruptors can induce

192   effects such as activation in the expression of different cytokines in the different hematopoietic lineages,

193   (Chighizola and Meroni 2012; Kovats 2015; Liu et al. 2014). For example, Liu et al demonstrated that

194   macrophages exposed to concentrations of 22 μg/mL of the endocrine disruptor bisphenol-A induce the

195   expression of IL-6 and TNF-α (Liu et al. 2014). On the contrary, at concentrations of 1, 10 and 100 μg/mL,

196   DBP, BBP and DEHP a decrease in the number of hematopoietic progenitors was observed (Figure 4a-c).

197   This is important since the presence of phthalates in blood and umbilical cord serum has been demonstrated

198   (Huang et al. 2014; Liu et al. 2014). The negative effect of phthalates on hematopoietic progenitors has been

199   reported in cultures initiated with CD34^{+} cells in the presence of DEHP for 72 h, where a significant reduction

200   of 57.4% was reported in the number of colonies formed compared to the control (Manz et al. 2014).

201

202   **Conclusions**

203   We demonstrated that the presence of DBP, BBP and DEHP phthalates affects the *in vitro* expansion of

204   human hematopoietic stem cells isolated from the umbilical cord blood, the DBP being the most cytotoxic of

205   the phthalates tested. Considering the widespread exposure to phthalates in the population, future molecular

206   studies will be necessary to understand the effect of phthalates on hematopoietic stem-cell transplantation.

207   Our findings are expected to open new research horizons to investigate the effects of the endocrine disruptors

208   on the transplantation of hematopoietic stem cells.

209

210   **Acknowledgments**

216

217 **References**

218 Andersen HR et al. (1999) Comparison of short-term estrogenicity tests for identification of hormone-
219   disrupting chemicals Environmental health perspectives 107:89
220 Andrade-Zaldívar H, Kalixto-Sánchez M, Barba de La Rosa A, De León-Rodríguez A (2014) Expansion of
221   CD34+ human hematopoietic cells from umbilical cord blood using roller bottles Revista Mexicana
222   de Ingeniería Química 13
223 Andrade PZ, Santos Fd, Cabral J, Silva CL (2015) Stem cell bioengineering strategies to widen the
224   therapeutic applications of haematopoietic stem/progenitor cells from umbilical cord blood Journal
225   of tissue engineering and regenerative medicine 9:988-1003
226 Api A (2001) Toxicological profile of diethyl phthalate: a vehicle for fragrance and cosmetic ingredients Food
227   and Chemical Toxicology 39:97-108
228 Braun JM, Sathyanarayana S, Hauser R (2013) Phthalate exposure and children's health Current opinion in
229   pediatrics 25:247
230 Cairo MS, Wagner JE (1997) Placental and/or umbilical cord blood: an alternative source of hematopoietic
231   stem cells for transplantation Blood 90:4665-4678
232 Chighizola C, Meroni PL (2012) The role of environmental estrogens and autoimmunity Autoimmunity
233   reviews 11:A493-A501
234 De León A, Mayani H, Ramírez OT (1998) Design, characterization and application of a minibioreactor for
235   the culture of human hematopoietic cells under controlled conditions. In: Cell Culture Engineering
236   VI. Springer, pp 127-138
237 Genuis SJ, Beesoon S, Lobo RA, Birkholz D (2012) Human elimination of phthalate compounds: blood,
238   urine, and sweat (BUS) study The Scientific World Journal 2012
239 Hays SM, Aylward LL, Kirman CR, Krishnan K, Nong A (2011) Biomonitoring equivalents for di-isononyl
240   phthalate (DINP) Regulatory Toxicology and Pharmacology 60:181-188
241 Huang Y et al. (2014) Phthalate levels in cord blood are associated with preterm delivery and fetal growth
242   parameters in Chinese women PloS one 9:e87430
243 Kavlock R et al. (2002) NTP center for the evaluation of risks to human reproduction: phthalates expert panel
244   report on the reproductive and developmental toxicity of di (2-ethylhexyl) phthalate Reproductive
245   toxicology 16:529-653
246 Kostić IS, Anđelković TD, Anđelković DH, Cvetković TP, Pavlović DD (2016) Determination of di (2-
247   ethylhexyl) phthalate in plastic medical devices Hemijska Industrija 70:159
248 Kovats S (2015) Estrogen receptors regulate innate immune cells and signaling pathways Cellular
249   immunology 294:63-69
250 Krüger T, Cao Y, Kjærgaard SK, Knudsen LE, Bonefeld-Jørgensen EC (2012) Effects of phthalates on the
251   human corneal endothelial cell line B4G12 International journal of toxicology 31:364-371
252 Latini G (2005) Monitoring phthalate exposure in humans Clinica Chimica Acta 361:20-29
253 Latini G, De Felice C, Presta G, Del Vecchio A, Paris I, Ruggieri F, Mazzeo P (2003) In utero exposure to di-
254   (2-ethylhexyl) phthalate and duration of human pregnancy Environmental health perspectives
255   111:1783
256 Liu Y, Mei C, Liu H, Wang H, Zeng G, Lin J, Xu M (2014) Modulation of cytokine expression in human
257   macrophages by endocrine-disrupting chemical Bisphenol-A Biochemical and biophysical research
258   communications 451:592-598
259 Louis KS, Siegel AC (2011) Cell viability analysis using trypan blue: manual and automated methods. In:
260   Mammalian Cell Viability. Springer, pp 7-12
261 Main KM et al. (2006) Human breast milk contamination with phthalates and alterations of endogenous
262   reproductive hormones in infants three months of age Environmental health perspectives 114:270
263 Manz P, Cadeddu R-P, Wilk M, Fritz B, Haas R, Wenzel F (2014) Impact of Di (2-ethylhexyl) phthalate on
264   migration rate of human promyelocytic leukemia cells (HL-60) Clinical hemorheology and
265   microcirculation 58:241-246
266 Mayani H et al. (1998) Kinetics of Hematopoiesis in Dexter-Type Long-Term Cultures Established from
267   Human Umbilical Cord Blood Cells Stem Cells 16:127-135
268 Meeker JD, Sathyanarayana S, Swan SH (2009) Phthalates and other additives in plastics: human exposure
269   and associated health outcomes Philosophical Transactions of the Royal Society B: Biological
270   Sciences 364:2097-2113
271 Pereira C, Clarke E, Damen J (2007) Hematopoietic colony-forming cell assays. In: Stem Cell Assays.
272   Springer, pp 177-208

273 Phelan K, May KM (2007) Basic techniques in mammalian cell tissue culture Current protocols in cell
274   biology:1.1. 1-1.1. 22
275 Sarma NJ, Takeda A, Yaseen NR (2010) Colony forming cell (CFC) assay for human hematopoietic cells
276   Journal of visualized experiments: JoVE
277 Shaz BH, Grima K, Hillyer CD (2011) 2-(Diethylhexyl) phthalate in blood bags: is this a public health issue?
278   Transfusion 51:2510-2517
279 Singh A, Lawrence W, Autian J (1972) Teratogenicity of phthalate esters in rats Journal of Pharmaceutical
280   Sciences 61:51-55
281 Staples CA, Peterson DR, Parkerton TF, Adams WJ (1997) The environmental fate of phthalate esters: a
282   literature review Chemosphere 35:667-749
283 Thomas JA, Thomas MJ, Gangolli SD (1984) Biological effects of di-(2-ethylhexyl) phthalate and other
284   phthalic acid esters CRC critical reviews in toxicology 13:283-317
285 Tranfo G, Paci E, Pigini D, Bonanni RC, Capanna S, De Carolis C, Iavicoli S (2014) Phthalate metabolites in
286   amniotic fluid and maternal urine samples Journal of Environmental Protection 5:1411
287 Wognum B, Yuan N, Lai B, Miller CL (2013) Colony forming cell assays for human hematopoietic
288   progenitor cells. In: Basic Cell Culture Protocols. Springer, pp 267-283

289

290

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)

319 DBP treatments did show a significant decrement at 10 and 100 μg/mL. n=3 (p<0.05). B) Cultures exposed to

320 100 μg/mL BBP showed a significant decrement. n=3 (p<0.05). C) Treatments with DEHP showed a

321 significant decrement in the amount of CFUs exposed to 100 μg/mL compared to controls (p<0.05) n=2. D)

322 Treatments with DEP did not show any significant difference between treatments and controls.

323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359

360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
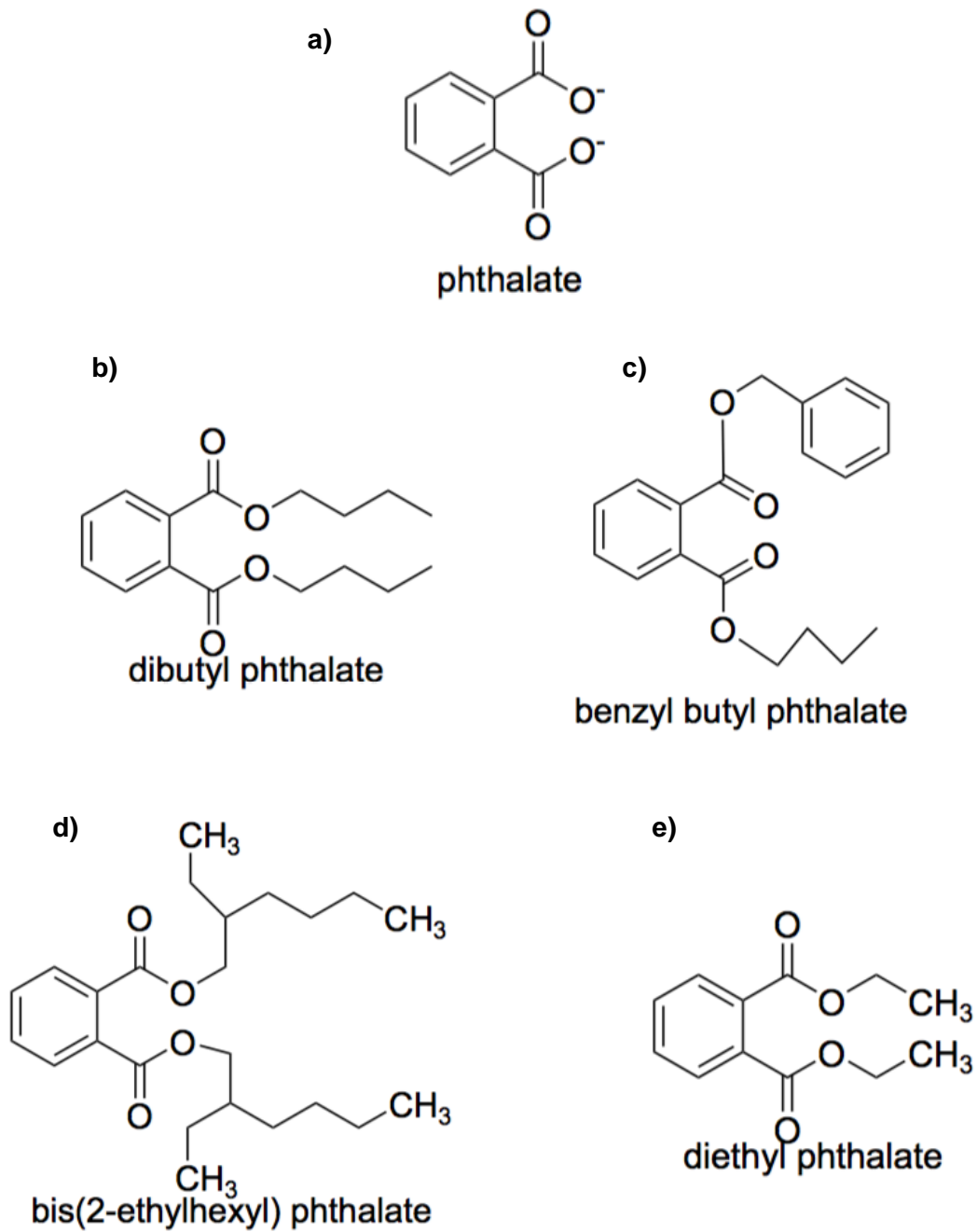397
398
399
400
401
402
403
404
405
406

a)

phthalate

b)

dibutyl phthalate

c)

benzyl butyl phthalate

d)

CH₃

CH₃

CH₃

CH₃

bis(2-ethylhexyl) phthalate

e)

CH₃

CH₃

diethyl phthalate

Fig. 1

407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
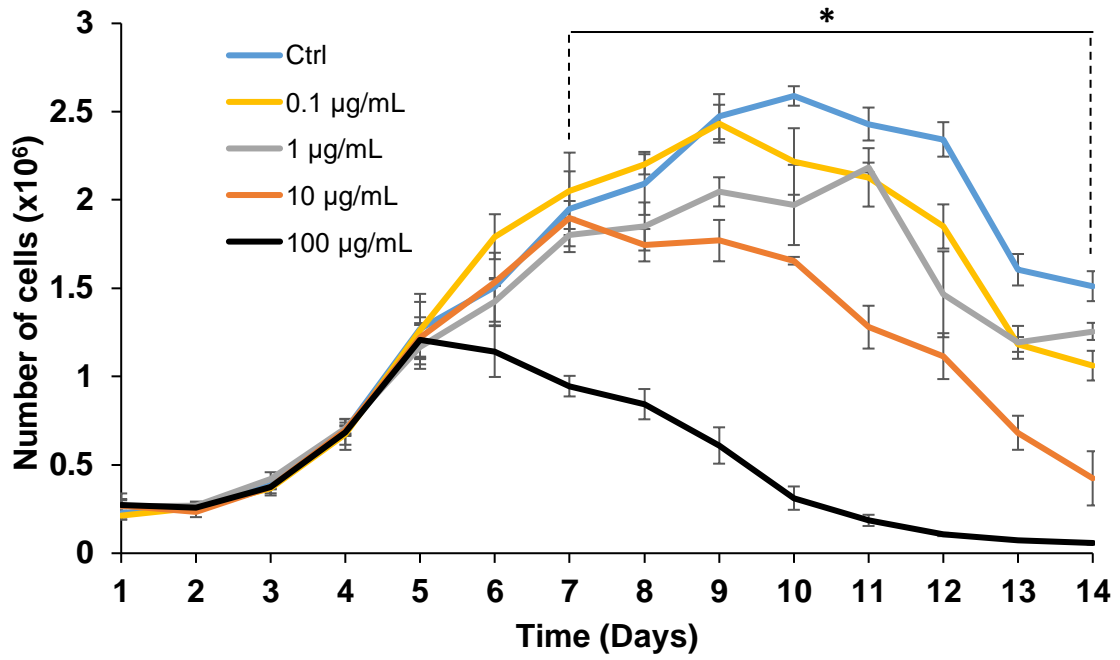437
438
439
440
441
442
443
444
445
446
447
448
449
450

Fig.2

451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
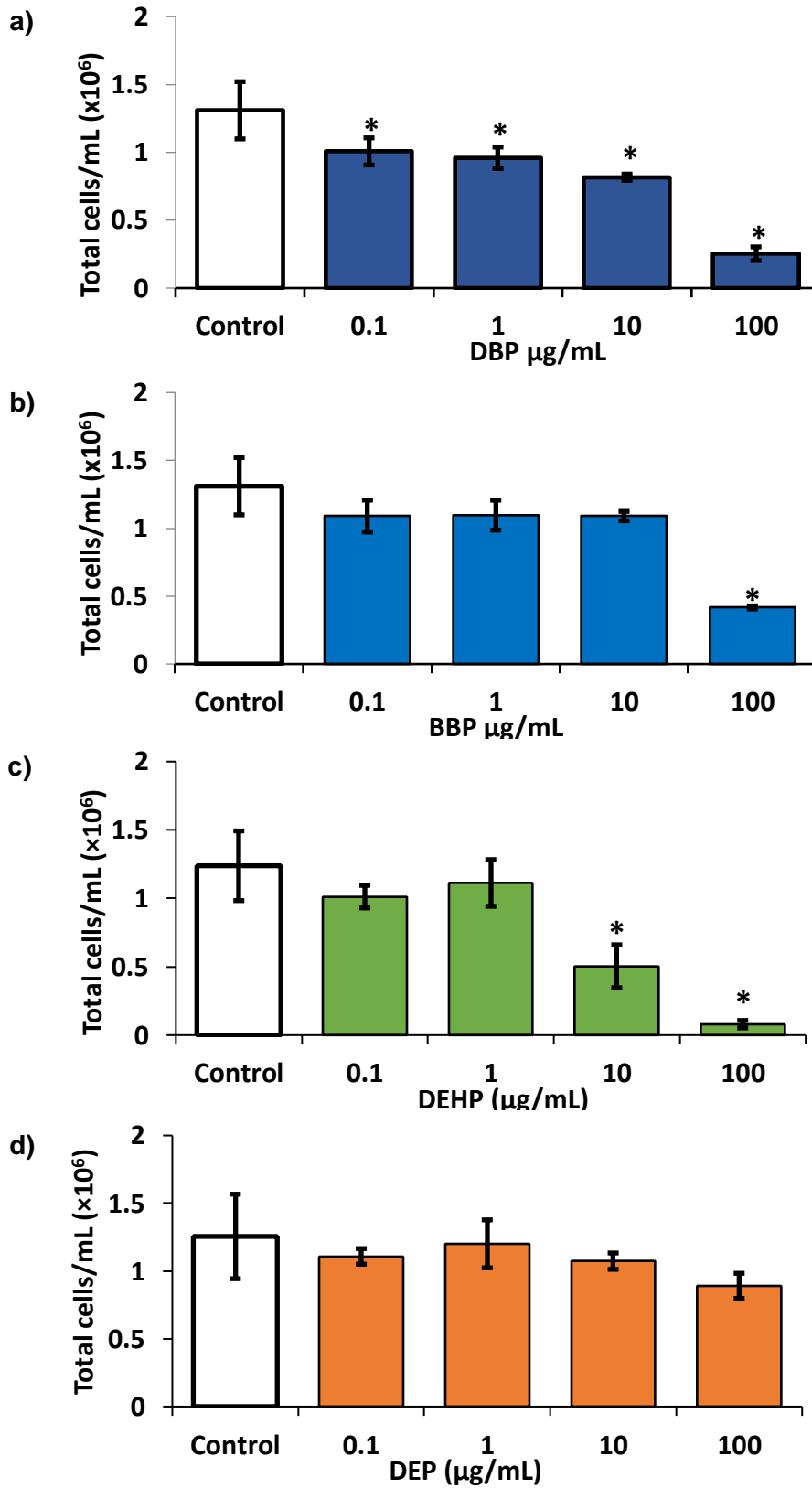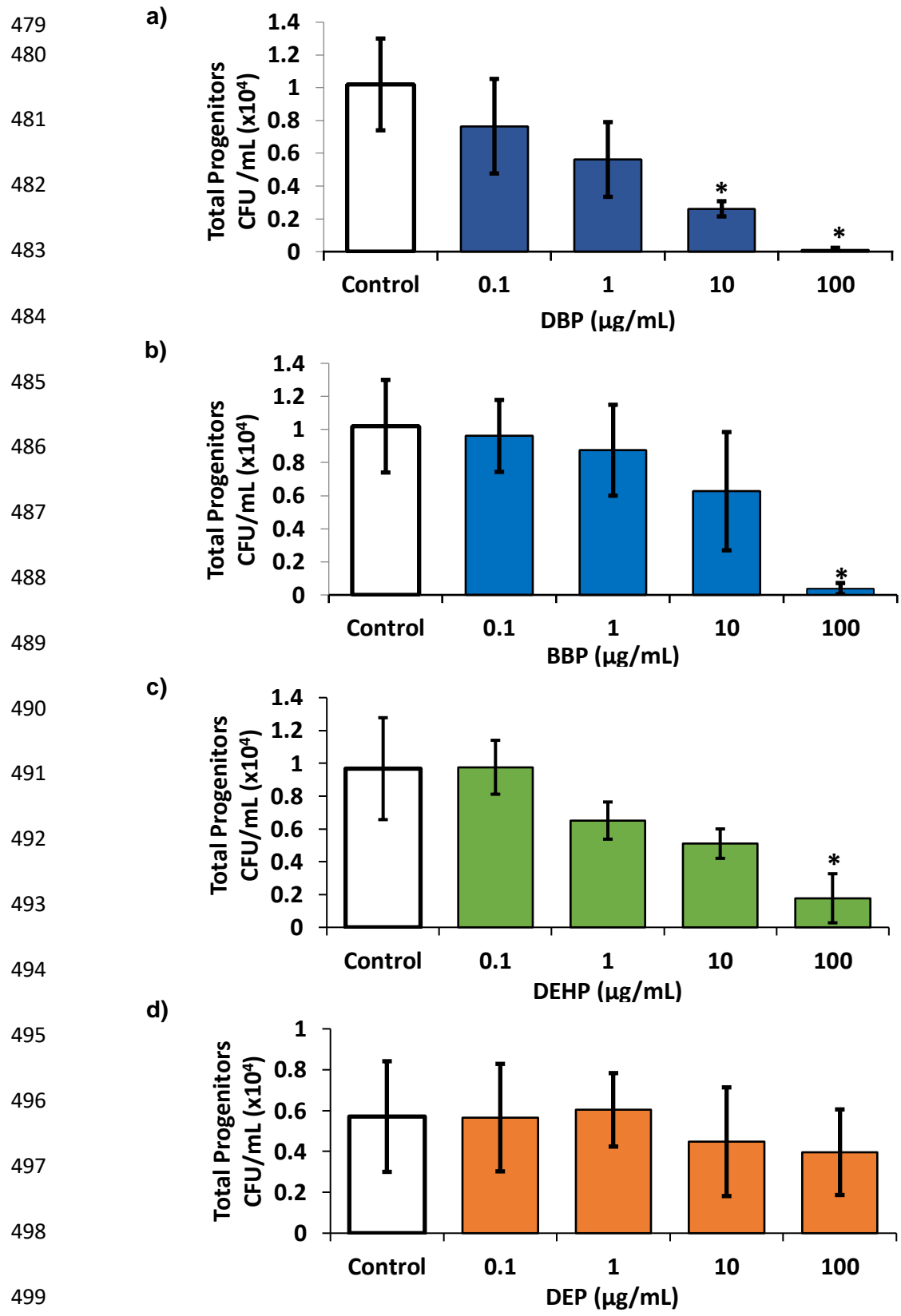469
470
471
472
473
474
475
476
477
478

Fig. 3

479
480

481

482

483

484

485

486

487

488

489

490

491

492

493

494

495

496

497

498

499

500

a)

b)

c)

d)

Fig. 4