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RESEARCH ARTICLE

Di (2-ethylhexyl) phthalate induces cardiac disorders in BALB/c mice

Ines Amara[1] · Rim Timoumi[1] · Emna Annabi[1] · Fadwa Neffati[2] · Mohamed Fadhel Najjar[2] · Chayma Bouaziz[1] · Salwa Abid-Essefi[1]

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Abstract

Because of the extensive use of phthalates for domestic, medical, and industrial applications, the evaluation of their toxic effects is of major concern to public health. The aim of the present study was to assess the propensity of di (2-ethylhexyl) phthalate (DEHP), one of the most used phthalates, to cause oxidative cardiac damage in mice. DEHP was administered intraperitoneally at doses of 5, 50, and 200 mg/kg body weight for 30 consecutive days in BALB/c mice. We assessed the effect of DEHP on cardiac injury using biochemical profile (such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine phosphokinase (CPK), total cholesterol (T-CHOL), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)), parameters related to myocardiac oxidative stress, such as malondialdehyde (MDA) level, protein carbonyl (PC) concentration, and DNA fragmentation. In addition, we evaluated antioxidant status; enzymatic (catalase (CAT) and superoxide dismutase (SOD) activities) and non-enzymatic (protein-bound sulfhydryl concentration (PSH)) antioxidants. Acetylcholinesterase (AChE) activity and histopathological changes were also assessed in heart mice treated with DEHP. Our results showed that DEHP induced an elevation of serum marker enzymes and perturbated the lipid profile. In addition, this phthalate increased lipid peroxidation, protein carbonyl levels, and DNA fragmentation in the heart in a dose-dependent manner. Antioxidant status was also perturbated by the increase of the CAT and SOD activities and the decrease of the protein-bound sulfhydryl concentration. AChE activity was also inhibited in the heart following the treatment with DEHP. These biochemical alterations were also confirmed by histopathological changes. Increased free radical production at various doses of DEHP would result in impairment of the redox status leading to an enhanced dose-dependent cardiotoxicity.

Keywords Phthalate · Di (2-ethylhexyl) phthalate · Oxidative stress · Lipid peroxidation · Genotoxicity · Cardiotoxicity

Introduction

Because of their widespread use, phthalates pose a major scientific and public health hazard. They are defined as endocrine-disrupting chemicals (EDCs). In fact, these phthalates are exogenous compounds able to interact with the endocrine system and modify its functioning. The downstream effects arise from their interaction with hormone, orphan, or nuclear receptors or interaction with steroid biosynthesis and metabolism pathways (Diamanti-Kandarakis et al. 2009).

Many reports associate EDC exposure to many adverse human health effects, such as cardiovascular conditions. Moreover, several epidemiological studies have demonstrated that the exposure to EDC can cause hypertension, coronary artery alteration, atherosclerosis, and myocardial infarction (Melzer et al. 2010; Khalil et al. 2013).

Di (2-ethylhexyl) phthalate (DEHP) is one of the major useful esters as plasticizer which increases flexibility and elasticity of high-molecular weight polymer. DEHP is a highly hydrophobic compound, and the non-covalent bond that establishes with the polyvinyl chloride (PVC) polymer is very weak. Consequently, it can leach out from the polymer material and, then, can be absorbed through skin contact, ingestion, or inhalation. DEHP can also release directly into the body from medical products, given that this plasticizer is widely used in medical devices,

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✉ Salwa Abid-Essefi
salwaabid@yahoo.fr

[1] Faculty of Dental Medicine, Laboratory for Research on Biologically Compatible Compounds, University of Monastir, LR01SE1, Rue Avicenne, 5000 Monastir, Tunisia

[2] Laboratory of Biochemistry-Toxicology, University of Monastir, Monastir University Hospital, Monastir, Tunisia

such as in transfusion bags holding blood and plasma, intravenous fluid tubing associated with their administration, umbilical catheters, hemodialysis tubing, respiratory masks, and examination gloves (Green et al. 2005).

Therefore, some patients undergoing various medical interventions are exposed to high doses of DEHP. In this context, Doull et al. (1999) estimate that long-term exposures of dialysis patients reach 457 μg DEHP/kg/day. The exposure of developing fetuses can reach up to 20 mg DEHP/kg/day, which is a critical rate for their development. Blood bags are an important source of exposure to DEHP, with exposure levels of 0.7 mg/kg/time in transfused persons. Previous studies documented that DEHP is cytotoxic in different cell lines (Kismali et al. 2017; Ma et al. 2018) and showed, also, that this phthalate is deleterious in various organs, like the reproductive tract, liver, lungs, and heart (Tickner et al. 2001).

Although the studies investigating the effect of DEHP on cardiotoxicity are not numerous, few of them have tested its effect on cardiomyocytes. They demonstrated that exposure to DEHP leads to a decrease of connexin-4 protein expression which pushes chick embryonic cardiomyocytes to decrease their contractile function. The mechanism behind the asynchronous cell beating is due to a decrease of cardiac mechanical and electrical activity (Gillum et al. 2009). DEHP exposure was related to modifications in the variability of heart rate, enhanced cardiovascular reactivity, and prolonged blood pressure recovery. Alterations in cardiac gene expression were also observed. These modifications in autonomic balance often precede clinical manifestations of hypertension, atherosclerosis, and conduction abnormalities (Jaimes et al. 2017). Since this phthalate crosses the placental barrier, in utero exposure has been shown to induce a decrease in arterial blood pressure in male offspring (Martinez-Arguelles et al. 2013). In rats, DEHP causes ventricular dysfunction of the heart. Indeed, this phthalate increases the expression of genes associated with esterification, β-oxidation, fatty acid transport, and mitochondrial import. Such an increase leads to increased need of cardiomyocytes for energy production which makes these cells more dependent on fatty acids. DEHP also downregulated the enzymes responsible for glucose oxidation (Posnack et al. 2011). In this context, the post-mitotic cells that compose the heart tissue are very sensitive to oxidative attacks, since cardiomyocytes draw their energy preferentially from fatty acids (Sohal and Weindruch 1996). Many cardiovascular diseases, like hypertension, atherosclerosis, and heart failure, can be induced in cardiac tissue following an overproduction of reactive oxygen species (ROS) (Heistad 2006). ROS directly modify central proteins responsible for excitation–contraction coupling and, thus, alter the contractile function. Moreover, they lead to remodeling of the extracellular matrix by activating the matrix metalloproteinases and stimulating the proliferation of cardiac fibroblast. ROS can also activate a variety of transcription factors and cause apoptosis (Tsutsui et al. 2011). Therefore, the present study was carried out to determine the effects of DEHP exposure on the oxidative status in the cardiac tissue.

Materials and methods

Chemicals

DEHP, thiobarbituric acid (TBA), and pyrogallol were purchased from Sigma-Aldrich (St. Louis, MO). 2, 4-Dinitrophenylhydrazine (2,4-DNPH) and guanidine were obtained from VWR International (Fontenaysous-Bois, France). Low Melting Point Agarose (LMA) and Normal Melting Point Agarose (NMA) were purchased from Sigma (St. Louis, MO). The rest of the used products were of analytical grade.

Animals and experimental design

Twenty-four male mice of BALB/c strain (sexual, St. Doulchard, France) were used (weighing on average 20 ± 0.3 g; 6 weeks old). These animals were fed with a standard granulated food and drinking water and were randomly grouped into four groups of six rats each and treated as follows:

Group 1: mice received intraperitoneally corn oil, considered as negative controls.
Group 2: mice received intraperitoneally DEHP at 5 mg/kg body weight (b.w.) everyday from day 1 until day 30, the sacrifice day.
Group 3: mice received intraperitoneally DEHP at 50 mg/kg (b.w.) everyday from day 1 until day 30.
Group 4: mice received intraperitoneally DEHP at 200 mg/kg (b.w.) every day for 30 days.

The DEHP doses used in the present study (5, 50, and 200 mg/kg b.w./day) ranked from dose levels reflecting the mean real human daily exposure and the NOAEL (no observed adverse effect level) to a relatively high DEHP level that is still inferior to LD_{50} in mice (Kavlock et al. 2002; Thomas et al. 1978).

After 30 days of treatment, all the mice were euthanized by cervical dislocation and the hearts were removed. In order to eliminate the superficial blood, these hearts were washed in ice-cold phosphate-buffered saline (PBS) solution pH 7.4. Red blood cells were also removed with an ice-cold PBS wash after slicing the hearts.

Preparation of heart extracts

10 mM Tris–HCl (pH 7.4) was used to homogenize obtained hearts with a Potter (glass Teflon). Heart suspensions were centrifuged at 12,000 rpm for 30 min at 4 °C, and a clear supernatant was collected which is used to determine the protein concentration by the Bradford's colorimetric assay.

Determination of biochemical parameters

The biochemical parameters aspartate aminotransferase (AST), alanine amino-transferase (ALT), lactate dehydrogenase (LDH), creatinine phosphokinase (CPK), total cholesterol (T-CHOL), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined in the clear supernatant serum with the biochemical parameter counter (C×9 PRO; Beckman, Switzerland). Results were expressed as international units per liter (UI/L) or mmol per liter (mmol/L).

Determination of protein carbonyl contents

Quantification of protein carbonyl (PC) was done following the method of Mercier et al. (2004). In brief, trichloroacetic acid (20%) is used to precipitate proteins. From this precipitate, the carbonyl group is derivatized with 2,4-dinitrophenylhydrazine, which gives a stable dinitrophenylhydrazone product. Then, this stable product was quantified spectrophotometrically at 370 nm after suspension in guanidine hydrochloride (6 M). PC concentration was quantified using the molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

Determination of lipid peroxidation products

Lipid peroxidation in the heart tissue was estimated spectrophotometrically by measuring malondialdehyde (MDA) according to the method established by Ohkawa et al. (1979). The absorbance was measured at 546 nm. According to a standard curve, the MDA content was determined and results were expressed as nmol of malondialdehyde/mg of proteins.

DNA damage assessed by the comet assay

The nuclei isolated by homogenization are spread on ordinary microscope slides covered with agarose. Electrophoresis was, then, applied under alkaline conditions at 4 °C in the dark for 30 min at 25 V. About 100 nuclei per slide were visualized after staining with ethidium bromide. The results are obtained after a score calculation based on the degree of damage to the DNA which is relative to the tail fluorescence intensity (Collins et al. 1996).

Measurement of catalase activity

Catalase (CAT) activity was determined by a spectrophotometric method. It consists to follow the H_2O_2 decomposition over time at 240 nm (Acuna et al. 2009). One unit of CAT activity was referred to the μmoles of H_2O_2 consumed/min/mg of protein.

Measurement of superoxide dismutase activity

The activity of superoxide dismutase (SOD) was measured based on the method of Marklund and Marklund (1974). In the presence of the superoxide radical ($O_2^{\cdot -}$), the pyrogallol is autoxidized and can be quantified spectrophotometrically at 440 nm. The SOD, being an enzyme that catalyzes dismutation of $O_2^{\cdot -}$, will inhibit the autoxidation of pyrogallol. Thus, the activity of this enzyme was estimated by the amount of enzyme necessary to inhibit 50% of pyrogallol autoxidation and was expressed as enzyme unit/mg of protein.

Measurement of protein-bound sulfhydryl contents

Protein-bound sulfhydryl concentration (PSH) was measured by the method of Sedlack and Lindsay (1968) by eliminating the non-protein sulfhydryl (NPSH) content from the total sulfhydryl content (TSH). The amount of TSH in the heart homogenate is relative to the yellow color intensity of the TNB derivative measured spectrophotometrically at 421 nm. This derivative is generated following the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols present in the samples (Sedlack and Lindsay 1968). Results were reported as μg PSH/mg of protein.

Measurement of acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was measured in heart tissues according to Ellman et al. (1961). Measurement of this activity is based on the degradation of acetylcholine iodide as a substrate of AChE. The results were expressed as μmol acetylthiocholine hydrolyzed/min/mg protein.

Histopathological examination

Histological assessment of the heart tissues was performed on separate groups of mice. Briefly, mice hearts were removed at the end of the experimental period, were cut sagittally into two halves, and were fixed in immersion containing 15% formaldehyde at room temperature for 24 h. After dehydration using graded ethanol, pieces of hearts were embedded in paraffin. Paraffin sections (5–6 μm) were cut using a rotary microtome. The sections were stained with hematoxylin and eosin and analyzed with light microscopy. Each heart slide was examined,

and the severity of the changes observed were scored as follows: normal appearance (−), mild (+), moderate (++), and severe (+++).

Statistical analysis

Each experiment was done three times separately, and data were expressed as the mean ± SD of the means. To determine differences between groups, we used one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Results

Assessment of DEHP effects on biochemical parameters and lipid profile in serum

The measurements of enzymatic marker levels in cardiac tissues following DEHP treatment are indicated in Table 1. Compared to control, DEHP at different doses (5, 50, and 200 mg/kg) induced a significant increase in LDH, AST, ALT, ALP, and CPK levels.

Table 2 shows the effect of DEHP on myocardial lipids, such as TC, TG, LDL-C, and HDL-C. DEHP-induced myocardial infarcted mice promoted a significant increase ($P < 0.05$) in the concentrations of TC, TG, and LDL-C and a significant decrease in HDL-C in cardiac tissues when compared to control mice.

Therefore, DEHP disturbed significantly the homeostasis of cardiac tissues by altering biochemical parameters in mice.

Estimation of lipid peroxidation

As reported in Fig. 1, DEHP treatment (5, 50, and 200 mg/kg) increased MDA levels in mice cardiac tissues. This increase indicates an enhancement in the lipid peroxidation in cardiac tissues. It passed from 3.93 ± 0.85 nmol MDA/mg of proteins in the control group to reach 11.72 ± 0.84 nmol MDA/mg of proteins at 200 mg/kg b.w. of DEHP.

Estimation of protein oxidation

DEHP exposure elevated significantly the PC level as compared with the control group (Fig. 2). This elevation was a dose-dependent manner, and it passed from 0.6 ± 0.13 nmol of carbonyl group/mg of proteins in the control group to 1.43 ± 0.12, 2.27 ± 0.22, and 3.68 ± 0.37 nmol of carbonyl group/mg of proteins in groups treated with DEHP at 5, 50, and 200 mg/kg bw, respectively.

DNA fragmentation

The present results of alkaline comet assay demonstrated that the cardiomyocyte DNA of mice exposed to DEHP at doses of 5, 50, and 200 mg/kg b.w. for 30 days was increased compared to the control group (Fig. 3). The increases in DNA damage indicated that this phthalate could have a genotoxic risk in cardiac cell nuclei.

Heart antioxidant status

Antioxidant enzyme activities

DEHP treatment led to a significant increase in SOD and CAT activities in mice cardiac tissues (Figs. 4 and 5). SOD activities were between 6.10 ± 1.00 U SOD/mg of proteins in the control group and 30.81 ± 2.27 U SOD/mg of proteins in the group treated with the highest dose of DEHP (200 mg/kg). CAT activities were between 713.58 ± 228.38 μmol H_2O_2/min/mg of proteins in the

Table 1 Effect of DEHP at different doses on biochemical tissues markers in male BALB/c mice treated with DEHP by intraperitoneal route

	DEHP (mg/kg body weight)							
	0		5		50		200	
Index	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
LDH (UI/L)	590	1	786	7.87***	817.66	17.46 a***	833.83	8.83***
AST (UI/L)	148.9	3.36	201.21	12.14***	221.37	5.67 a***	238.2	2.83 c***
ALT (UI/L)	8.1	0.1	11.5	0.78**	13.24	0.525***	14.9	0.96 b***
ALP (g/L)	47	4.58	55.33	4.5	62	3**	70.96	1.94 b***
CPK (UI/L)	12,301	0.8	14,568	0.583*	20,398	1.075 c***	26,794	0.564 c, f***

Values are expressed as mean ± SD (six animals were treated per group)

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, values are significantly different from control group; a $P < 0.05$, b $P < 0.01$, and c $P < 0.001$, values are significantly different from group treated with 50 mg/kg of DEHP; d $P < 0.05$, e $P < 0.01$, and f $P < 0.001$, values are significantly different from group treated with 200 mg/kg of DEHP

Table 2 Lipid profile (total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C)) and atherogenic index (AI) in serum of control and treated mice with DEHP

	DEHP (mg/kg body weight)							
	0		5		50		200	
Index	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
TC (mmol/L)	1.43	0.31	1.64	0.12	2.07	0.087**	2.23	0.055 a**
TG (mmol/L)	0.63	0.199	1.033	0.115*	1.24	0.04**	1.37	0.05 a***
HDL (mmol/L)	0.403	0.011	0.316	0.015***	0.27	0.01 b***	0.2	0.01 c, f***
LDL (mmol/L)	1.19	0.037	1.36	0.03**	1.44	0.03**	1.62	0.03 c, f***
AI	2.96	0.14	4.3	0.28**	5.34	0.08 a***	8.11	0.49 c, f***

Values are expressed as mean ± SD (six animals were treated per group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, values are significantly different from control group; a $P < 0.05$, b $P < 0.01$, and c $P < 0.001$, values are significantly different from group treated with 50 mg/kg of DEHP; d $P < 0.05$, e $P < 0.01$, and f $P < 0.001$, values are significantly different from group treated with 200 mg/kg of DEHP

control group and 3668.9 ± 178.59 μmol H_2O_2/min/mg of proteins in the group treated with the highest dose of DEHP (200 mg/kg).

Protein-bound sulfhydryl contents

Cardiac tissues' PSH levels decreased significantly following DEHP treatment (Fig. 6). PSH levels decreased from a basal value of 41.75 ± 4.69 μg PSH/mg of proteins in the control group to 27.65 ± 1.02, 20.39 ± 1.2, and 13.00 ± 2.74 μg PSH/mg of proteins in groups treated with DEHP at 5, 50, and 200 mg/kg body weight, respectively.

Heart AChE activity

As shown in Fig. 7, mice treated with DEHP induced a significant inhibition of cardiac tissues' AChE activity. The AChE activity increased from a basal level of 0.0095 ± 0.00068 μmol acetylcholine hydrolyzed/min/mg of proteins to reach 0.0024 ± 0.0013 μmol acetylcholine hydrolyzed/min/mg of proteins at 200 mg/kg b.w. of DEHP.

Histopathology of heart tissue Histological evaluation showed that the myocardium of the control group had a normal histo-architechture with intact myofibrils, clear transverse striations, and absence of hemorrhage or inflammation. DEHP exposure induced structural changes in this tissue, characterized by inflammatory cell infiltration, vascular alteration, appearance of cytoplasmic vacuolization, and loss of transverse striations (Fig. 8). Incidences of lesions in the experimental groups were scored in Table 3.

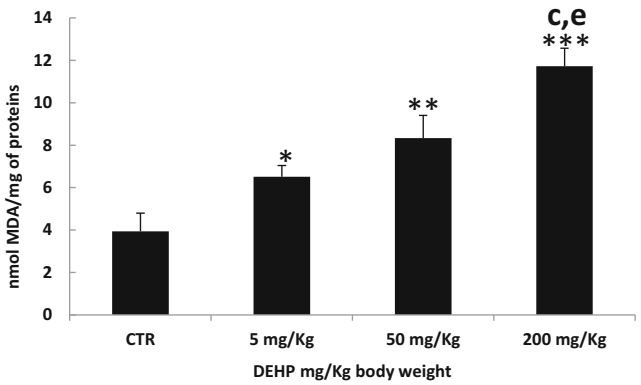

Fig. 1 Effect of DEHP treatment on malondialdehyde (MDA) (nmol MDA/mg of proteins) in cardiac tissues of experimental mice after treatment of 30 days. Values represent mean ± SD (six animals were treated per group); *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, values are significantly different from the control group; *c* $P < 0.001$, values are significantly different from group treated with 50 mg/kg of DEHP; *e* $P < 0.01$, values are significantly different from group treated with 200 mg/kg of DEHP

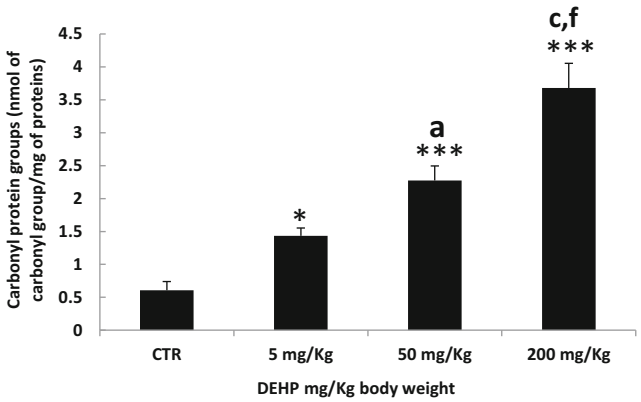

Fig. 2 Effect of DEHP treatment on carbonyl protein groups (nmol of carbonyl group/mg of protein) in cardiac tissues of experimental mice after treatment of 30 days. Values represent mean ± SD (six animals were treated per group); *$P < 0.05$ and ***$P < 0.001$, values are significantly different from the control group; *a* $P < 0.05$ and *c* $P < 0.001$, values are significantly different from group treated with 50 mg/kg of DEHP; *f* $P < 0.001$, values are significantly different from group treated with 200 mg/kg of DEHP

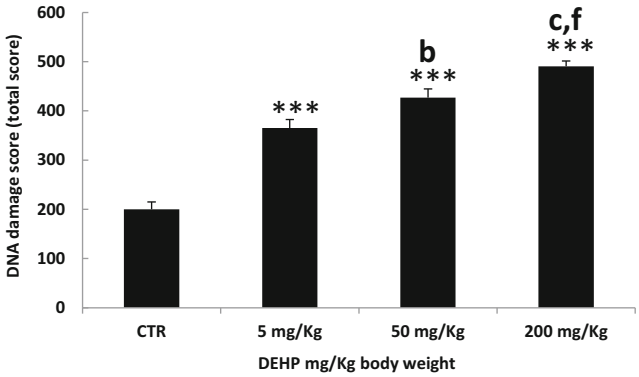

Fig. 3 Effect of DEHP treatment on DNA damage (total score) in cardiac tissues of experimental mice after treatment of 30 days. Values represent mean ± SD (six animals were treated per group); ***$P < 0.001$, values are significantly different from the control group; *b* $P < 0.01$ and *c* $P < 0.001$, values are significantly different from group treated with 50 mg/kg of DEHP; *f* $P < 0.001$, values are significantly different from group treated with 200 mg/kg of DEHP

Discussion

Phthalates, like DEHP, continue to be an irreplaceable plasticizer that is used to impart flexibility and elasticity to polyvinyl chloride products. These environmental xenobiotics are unavoidable contaminants. Exposure to DEHP was implicated in many toxicological outcomes in mammals, reproductive toxicity (Wang et al. 2017), neurotoxicity (Du et al. 2017), carcinogenicity (Chen et al. 2017), hepatotoxicity (Ha et al. 2016), and lipid metabolism disruption (Pradhan et al. 2018). Unfortunately, the mechanism underlying DEHP-induced cardiotoxicity is not established. It is well known that oxidative damage is one of the major lesions of heart tissue. The present study was undertaken to determine the effect of DEHP on induction of oxidative stress and cardiotoxicity in mice.

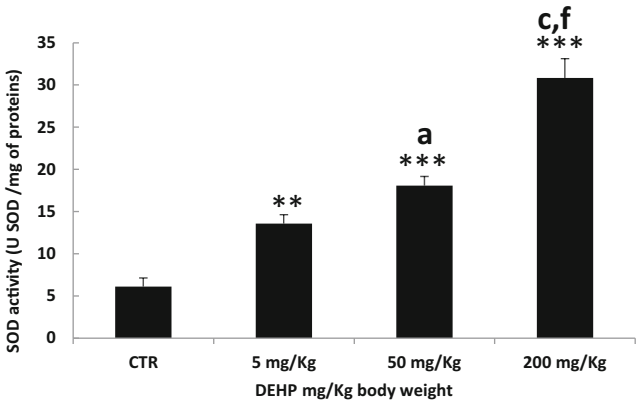

Fig. 4 Effect of DEHP treatment on superoxide dismutase (SOD) activity (U SOD/mg of proteins) in cardiac tissues of experimental mice after treatment of 30 days. Values represent mean ± SD (six animals were treated per group); **$P < 0.01$ and ***$P < 0.001$, values are significantly different from the control group; *a* $P < 0.05$ and *c* $P < 0.001$, values are significantly different from group treated with 50 mg/kg of DEHP; *f* $P < 0.001$, values are significantly different from group treated with 200 mg/kg of DEHP

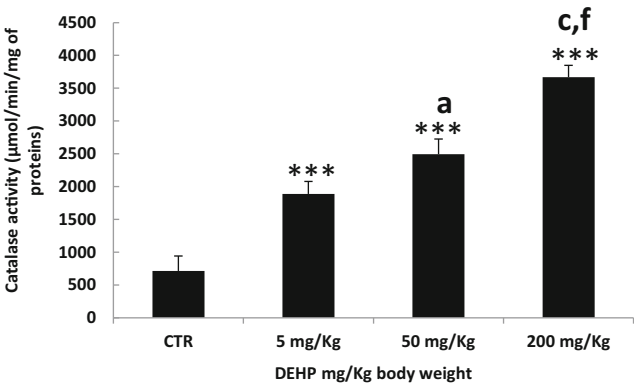

Fig. 5 Effect of DEHP treatment on catalase (CAT) activity (µmol/min/mg of proteins) in cardiac tissues of experimental mice after treatment of 30 days. Values represent mean ± SD (six animals were treated per group); ***$P < 0.001$, values are significantly different from control group; *a* $P < 0.05$ and *c* $P < 0.001$, values are significantly different from group treated with 50 mg/kg of DEHP; *f* $P < 0.001$, values are significantly different from group treated with 200 mg/kg of DEHP

Treatment with DEHP at different doses (5, 50, and 200 mg/kg) resulted on the onset of myocardial damage as evidenced by significant increase in serum LDH, AST, ALT, ALP, and CPK levels which constitute important indices to evaluate the severity of myocardial infarction (Sheela-Sasikumar and Shyamala-Devi 2000). In fact, the damage of myocardial cells due to insufficiency of oxygen supply or glucose disrupts the integrity of the cell membrane which makes it more porous and, thus, facilitates the leakage of these enzymes. High risk of myocardial infarction is usually accompanied by abnormalities in the lipid profile. In fact, increased level of circulating cholesterol and its accumulation in the heart tissue is the major cause of cardiovascular damage (Mediene-Benchekor et al. 2001). Results from the present study showed that DEHP treatment increased levels of TC, TG, and LDL-C and decreased levels of HDL-C. A strengthening of lipid biosynthesis could explain the observed changes

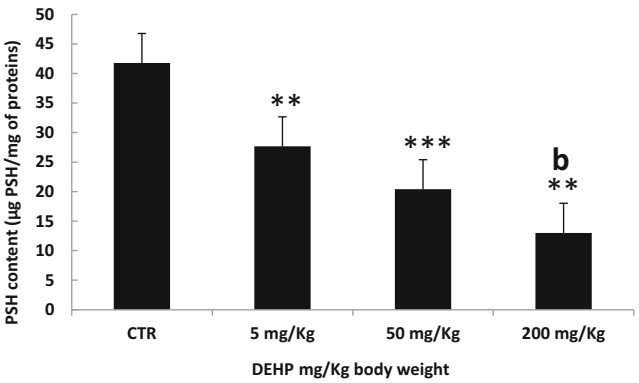

Fig. 6 Effect of DEHP treatment on protein-bound sulfhydryl (PSH) content (µg of PSH/mg of proteins) in cardiac tissues of experimental mice after treatment of 30 days. Values represent mean ± SD (six animals were treated per group); **$P < 0.01$ and ***$P < 0.001$, values are significantly different from control group; *b* $P < 0.01$, values are significantly different from group treated with 50 mg/kg of DEHP

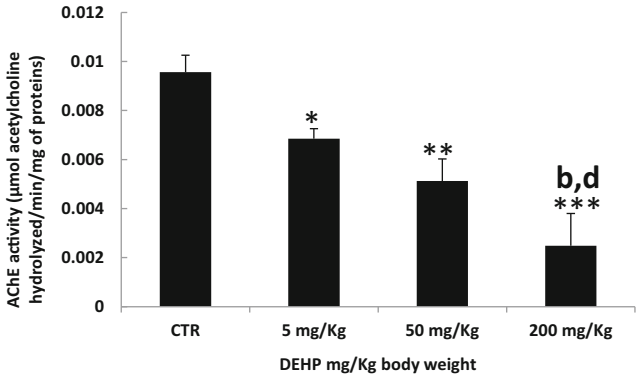

Fig. 7 Effect of DEHP treatment on acetylcholinesterase (AChE) activity (µmol of acetylcholine hydrolyzed/min/mg of proteins) in cardiac tissues of experimental mice after treatment of 30 days. Values represent mean ± SD (six animals were treated per group); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, values are significantly different from control group; *b* $P < 0.01$, values are significantly different from group treated with 50 mg/kg of DEHP; *d* $P < 0.05$, values are significantly different from group treated with 200 mg/kg of DEHP

in lipid levels. Studies in patients have shown that the increase in serum triglycerides linked to a decrease in HDL-C levels is a predisposing factor for cardiovascular risk (Patsch 1993). Our results show that in mice, DEHP increases TG levels and decreases HDL-C, which could increase the risk of predisposition to cardiovascular damage. In this study, in addition to changes in lipid profile in mice treated with DEHP, we examined the levels of atherogenic index (AI). AI represents the ratio of LDL-C to HDL-C which is a pertinent index of cardiovascular diseases (Panagiotakos et al. 2003). This index

Table 3 Grading of the histopathological alterations in the heart of mice exposed to different concentration of DEHP: (−) none, (+) mild, (++) moderate, (+++) severe

Histopathological alterations	DEHP (mg/kg body weight)			
	0	5	50	200
Inflammation	–	+	++	+++
Vascular damages	–	+	++	+++
Fragmentation of myofibrils	–	–	+	+++
Loss of transverse striations	–	–	+	+++
Appearance of vacuoles	–	+	++	+++

is importantly increasing in atherosclerosis (Acay et al. 2014). Our results demonstrated that DEHP exposure increased the AI which is a strong marker to predict the risk of high cardiovascular diseases. In fact, AI reflects the true relationship between protective and atherogenic lipoprotein (Dobiášová et al. 2011). The increase of AI index suggests a cardiotoxic effect of this phthalate.

In relation to DEHP inducing heart damage, it was known that the heart is very sensitive to oxidative damage due to its very active oxidative metabolism and fewer antioxidant defenses compared to other organs (Posnack 2014). Among the important manifestations of cellular oxidative damage is lipid peroxidation. Accumulating evidence suggests that excess generation of free radicals can also damage cell membranes or macromolecules and increase cell membrane permeability (Sunmonu and Afolayan 2010). In our study, malondialdehyde

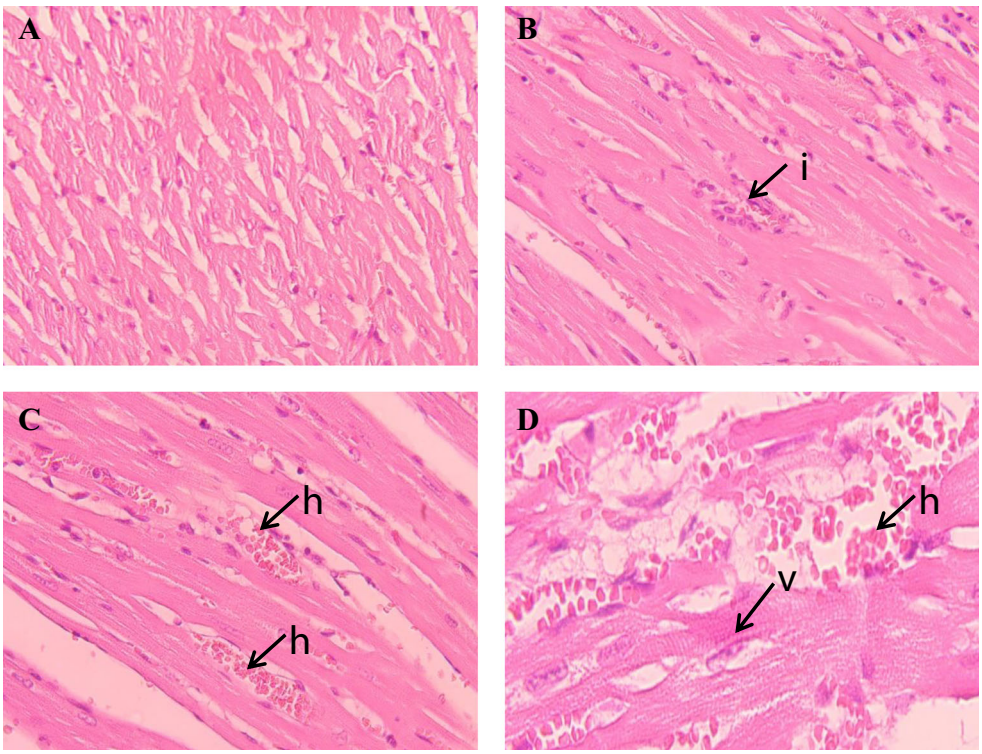

Fig. 8 Histopathology of hematoxylin and eosin (H and E, 400×)-stained mice heart tissue of DEHP-treated groups. **a** Control group: normal histology appearances, continuous structure of myofibrils, clear transverse striations, and absence of inflammatory cell infiltration. **b** Treated mice with DEHP (5 mg/kg): inflammatory cell infiltration (*i*) between myocardial bundles. **c** Treated mice with DEHP (50 mg/kg): focal hemorrhage (*h*). **d** Treated mice with DEHP (200 mg/kg): marked hemorrhage (*h*), cytoplasmic vacuolization of cardiac muscle cells (*v*), and disarrangement of fibers

(MDA) level was elevated in the heart tissue of myocardial injured mice which is an indication of intensified free radical production in this tissue. These free radicals initiate lipid peroxidation of the membrane-bound polyunsaturated fatty acids and lead to the loss of membrane structural and functional integrity. This finding supports the hypothesis that the mechanism of DEHP cardiotoxicity is related to the oxidative stress. Further evidences of DEHP-induced oxidative damage in heart were revealed as assessed by enhanced levels of protein carbonyl levels. Jawalekar et al. (2010) associated the accumulation of oxidized proteins with pathological conditions including cardiovascular diseases which might impair myocardial cell function. In addition to cellular lipids and proteins, free radicals can also affect cellular DNA. In fact, oxidative stress products, such as MDA and carbonyl protein, have been identified to bind to DNA leading to its damage and mutations (Eder et al. 2006; Eckl et al. 1993; Marnett et al. 1985; Kasai et al. 1982). Therefore, it is possible that the observed DNA fragmentation in the heart of DEHP-treated mice assessed by comet assay is a consequence of ROS generation. Additionally, lipid peroxidation and protein oxidation seem to be responsible for DEHP-induced DNA damage as evidenced by a dose-dependent correlation between MDA and carbonyl protein content and DNA damage score. Similar results have been reported in embryonic kidney cells treated with DEHP, which showed that this phthalate induced oxidative DNA damage (Wang et al. 2015). In normal conditions, the generation of reactive metabolites and their damaging effects is controlled by endogenous antioxidant enzymes. Among them, SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, which is subsequently converted to water by CAT and GPx (Chaâbane et al. 2016). We found that the myocardial activities of these enzymes increased significantly in DEHP-treated mice. This might reflect an adaptive mechanism to the generated free radicals. Nevertheless, these increases were insufficient to protect myocardiocytes from free radicals. In fact, we found that the levels of MDA and PC, the main markers of oxidative stress, were significantly increased compared with the control.

Non-enzymatic antioxidants, such as sulfhydryl groups, constitute a second line of cellular defense against free radicals. These thiols formed a class of organic sulfur derivatives, characterized by their containing of sulfhydryl residues present in biological systems (Mello et al. 2015). Sulfhydryl groups are essential for maintaining the structure and function of proteins, enzymes, as well as membranes. They also regulate the cellular redox status by coordinating the antioxidant defense network (Mateen et al. 2016). Our data demonstrate an elevation in protein sulfhydryl content. Given that protein sulfhydryl groups are an important target of oxidative stress, it is possible that the decrease in their rate induced by DEHP ultimately leads to cellular damage. In fact, the oxidation of proteins may be expected to lead to dramatically altered redox status, with subsequent altered enzymatic function especially those involved in cell death (Han et al. 2006). In addition to these biological markers, we evaluated AChE activity to assess heart function. Given an important component of the cardiac cholinergic system, AChE controls the acetylcholine levels and, thus, regulates cardiac parasympathetic responses (Hoover et al. 2004). In fact, acetylcholine (ACh) is known to regulate the cardiac redox environment and, thus, suppress ROS generation during oxidative stress (Sun et al. 2014). At the level of the cholinergic neuro-effector junctions, the enzyme AChE completed the signaling action by rapidly catalyzing the hydrolysis of acetylcholine (Lefkowitz et al. 1996). Previous studies suggest that ROS generation could be implicated in AChE inhibition (Tsakiris et al. 2000). In this work, DEHP treatment was demonstrated to significantly reduce AChE activity in a dose-dependent manner in mice heart tissues. The present inhibition of AChE activity may be the consequence of oxidative stress generated by this phthalate in mice hearts. These biochemical modifications caused by DEHP were related to histological alterations. Our histopathological analysis revealed that this phthalate induced structural alteration characterized by lymphoid infiltration around blood vessels, perinuclear vacuolization of cardiac muscle cells, interfibrillar hemorrhage, and disarrangement of the myocardium bundles. This might result from an increased ROS generation in the heart tissue.

Conclusion

To our knowledge, this is the first study that has evaluated the DEHP alteration of redox state in the heart tissue of experimental mice. In summary, the results of our study indicated that the administration of DEHP induced a change in biochemical indices and lipid profile which suggests that exposure to DEHP can induce heart injury in mice. DEHP exposure was found also to create a state of oxidative stress and inhibit the cardiac AChE activity. The toxic effects of DEHP occurred probably through ROS generation, leading to many alterations of different cardiomyocyte components.

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