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Toxicity of Dibutyl phthalate (DBP) toward isolated human blood lymphocytes: Apoptosis initiated from intracellular calcium enhancement and mitochondrial/lysosomal cross talk

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ABSTRACT

Dibutyl phthalate (DBP) is a phthalate ester with wide application in industrial products, so human exposure can happen in workplaces and environment. Conflicting results have been acquired in researches which measured the influences of phthalates contact on immune responses in laboratory animals. Nevertheless, the straight influence of DBP on human lymphocytes and entire mechanisms of its effect against these cells continue to be unexplored. The major purpose of present research was to evaluate the mechanisms which lead to the DBP toxicity on human lymphocytes using accelerated cytotoxicity mechanisms screening (ACMS) technique. Cell viability was determined following12h incubation of lymphocytes with 0.05–1 mM DBP, and mechanistic parameters were assessed after 2, 4 and 6 h of lymphocyte treatment with ½ the IC5012h (0.3 mM), the IC5012h (0.6 mM) and twice the IC5012h (1.2 mM) of DBP. The $IC50_{12\ h}$ of a chemical/toxicant is defined as concentration that kills 50 % of cells after 12 h of exposure. The results indicate that DBP exerts toxic effects on isolated human lymphocytes, probably through mitochondrial and lysosomal damage induced by glutathione depletion and oxidative stress. In this study, suppression of cytokines (IL2, INF-gamma and TNF-alpha) production and increase in intracellular calcium were also related to DBP induced lymphocyte toxicity.

1. Introduction

Dibutyl phthalate (DBP) is a phthalate ester and a plasticizer, with a broad spectrum application mostly in manufacturing of wide range of nutritional formulas and medical apparatuses. Due to this wide spectrum of industrial and public application, humans are believed to be exposed to DBP in both industrial and living places via occupational exposure, or indirectly as a pollution in contaminated water, air and foodstuffs [1,2]. Inhalation, ingestion, intravenous injection, and skin contact are existent routs of exposure. Phthalates are detected in microwave prepared food, drinking water, [3], infant milk and formula [4,5], toys [6], medications and nutritional supplements [6,7]. Dust originated from building materials or vapors released from indoor fragrances and furniture are other major sources of phthalate exposures [8,9].

DBP is recognized by European Chemicals Agency (ECHA) as a substance of very high concern (SVHC) due to its wide dispersive uses, high volume of release in the environment and also probable toxic properties [10]. Based on this report, less than 22 million pounds of DBP was manufactured in 2007 which has been likely dispersed in the environment. So far the research about phthalates toxicity has been focused on potential adverse effects on reproductive system and their carcinogenicity. Intriguing, a former research proposed a link among breast cancer and phthalate exposure [11], although this has not yet been completely verified. Another study indicated that DBP could induce contact dermatitis [12] and enhanced sensitization [13]. However, phthalates could also modulate the immune system. Epidemiological studies also confirm an association between phthalate exposure and respiratory and dermatologic allergic signs eczema and asthma [14,15], however the implication of these findings is limited due to lack of objective exposure data [16]. Animal studies evaluating the effect of phthalates on immune system have shown controversial results. Studies to investigate the potential of phthalates to show adjuvant properties have demonstrated both immunosuppressive and adjuvant effects [17]. However, the direct effects of DBP on immune system cells such as lymphocytes remain to be elucidated. Lymphocytes take part in both

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adaptive and innate immunity, so this investigation intended to assess the influence of DBP on the lymphocytes viability, ROS formation, production of different cytokines and integrity of key organelles of human blood lymphocytes. Our results demonstrate that DBP reduce human lymphocytes viability via ROS production and following damage to subcellular components and macromolecules.

2. Methods and materials

2.1. Chemicals

Trypan blue, Dibutyl phthalate (DBP), Rhodamine123, 2′,7′-dichlorofuorescin diacetate (DCFH-DA), N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), bovine serum albumin (BSA), OPA (*o*-Phthalaldehyde), acridine orange, NEM(N-ethylmaleimide) and trichloroacetic acid were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany). Ficoll-paque PLUS was obtained from Ge Healthcare Bio-Science Company. FBS (Fetal Bovine serum) and RPMI1640 were purchased from Gibco, Life Technologies, Grand Island, NY.

2.2. Isolation of human lymphocyte

Blood samples were collected from healthy, non-smoking volunteers (age range 18–30 years) that have not consumed any immunomodulators drugs. Lymphocytes isolation was performed by standard ficoll paque procedure and cold-aggregation purification [18]. For cell viability assay test, lymphocytes were incubated with 0.05–1 mM DBP for 12 h. Cells were treated with 0.3, 0.6 and 1.2 mM of DBP and other parameters were measured at 2, 4 and 6 h. Each test was replicated at least 5 times.

2.3. Cell viability assay

Lymphocytes were seeded in 96well plate at density of 1×10^4 cell/well and exposed to different levels of DBP. 20 μL of cells suspension was mixed with 20 μL of 0.4 % trypan blue and loaded on a hemocytometer lam. IC50 (Half Maximal Inhibitory Concentration) was measured following calculation of cell viability using light microscope.

2.4. Measurement reactive oxygen species (ROS)

Following treatment of human lymphocytes for various time intervals (2, 4 and 6 h), 500 μL of DCFH-DA (10 μM) was loaded on cell and incubated at 37℃ for 20 min. for detection of DCF fluorescence, Shimadzu spectrofluorometer (RF5000U) with excitation and emission wavelength respectively 495 and 530 nm was employed [19]

2.5. Mitochondrial membrane potential (MMP) assessment

0.5 mL of rhodamine123 (1 μM) was added to cell suspension and incubated for 15 min. Then the cells were washed and fluorescence intensity of rhodamine123 was recorded using a Shimadzu spectrofluorometer (RF5000U) with 470 nm and 540 nm excitation and emission wavelength [20].

2.6. Lipid peroxidation

TBA reagent (TCA 15 %, TBA 0.37 % and HCl 2.5 N) incubated with lymphocytes lysate for 60 min in hot water (90℃). Samples absorbance was recorded at 532 nm (DU-7 Beckman spectrophotometer) and amount of TBA-MDA was calculated based on its calibration curve [21].

2.7. lysosomal membrane damage

Human lymphocytes were incubated with 100 μL acridine orange (5 μM) at 37℃ for 10 min. Shimadzu spectrofluorometer (RF5000U) with excitation and emission wavelength respectively 470 nm and 540 nm was used for detection of acridine orange fluorescent intensity [22].

2.8. GSH and GSSG measurement

Cell lysate was produced from DBP-treated lymphocytes by adding 0.5 mL of TCA 10 % and following centrifugation at 11,000×g for 2 min. The levels of reduced and oxidized glutathione (GSH and GSSG), were determined according to Hissin procedure [23]. Glutathione level was measured utilizing fluorescence intensity at 350 and 420 nm (excitation and emission).

2.9. Activity of caspase-3

Activity of Caspase-3 in human lymphocytes was assessed by Sigma's kit (CASP-3-C). The kit work based on hydrolyze of caspase-3 substrate (Ac-DEVD-*p*NA) in cell lysate which result in freeing of *p*-nitroaniline moiety. Concentration of *p*-nitroaniline was calculated after recording of its absorbance at 405 nm in the samples.

2.10. Proteolysis assessment

Interaction of free amino groups with OPA, in attendance of 2-mercaptoethanol was the method that used for proteolysis detection. 50 μL cells lysate supernatant was incubated with 1 mL of OPA reagent in room temperature and after 2 min, absorbance was recorded using a Beckman DU-7 spectrophotometer at 340 nm [24].

2.11. Cytokines measurement

The Quantikine (RRD Systems, Minneapolis, MN, USA) ELISA kits was utilized to assess the levels of TNF-alpha (TNF-α), Interferon-gamma (IFN-γ) and interleukin-2 (lL2) in supenatant of human lymphocytes.

2.12. Measurement of intracellular Ca2+

2 μM Fluo-3/AM (Calbiochem; Bad Soden, Germany) was made by dissolving dye in CaCl2 (5 mM) containing Ringer solution and added to human lymphocytes. Then, a Shimadzu spectrofluorometer (RF5000U) was utilized to determine Ca2+-dependent fluorescence intensity.

2.13. Quantifying cellular apoptosis

Apoptosis was measured using *BioVision* Annexin V-FITC Apoptosis Detection Kit. Isolated lymphocytes were incubated with FITC-conjugated annexin V and PI following the kit recipes (Biovision, USA). A Becton–Dickinson flow cytometer equipped with an argon ion laser for excitation (488 nm) was used to analyze samples.

2.14. Statistical analysis

GraphPad Prism 5 (Graphpad Software, La Jolla, CA) was utilized for statistical analysis. The normality of the data was assessed using the Kolmogorov-Smirnov test. One way analysis of variance followed by post hoc Tukey test performed for cell viability and caspase-3 activity data. Two way analysis of variance followed by post hoc Bonferroni test carried out for other data. Results are depicted as the mean ± standard error of the mean and a 5 % or less probability was assumed as statistically significant.

3. Results

3.1. Effect of DBP on human lymphocytes viability

As demonstrated in Fig. 1, DBP decrease lymphocytes viability in a

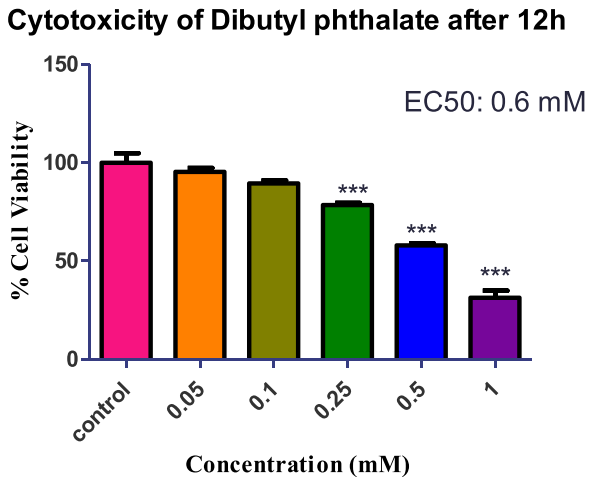


Fig. 1. Viability of human lymphocytes following treatment with DBP for 12 h. DBP caused dose-dependent decrease in viability of lymphocytes. However significant decrease was observed at concentration greater than 0.1 mM. ***P < 0.001.

concentration-dependent manner at concentration higher than 0.1 mM. The $IC50_{12\ h}$ of DBP was 0.6 mM which calculated based on a regression plot of different concentrations (data and curves not shown).

3.2. ROS assessment

Fig. 2 demonstrates alteration of human lymphocyte ROS production following treatment with different concentrations of DBP (0.3, 0.6 and 1.2 mM). As presented in Fig. 2, change in ROS levels was measured in different time intervals (2, 4 and 6 h) using DCFH-DA. DBP significantly

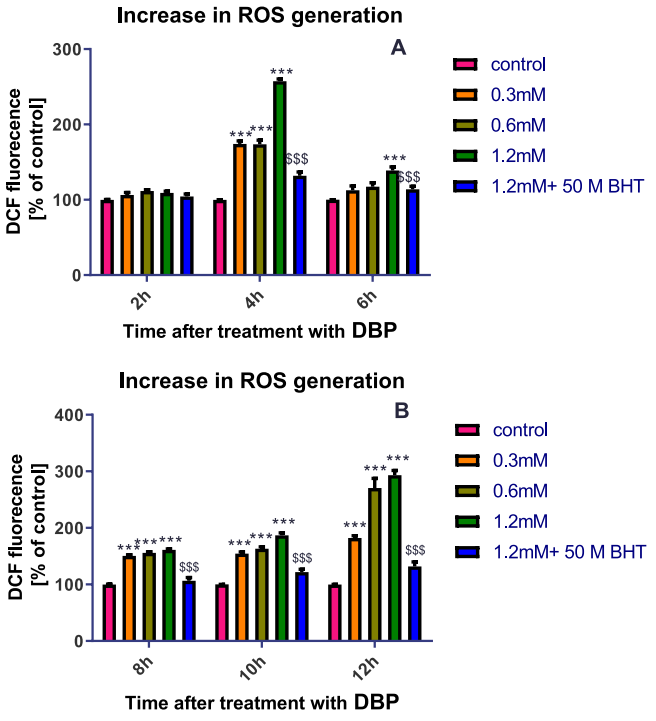


Fig. 2. ROS generation. Induction of ROS by DBP was significant (P < 0.05) at all concentrations at 4 h and only at highest concentration at 6 h in comparison with control (B), but constant significant (P < 0.05) promotion in ROS was notified until 12 h (A). DBP-induced ROS generation in lymphocytes was prevented by BHT. *P < 0.05, **P < 0.01 and ***P < 0.001, $$$: significant difference with 1.2 mM DBP.

(P < 0.05) promoted ROS generation in all applied concentrations at 4 h after exposure to the lymphocytes. Then we faced a decrease at ROS formation specially 6 h after DBP treatment that only the highest concentration (1.2 mM) could retain a significant higher ROS level compared to untreated control lymphocytes (Fig. 2A). The change in formation of ROS also was monitored after 6 h and constant raises in ROS formation was again detected at 8, 10 and 12 h after treatment with DBP (Fig. 2B). DBP-induced ROS generation in human lymphocytes was prevented by an antioxidant, buyhylatedhydroxy toluene (BHT).

3.3. Mitochondrial membrane potential

As demonstrated in Fig. 3, DBP induced MMP collapse at different time intervals. Although decrease in MMP initiated 2 h after DBP exposure, this decrease was non-significant at 2 and 4 h. However at 6 h after treatment, DBP significantly (P < 0.05) declined MMP at two higher levels (0.6 and 1.2 mM). Pre-incubation by Cyclosporine A, a blocker of mitochondrial permeability transition (MPT) pores, buyhylatedhydroxy toluene (BHT), an antioxidant and chloroquine, an intralysosomal pH enhancer prevented the collapse in MMP in human lymphocytes.

3.4. Lipid peroxidation

The influence of DBP on peroxidation of lipid depicted in Fig. 4. Significant (P < 0.05) promotion in TBARS generation was notified only 2 h after exposure of cells with two higher concentration of DBP (0.6 and 1.2 mM). Significant (P < 0.05) increases in TBARS generation (with 0.6 and 1.2 mM) continue until 6 h following treatment. Pre-incubation of lymphocytes with BHT prevented raise of TBARS following treatment with DBP.

3.5. Lysosomal membrane destabilization

Although significant (P < 0.05) lysosomal damage was observed with all concentration of DBP after 4 and 6 h, at shorter time of 2 h after treatment only 1.2 mM of DBP produced significant (P < 0.05) lysosomal membrane damage. Damage of lysosomal membrane by DBP, stopped via pre-incubation of lymphocytes with 100 μM chloroquine (an intralysosomal pH enhancer), buyhylatedhydroxy toluene (BHT) and Cyclosporine A (Fig. 5).

3.6. GSH and GSSG content

2 h following exposure to DBP, fall in intracellular GSH level and increase in extracellular GSSG level were detected in isolated lymphocytes. The level of Intracellular GSH significantly (P < 0.05) declined at 4 and 6 h after incubation of lymphocytes with all DBP concentrations as

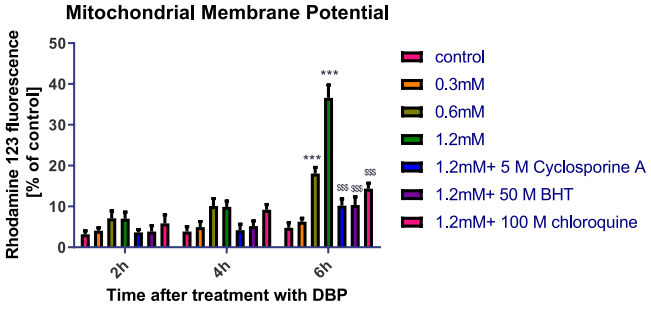


Fig. 3. Mitochondrial membrane potential (MMP). Significant (P < 0.05) fall in lymphocyte MMP was found only after 6 h exposure to DBP. Following 6 h incubation, 0.6 and 1.2 mM DBP cause significant (P < 0.05) collapse in MMP. Cyclosporine A, BHT and chloroquine inhibited MMP collapse by DBP in lymphocytes. ***P < 0.001, $$$: significant difference with 1.2 mM DBP.

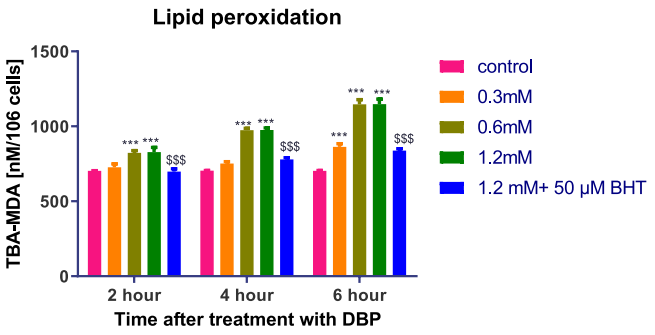


Fig. 4. Lipid peroxidation. After 2, 4 and 6 h exposure, IC_{50} and $2IC_{50}$ of DBP induced significant ($P < 0.05$) peroxidation of lipid in human lymphocytes. DBP-induced lipid peroxidation in lymphocytes prevented by BHT. *P < 0.05, **P < 0.01 and ***P < 0.001, $$$: significant difference with 1.2 mM DBP.

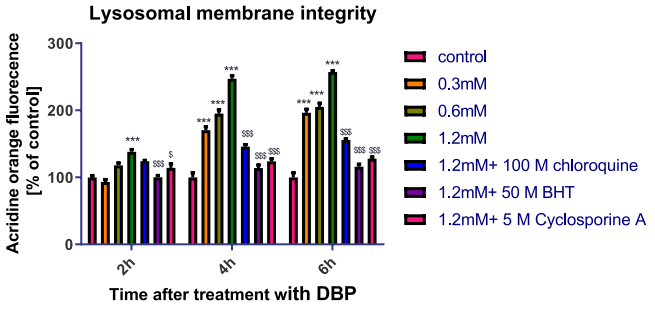
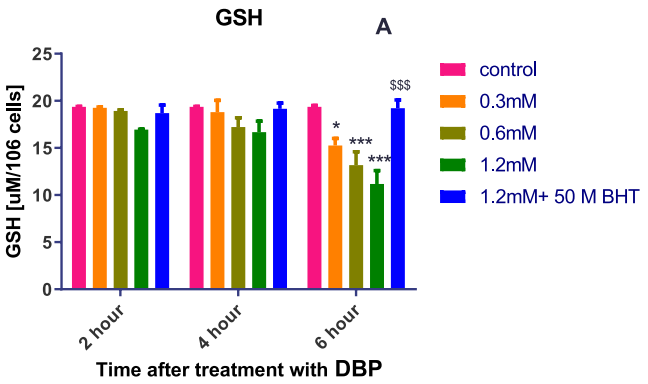


Fig. 5. Lysosomal membrane integrity. 4 and 6 h after treatment all concentration of DBP caused significant ($P < 0.05$) lysosomal membrane leakage, but only highest concentration of DBP caused significant ($P < 0.05$) leakage of lysosomal membrane at 2 h. BHT, Cyclosporine A and chloroquine prevented DBP-induced lysosomal membrane leakage. ***P < 0.001, $$$: significant difference with 1.2 mM DBP.



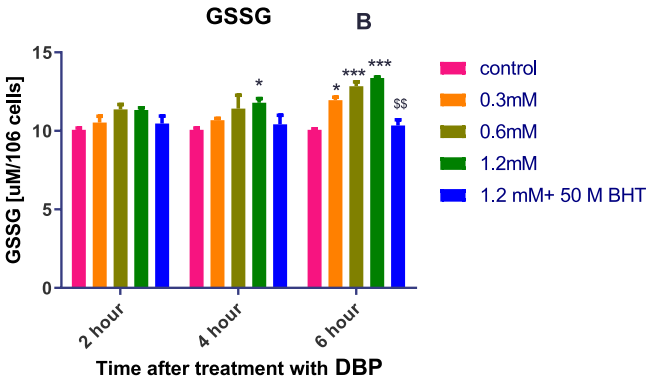


Fig. 6. Levels of glutathione. 4 h following exposure to DBP, significant ($P < 0.05$) collapse in lymphocyte intracellular GSH (part A) and raises in lymphocytes extracellular GSSG (part B) was observed. DBP-induced alteration in GSH and GSSG levels was prevented by BHT. *P < 0.05, **P < 0.01 and ***P < 0.001, $$$: significant difference with 1.2 mM DBP.

demonstrated in Fig. 6A. Meantime, as displayed in Fig. 6B, significant ($P < 0.05$) increase in extracellular GSSG level of lymphocytes was also observed at 4 and 6 h after incubation with all concentrations of DBP. Alteration of GSH and GSSG levels were inhibited via pretreatment of cells with BHT.

3.7. Caspase-3 activity

The activity of Enzyme (Caspase-3) was determined 6 and 12 h following exposure of human lymphocytes to 0.6 mM (IC_{50}) of DBP. Significant ($P < 0.05$) raise was found (Fig. 7A) after 6 h exposure of human lymphocytes to DBP, furthermore increased activity of caspase-3 remained significant ($P < 0.05$) compared to untreated control until 12 h.

3.8. Intracellular Ca2+

Fig. 7B demonstrate that DBP increase level of intracellular Ca2+ in isolated lymphocytes in a time-dependent manner. But only 6 h after exposure to 0.6 mM of DBP, significant ($P < 0.05$) raise in lymphocytes cytoplasmic Ca2+ level was detected.

3.9. Proteolysis

Fig. 8 shows the cellular proteolysis in human lymphocytes following exposure to DBP that was measured relying on interaction of free amine groups with OPA. At all times, significant ($P < 0.05$) promotion in released primary amine was noticed with 2IC50 of DBP (1.2 mM), nevertheless DBP (0.6 mM) evoked significant ($P < 0.05$) raises of free amine groups only at 4 and 6 h following exposure (Fig. 8).

3.10. Determination of cytokines

Following 12 and 24 h exposure to DBP, the amounts of TNF-alpha, IFN-γ and IL_2 were measured in isolated lymphocytes using ELISA kits. As illustrated in Fig. 9, 0.3 and 0.6 mM of DBP significantly ($P < 0.05$) reduced all cytokines generation at 12 and 24 h time intervals in comparison with untreated control.

3.11. Cellular apoptosis

Staining of untreated group with annexin V and PI showed that 91 % of lymphocytes were survived. Anyway, 0.6 mM (IC50) of DBP causes nearly 55 % apoptotic death of isolated lymphocytes (Fig. 10).

4. Discussion

As lymphocytes are important components in immune system, the influence of DBP on isolated human lymphocytes viability as well as the effect of DBP on different oxidative stress related factor and different cell organelles were investigated in this research. Moreover, to demonstrate the participation of other paths in the adverse effect of DBP on lymphocytes, the potential preventive influences of an antioxidant (butylated hydroxytoluene [BHT]), a blocker of MPT pores (cyclosporine A), and an intralysosomal pH booster (chloroquine) were also examined according to prior research[18].

Dibutyl phthalate was found in the serum of young Puerto Rican girls with premature breast development at 15–276 ug/L [25]. The degradation product of dibutyl phthalate, mono-isobutyl phthalate, was detected in the serum of female and male seniors from Uppsala, Sweden at concentrations of 2.7–1820 and 3.22–1390 ng/mL, respectively [26].

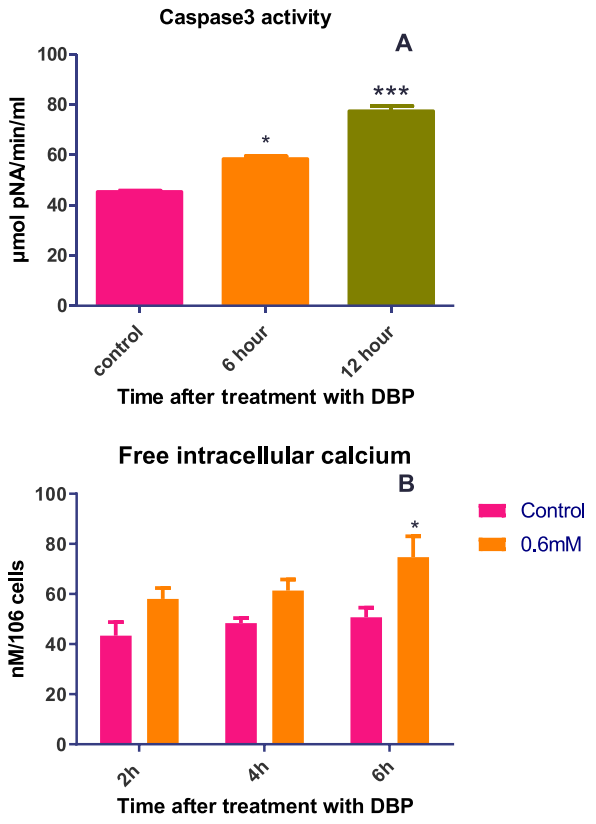


Fig. 7. Activity of caspase-3. Following 6 and 12 h exposure to IC_{50} (0.6 mM) of DBP, significant ($P < 0.05$) raise in human lymphocytes caspase-3 activity was observed (A). DBP increase lymphocytes intracellular Ca2+ in time-dependent manner. But significant ($P < 0.05$) increase in intracellular Ca2+ was observed in lymphocytes only after 6 h exposure to DBP (B). *P < 0.05, **P < 0.01 and ***P < 0.001.

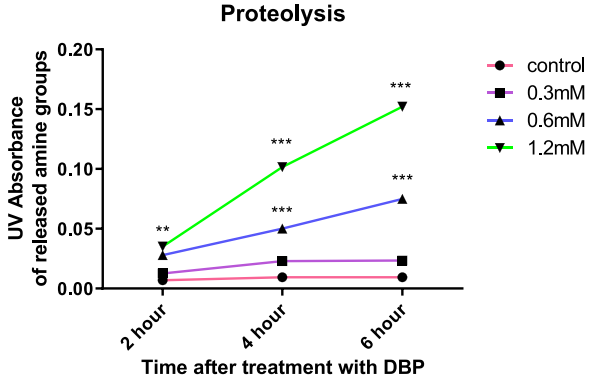


Fig. 8. Proteolysis. With highest concentration of DBP (1.2 mM) significant ($P < 0.05$) raise in released amino groups was detected at different times, but 0.6 mM DBP caused significant ($P < 0.05$) freeing of amino groups, only at 4 and 6 h following incubation. *P < 0.05, **P < 0.01 and ***P < 0.001.

To investigate the effects of DBP, we used ACMS technique that is an accepted method for mechanistic study of metals and chemicals. A major assumption with ACMS is that high dose/short time (in vitro) simulates low dose/long time (in vivo) [27]. In present study, DBP (0.25, 5 and 1 mM) significantly reduced human lymphocytes viability through induction of apoptotic cell death within 12 h treatment. DBP-induced cell death in human lymphocytes possibly associated with caspase-3, as it was proposed that DBP caused cell death in pancreatic alpha cells through promotion of caspase-3 activity[28], which has a pivotal role in

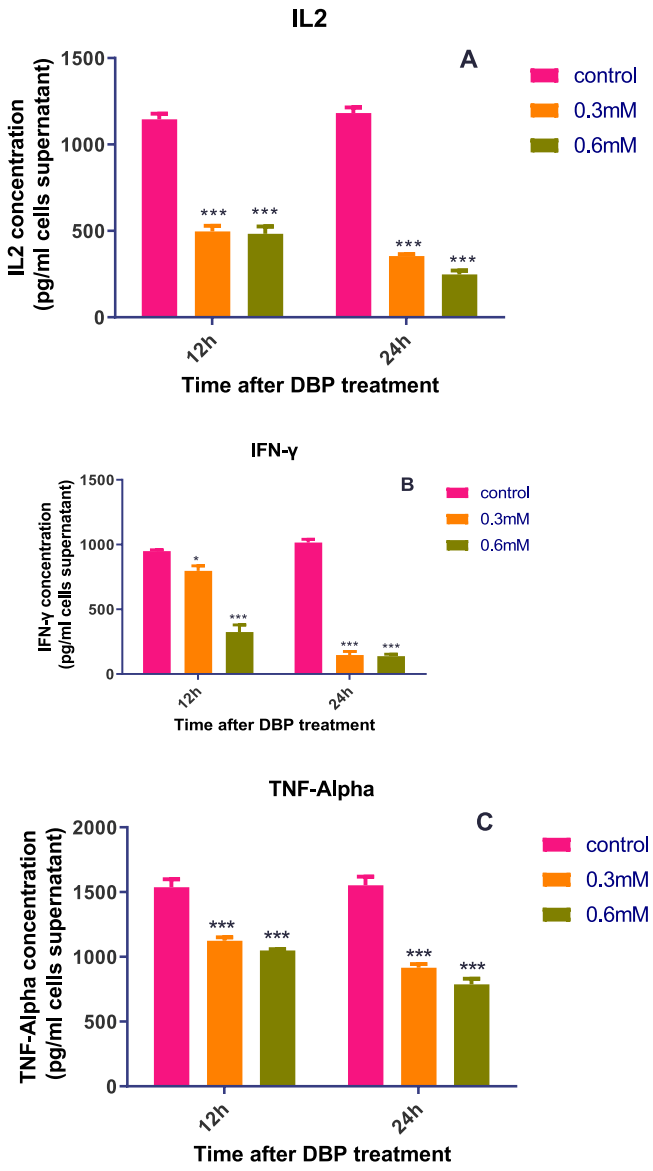


Fig. 9. Cytokines generation. A significant ($P < 0.05$) decrease in cytokines generation was found with 0.3 and 0.6 mM of DBP at 12 and 24 h (9 A: IL2, B: IFN-γ, C: TNF-alpha). *P < 0.05, **P < 0.01 and ***P < 0.001.

cell death [29]. To explore the participation of caspases in cell death produced by DBP in human lymphocytes, caspase-3 activity was measured in these cells. Activity of caspase-3 raised in human lymphocytes within 12 h incubation with DBP. In accordance with our results, after 12 h and/or 24 h treatment, 1–100 µg/mL (0.359 mM) Di-n-butyl phthalate, and its metabolites show various potential for induction of apoptosis in human peripheral blood mononuclear cells via increase in caspases-3, −8, −9 activities [30].

Some functions of lymphocytes, including the DNA synthetic response, are sensitive to ROS generation. The phthalates-induced generation of ROS has been demonstrated in different cells, including cultured bovine peripheral lymphocytes [31] and rat insulinoma cells [32]. In present study, constant enhancement in generation of ROS was observable following 6 h exposure to DBP. When ROS formation exceed antioxidant capacity of cell, it leads to oxidative stress which foster macromolecules damage. Such effects has found by Sicińska et al., which investigated the effects of di-n-butyl phthalate (DBP), butylbenzyl phthalate (BBP) and their metabolites on PBMC in the concentrations range of 0.1–10 µg/mL for 24 h. All studied phthalates induced DNA

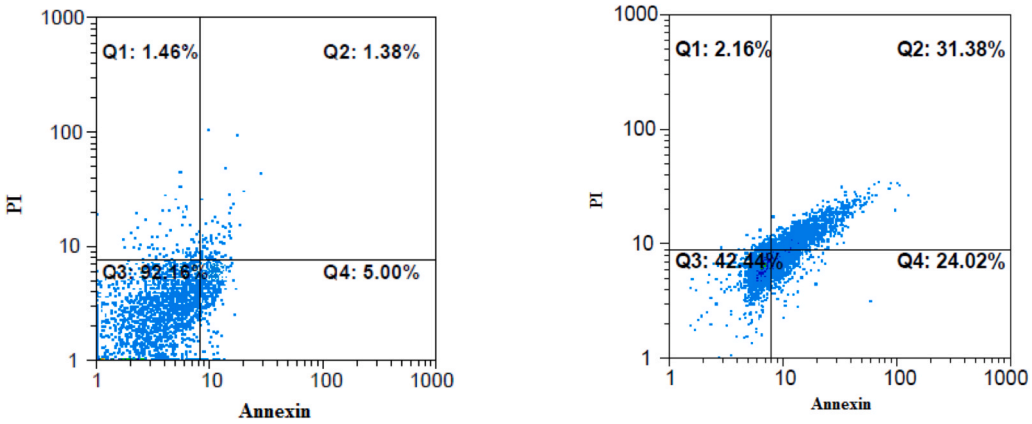


Fig. 10. Type of lymphocytes death (apoptosis and necrosis) A: before and B: after 12 h exposure to 1.2 mM of DBP.

single and double strand-breaks and more strongly oxidized purines than pyrimidines. Analyzed compounds caused an increase of total ROS level, while hydroxyl radical was generated mostly by DBP and BBP [33].

In present study, intracellular GSH collapse and extracellular GSSG rise were detected. In the role of a key intracellular antioxidant, glutathione serves as the most important scavenger of reactive oxygen species. Best function of immune system requires a finely balanced level of glutathione in lymphoid cells. Depletion of the intracellular glutathione pool, has significant influences on a variety of lymphocyte functions [34,35]. In general, few published researches have studied the effect of phthalates on human lymphocytes cytokines secretion. When cell lines were utilized making it difficult to interpret results and their possible significance for healthy human individuals. In our study, DBP reduce TNF-alpha, IFN-γ and IL_2 levels. Decreasing production of such cytokines can influence on functions of immune system and causes disruption of defense against infectious agents and malignant cells. Human lymphocytes metabolize Diethyl phthalate (DEP) and Di-n-butyl phthalate (DnBP) to their relevant monoester. They affect cytokine secretion by immune cells include T cells and monocytes/macrophages. Although DEP induces liberation of LDH by monocytes, the changed pattern of cytokine secretion did not appear to be a consequence of cell death [36]. Hence it is probable that the phthalates are absorbed and metabolized by lymphocytes, and that they affect cellular signaling that control production of cytokine. It have been suggested that phthalates affect PG production and signaling due to similarity of their structure to that of PGs [37]. PGs on the other hand, have been demonstrated to induce a cytokine secretion pattern alike to that observed in phthalate-treated monocytes/macrophages [38,39]. In our study, raise in intracellular calcium was detected after incubation of human lymphocytes with DBP. Consistent with our results, Sicińska observed an increased Ca^{2+} level in PBMCs incubated with phthalate compounds, and particularly with DBP and BBP [30]. Calcium has different functions and roles in lymphocytes. Apart from that, substantial increase in intracellular calcium can induce oxidative stress probably through MPT pore opening and freeing of cytochrome c that in turn trigger cell death cascades. So, it can be suggested that DBP inflicts such effects on intracellular calcium and cytokines production through acting on prostaglandin receptors.

Emptying of glutathione following generation of considerable ROS, ultimately result in peroxidation of lipid and lymphocytes membrane destruction. Interestingly, in this study damage to cell membrane was observed, as peroxidation of lipid remarkably increased following treatment of cells with DBP. Remarkable lipid peroxidation has also been reported in a study that investigate the ecotoxicological effects of DBP using zebrafish (Danio rerio) [40]. Yan et al. reported that DBP produce extra ROS and peroxidation of lipids in mice allergic asthma [41]. Although mitochondria which are conduct cellular respiration, may adversely influenced by oxidative stress, it also able to be a key place for ROS production [42]. In some investigations mitochondria suggested as a subcellular target for phthalates, moreover collapse of the MMP proposed as a mechanism for phthalates adverse effects [32,43]. In a previous study, a reduction of the mitochondrial membrane potential were observed in PBMCs incubated with phthalate compounds, and particularly with DBP [30]. It has also been demonstrated that phthalate hydrolyzed and modified in mitochondria [44]. Thus in present investigation, the influence of DBP on MMP of lymphocytes was assessed and collapse of mitochondrial membrane potential observed after 6 h treatment. Inhibition of MMP Collapse via pre-incubation of cells with Cyclosporine A or BHT (an antioxidant) implied the connection between ROS generation and collapse of mitochondrial membrane potential in toxicity of DBP toward isolated human lymphocytes.

The mitochondrial membrane damage could further potentiate the DBP-induced oxidative stress via elevating mitochondrial H_2O_2 diffusion into the lysosomes and producing more lysosomal hydroxyl radicals. To explore the mechanism of Di(2-ethyhexyl) phthalate toxic effect on pancreatic beta cells (INS-1 cells) function, Yan She et al. demonstrated that DEHP increase lysosomal membrane permeability and reduce MMP in INS-1 cells [45]. In accordance with previous studies, DBP also lead to lysosomal membrane permeation and release of acridine orange to cytosol from damaged lysosome in present research. Injury of lysosome can induce through ROS generation and resulting in enhancement of oxidative stress. We investigated such possible connection between reduction of lysosomal membrane integrity and oxidative stress through pre-incubation of cell with BHT. Inhibition of lysosomal membrane leakage by BHT was found in our study proved the existence of such relationship. Interestingly in our study, intralysosomal pH enhancer (chloroquine) delayed mitochondrial membrane damage and MPT pore sealing agent (Cyclosporine A) also delayed lysosomal membrane destabilization stimulated by DBP.

Taken together, all these cellular events brought us to a final conclusion that there is a possible cross-talk between mitochondria and lysosomes, which is a possible mechanism of DBP toxicity toward human lymphocytes. DBP-induced generation of lymphocyte reactive oxygen species could directly impair lymphocyte mitochondria through MPT pore opening and interruption of electron transfer chain. mitochondria-derived hydrogen peroxide (H2O2) reached into lysosome, where it motivate a fenton type reaction (haber-weiss), a reaction which catalyzed by redox-active Fe2+/Fe3+ in lysosome. As demonstrated in Sicińska study [33], induction of this events by DBP, result in production of very reactive hydroxyl radical (HO•) which could cause lysosomal membrane permeation and freeing of cathepsins (digestive proteases). Although it was demonstrated in several studies that DBP inhibit cathepsin B [46,47], Hydroxyl radicals and other released proteases could unfasten the mitochondrial MPT pore, either through oxidation of circumambient thiol groups or via activation of pro-apoptotic proteins

(Bax or Bid) and enzymes such as PLA2 (phospholipase A2). Release of cytochrome c due to electron transfer chain disruption, further potentiates mitochondrial H_2O_2 generation and continues the cycle of mitochondrial/lysosomal oxidative stress cross-talk.

5. Conclusion

Regarding the results of present investigation, DBP decreases the viability of human lymphocytes via changing oxidative stress parameters, which in turn damage subcellular organelles and variety of macromolecules. We also demonstrate that alterations in secretion of cytokines, amount of intracellular calcium and mitochondrial/lysosomal cross-talk has an important contribution in adverse effects of DBP toward human lymphocytes. As a consequence, DBP may decrease performance of lymphocytes, which in turn incapacitate immune system in confronting malignant cells and infectious agents.

Ethical approval

This study was carried out in Shahid Beheshti University of Medical Science (SBMU) at the Faculty of Pharmacy and approved by Research Ethic Committee of SBMU (Number: IR. SBMU. RAM. REC. 1395. 7; Date: April 10, 2016). All donors were aware of this study and filled an approval form.

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CRediT authorship contribution statement

Mohammad Hadi Zarei: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Jalal Pourahmad:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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