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LACTATIONAL EXPOSURE TO DI-2-ETHYL HEXYL PHTHALATE (DEHP) INDUCES OXIDATIVE STRESS AND CAUSES NEURODEGENERATION IN HIPPOCAMPUS OF OFFSPRING FEMALE ALBINO RATS

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Abstract:

The purpose of this study was to determine the effect of DEHP on the hippocampus of F1 progeny female albino rats. Healthy mother rats were divided into four groups. The first group (control) was orally fed with olive oil–vehicle and the other three groups were treated with DEHP 1, 10 and 100 mg /kg bw/day via oral gavage from PND (Post natal day) 1 till 21. On PND 60 the offspring female rats were sacrificed, their hippocampus dissected and processed for biochemical analysis. Results showed that DEHP induced oxidative stress in hippocampus by increasing reactive oxygen species and decreasing antioxidant enzymes. Further, DEHP reduced the mRNA expression of ERα, β and decreased that of Bcl2. Construction of a protein-protein interaction map with ERα, β, Bcl2 and their downstream interacting partners confirmed the existence of a network, enriched for molecular functions. To conclude, lactational exposure to DEHP causes hippocampal neurotoxicity.

Keywords: Phthalates, Lipid peroxidation, Superoxide dismutase, estrogen receptor, Bcl-2, Nissl's granules.

Introduction

Di-(2-ethylhexyl) phthalate (DEHP), a plasticizer commonly used by industry to add flexibility to polyvinyl chloride products, is highly prevalent in the environment. DEHP is present in a wide variety of consumer products, including medical devices such as intravenous tubing, catheters, and dialysis bags. They are not covalently bound with the plastics in which they are mixed and easily leach into the environment [1]. Phthalates cross from maternal blood into the developing fetus via placental transfer and into neonates via breast milk. These exposures may affect the developing endocrine system, which is essential for diverse biological functions, including sexual development and reproductive functions in adults [2].

The major route of DEHP exposure is the ingestion of contaminated food, other materials and water [3]. In particular, children are considered to be more susceptible because they are more likely to be exposed to DEHP. According to Guo et al. [4], children's exposure to DEHP through suckling or chewing on toys or other materials can reach 85 µg/kg/day. DEHP is able to cross the placenta and pass into breast milk, exposure to DEHP during gestation and lactation has been of particular concern [5]. The developmental toxicity of phthalate esters has been analyzed in animals *in vivo* and *in vitro* tests such as whole embryo culture (WEC) and stem cell tests (EST).

Administration of high doses of DEHP to the pregnant rodents lead to embryonic lethality, malformations such as cleft palate, fusion of the sternebrae, adverse effects on sexual differentiation [6]. DEHP has also been observed to affect females, but research is lagging in comparison to the male [7].

Brain is sensitive to oxidative stress due to its high oxygen consumption. Phthalates have been shown to produce neurochemical alteration as well as behavior changes in learning, memory, motor and sexual behavior [8]. Hippocampus has long been known as a seat of learning and memory in human and other animals. ER plays an important role in the development of the hippocampus. Many *in vitro* and *in vivo* studies have demonstrated that estrogen modulates neuronal viability, regulates spine density, synapse number and synthesis of neurotropic factors [9]. Bcl-2, a cell survival factor has been identified as an estrogen responsive gene in the reproductive tissues [10]. Estradiol may directly up regulate this survival factor through receptor-mediated interactions with regions of the bcl-2 promoter. Bcl-2 acts upstream to prevent the activation of caspases, inhibits free radical formation, and regulates calcium sequestration [11]. ROS initiated stress, may be regulated by cell defense mechanisms that include enzymatic antioxidants such as SOD, GPx [12]. ROS are generated continuously in the nervous system during the normal metabolism and neuronal activity. In the primary cultures of cortical and hippocampal neurons, H_2O_2 depletes cellular adenosine triphosphate (ATP) content [13]. ATPases are lipid dependent membrane bound enzymes. Any perturbation in the activities of ATPases affects the membrane status by inflicting changes in the electrophysiological energetic and the normal homeostasis [14]. Na^+/K^+-ATPase is responsible for the generation of the membrane potential through the active transport of sodium and potassium ions in the CNS necessary to maintain neuronal excitability. It is present at high concentrations in brain, consuming about 40–50% of the ATP generated in this organ [15]. Ca^{2+}-ATPase is responsible for the fine tuning of intracellular calcium levels. Moreover, the role of Mg^{2+}-ATPase is to maintain high brain intracellular Mg^{2+}, changes of that may control rates of protein synthesis and cell growth [16]. The present study was designed to study the effect of lactational exposure to DEHP on various ATPases, antioxidant enzymes, and mRNA expression of estrogen receptor α, β, Bcl-2 and histology of hippocampus in the offspring female albino rats.

Materials and Methods

The present study approved by the institutional ethical committee (Ref No. IAEC No 01/01/10) utilized adult pregnant female albino Wistar rats weighing about 180-200g (age 60 days). The animals housed in clean polypropylene cages, in an air-conditioned environment with constant photoperiod of 12 hour light /dark cycle were fed with pellet diet free of soy estrogen (Gold Mohur Ltd., Mumbai, India) and drinking water *ad libitum*. Mother rats were divided into four groups, each consisting of six animals; Group I: Control, Group II: DEHP 1 mg /kg/day, Group III: DEHP 10 mg /kg/day, and Group IV: DEHP 100 mg /kg/day. From PND (Post natal day)-1 to PND-21, they received phthalate (DEHP) through oral gavage. Body weights of the pups and mother were monitored throughout the experimental period.

On the 60th day the offspring female pubertal wistar rats were sacrificed, hippocampus was dissected for the oxidative stressors, antioxidant enzymes, Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase and mRNA expression of ER-α, ER-β and Bcl2 in hippocampus was quantified. 2µg of the total RNA isolated using Tri Reagent (Sigma) (Chomczynski and Sacchi, 1987) was reverse transcribed using a commercial Superscript III first strand cDNA synthesis kit (Bio Rad, USA) according to the manufacturer's protocol. Quantitative-PCR was carried out in an MX3000p PCR system (Stratagene, Europe) using SYBR Green PCR master mix (Eurogentec, USA). The specificity of amplification for each primer pair was determined by melting curve analysis. The data were analyzed by comparative CT method and the fold change was calculated. The hippocampal tissue was homogenized in 0.1 mol/L Tris–HCl buffer, pH 7.4 and centrifuged at 12,000 rpm for 10 min and the supernatant was used for determining the biochemical parameters described below. Protein concentration of the homogenate was determined by Lowry method using BSA standards [17].

Various reactive oxygen species, ATPases and enzymatic, non-enzymatic antioxidants' levels were determined as previously described. Estimation of Lipid peroxidation (LPO) [18] and Hydroxyl radicals (•OH) [19], Superoxide dismutases (SOD) [20], Catalase (CAT) [21], glutathione peroxidase (GPx) [22], glutathione reductase (GR), [23], and glutathione S-transferase (GST) [24] were determined. (Na^+K^+) [25], (Ca^{2+} ATPase) [26], [27] Mg^{2+}ATPase were studied. Non-enzymatic antioxidant the vitamin E was estimated by the method [28]. The vitamin C was estimated by the method [29]. For histology, the animals were sacrificed by perfusion and tissue fixed with 10% formaldehyde. The paraffin blocks were cut into 10 µm thickness using rotary microtome and stained with Cresyl fast violet to demonstrate the Nissl's substance in the neurons and cell nuclei of hippocampus [30]. The data were statistically analyzed by one-way ANOVA, Students Newman Keul's (SNK) test and Duncan's multiple range tests using SPSS 7.5. The significance was considered at level of $p<0.05$.

To find out the overall effect of DEHP and the affected molecular pathway, we constructed a protein-protein interaction (PPI) network that included ERα β and its interacting partners, Bcl2, SOD, CAT, GPx, GST, GR and Na^+/K^+ ATPase. Information regarding the experimentally validated interacting partners of Erα (Esr1) and Er β (Esr2) was retrieved from the BioGrid database for the organism *Rattus novergicus*, while that of Bcl2 was not available. The PPI 6 network was constructed using STRING version 9 available at http://string-db.org/, one of the largest database derived from numerous sources such as experimental repositories or computational prediction methods. STRING takes proteins as its nodes, and assigns an edge between two proteins if they interact with one another. Moreover, an enrichment analysis was performed to identify the associated molecular functions and KEGG pathway. The significance level was set to $p<0.05$.

Statistical Analysis

The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Students Newman Keul's (SNK) test and Duncan's multiple range tests to assess the significance of individual variations between the control and treatment groups using a computer based software **(**IBMSPSS 20) the significance was considered at level of *p<0.05*.

Results

Body and organ weight

Figure 1 A significant decrease in body weight was observed in DEHP treated F1 progeny when compared to control animals. But organ weight did not show any changes in DEHP treated rats.

Histology

Figure 2 shows the histological study on hippocampus in various doses of DEHP treated rats (Cresyl Fast violet). A, B, C, and D show neuronal morphology of pyramidal cell layers of cornus ammonis1, 2 and 3 (CA1, CA2 and CA3) in the hippocampus of control rats. There was not much change in 1mg treated group, but 10 and 100 mg treated groups showed disturbed cell architecture in CA3, CA2 and dentate gyrus regions when compared to control groups. The neuronal shrinkage of hippocampal layer due to degeneration of pyramidal cell in 10 or 100 mg DEHP exposed rats was also observed.

Lipid peroxidation and hydroxyl radical production

The lipid peroxidation level and hydroxyl radical production were significantly increased in the hippocampus of 1mg, 10mg, 100mg DEHP treated rats when compared to control (Figure 3 A&B).

Hippocampal antioxidant enzymes

Figure 4 (A,B,C,D&E) provides data on antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S- transferase (GST) in hippocampus of DEHP treated rats. All the doses of DEHP exposed rats showed a marked reduction in the activities of SOD, CAT, GPX, GR, and GST in hippocampus.

Effect of DEHP on non-enzymatic antioxidant levels

Figure 5(A&B) provides data on hippocampus non enzymatic antioxidant concentration such as vitamin C and vitamin E in control and DEHP treated rats. The hippocampal non enzymatic antioxidant levels like vitamin C and vitamin E were significantly diminished in DEHP treated rats in a dose dependent manner

Effect of DEHP on ATPases

ATPase such as the Na^+/K^+, Mg^{2+} and Ca^{2+}-ATPase activities were significantly decreased in hippocampus of 1mg, 10mg, 100mg DEHP treated rats when compared to control rats Figure 6 (A.B.C.D).

Estrogen receptors and Cell survival protein

Figures 7.8.9 provide data on estrogen receptors α, β and Bcl2 in hippocampus of DEHP treated rats. All the doses of DEHP exposed rats showed decreased mRNA expression in hippocampus.

Protein-Protein Interaction

Proteins are vital macromolecules, at both cellular and systemic levels, but they rarely act alone. Diverse essential molecular processes within a cell are carried out by molecular machines that are built from a large number of protein components organized by their PPIs. Figure 10 shows the estrogen receptor α, β and anti-apoptotic protein Bcl-2 on its downstream interacting partners by constructing a protein-protein interaction (PPI) map with the help of STRING (9.01)

Discussion

Phthalates are widely used in plastics and consumer products that are ubiquitous environmental contaminants. DEHP has been shown to induce oxidative stress by disrupting internal anti-oxidant protective mechanisms [31]. Histological study also showed DEHP (10,100mg/kg) induced neuronal shrinkage of CA1, CA2, CA3, in the hippocampus. Degeneration of pyramidal cells and perturbed the hippocampal architecture has also been revealed by Cresyl fast violet staining (Figure 2). Smith et al. has shown that exposure to DEHP (10 mg/kg, intraperitoneally) from postnatal day 16 to postnatal day 22 reduced axonal markers in the CA3 distal stratum oriens (SO) and reduced cell density of both immature and mature neurons in the dentate gyrus (DG) and CA3, respectively, in male rats [32]. Oxidative stress and the formation of free radicals are the major factors involved in the cytopathology of many neurodegenerative disorders, where neuron displays oxidation and up-

regulation of oxidative defenses. We found that DEHP significantly increased hydroxyl radical and LPO production in the hippocampus in a dose dependent manner. DEHP induced oxidative stress during prenatal and early postnatal periods in newborn male rats [33]. Earlier studies also stated that DEHP can impair fetal brain development by affecting lipid profile in newborn male rats [34]. Increased free radical generation may have perturbed hippocampal morphology and induced neurodegeneration in the hippocampus in the present study.

Antioxidant enzymes are an essential part of the cellular defense against ROS. The effects of oxidative stress could be alleviated by antioxidant enzymes, such as SOD, GPx, CAT, GST and GR. SOD neutralizes the toxicity of oxygen by catalyzing the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen whereas GPx detoxifies hydrogen and lipid peroxides, and modulates redox-sensitive signaling pathways [35]. In the present study DEHP significantly decreased antioxidant enzyme levels, such as SOD, CAT, GPx, GST and GR in hippocampus of DEHP treated animals compared to the control group. Reduction of non-enzymatic antioxidants such as vitamin E and vitamin C were very evident from our data (Figure 5) The reduction of vitamin C in hippocampus of DEHP exposed rats may be due to the decrease in the recycling of ascorbic acid and that of vitamin E may be through subnormal scavenging of LPO in the hippocampus of DEHP exposed rats. Wyse et al studied the effects of ROS on this enzyme and it includes selective alterations of its active site [36]. The other ATPases were decreased in the hippocampus of DEHP exposed rats, Hence Ca^{2+} and Mg^{2+} ATPases are vulnerable to damage by oxy radicals (Figure 6). The decrease in the levels of sodium, potassium, calcium, and magnesium ATPases could be due to the enhanced lipid peroxidation by free radicals in DEHP treated rats.

17β-estradiol suppressed intracellular oxygen radicals and provided neuroprotection against oxidative stress [37]. DEHP significantly decreased the mRNA levels of both ER α and β in the hippocampus (Figure7, 8). DEHP may interfere with the neuroprotective effects of estrogen in hippocampus. DEHP is also thought to exert anti estrogenic effects on both ERα and β in the brain [38]. In addition, ER α and β may contribute to neuroprotection by increasing the expression of Bcl-2 in primary hippocampal neurons [39]. Further the data on mRNA expression of Bcl-2 by quantitative real time PCR showed that DEHP significantly decreased Bcl-2 expression compared to control (Figure 9).

Besides, we also studied the effect of DEHP induced decrease in ER α, β and Bcl-2 on its downstream interacting partners by constructing a protein-protein interaction (PPI) map. The proteins confirmed to interact with ER α and β were also included in the network (Figure 4). The PPI map reveals different types of interactions such as co-expression, co-existence and common neighborhood (or domain fusion) between the queried proteins. This supports the hypothesis that the increased levels of ROS observed are probably due to the result of perturbations in this network. To explore functional properties and identify converging pathways, the protein participants in the PPI map were subjected to functional enrichment analysis. Gene ontology (GO) annotations indicated that these proteins were enriched for molecular functions such as 'ligand-dependent nuclear receptor binding' and 'transcription factor binding' and were participating in a variety of biological processes such as 'female genitalia development' and 'gland morphogenesis' (Table 3). However, no particular KEGG pathway was found to be significantly enriched. The current study is first of its kind which reveals that lactational exposure to DEHP disturbed the hippocampal morphology by increasing ROS production, decreasing antioxidant enzymes and ATPases like Na^{+}/K^{+} -ATPase, Ca^{2+}/ Mg^{2+} -ATPase, and also by decreasing the estrogen receptor expression and Bcl2. Thus this study highlights the damaging effect of DEHP on the hippocampal neurons of the female offspring albino rats.

Conclusion

To conclude, the present study reveals that DEHP induced neurotoxicity by disrupting internal antioxidant system that may impair the hippocampus of female offspring rats.

Declaration of Interest.

The author reports no conflict of interest.
The financial assistance provided by UGC under SAP-DRS-III programme, is gratefully acknowledged.

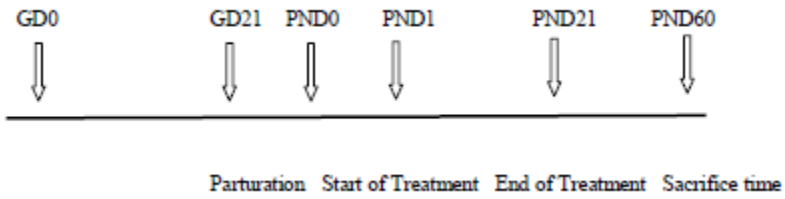
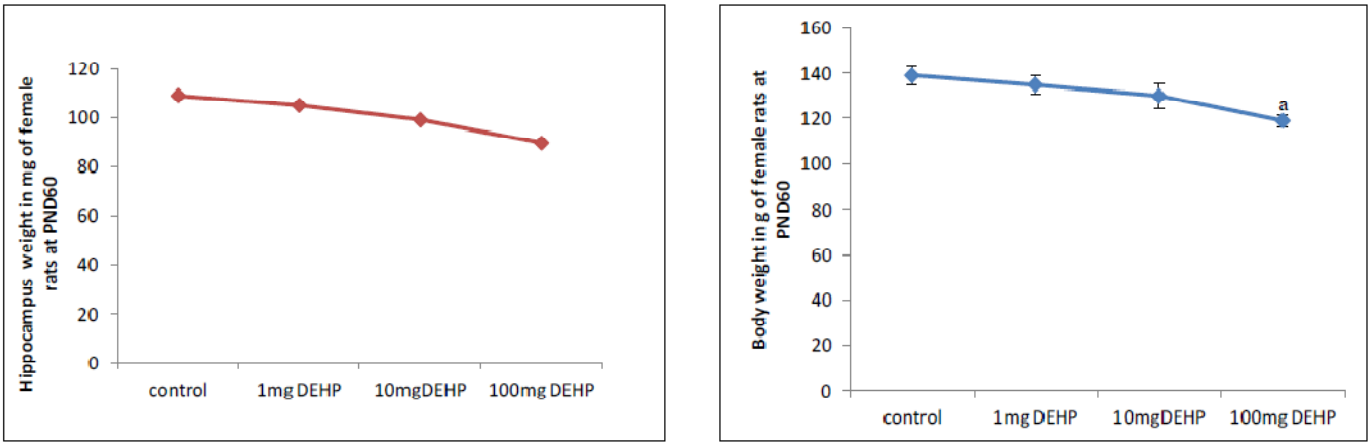
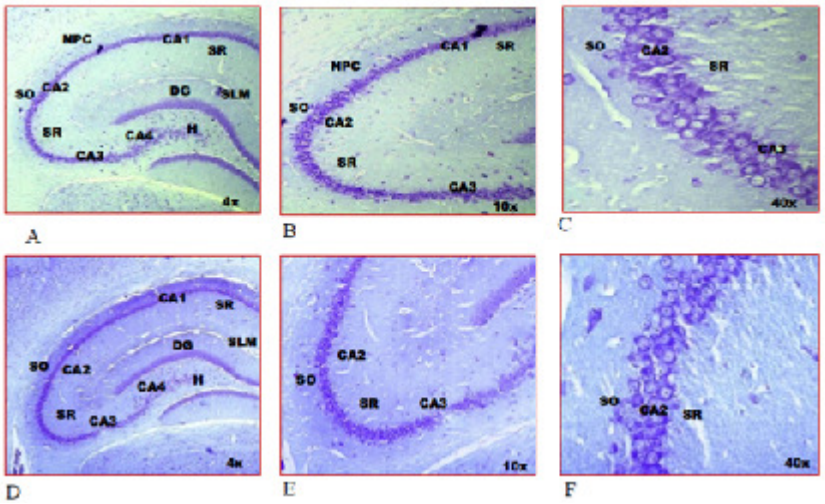


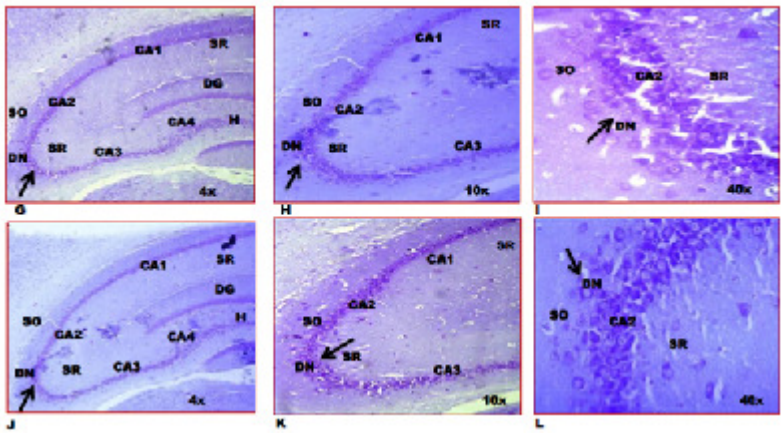
Fig.1. Effect of Lactational exposure to DEHP on hippocampus weight (A) and (B) body weight of female F1 progeny Wistar rats.



Each bar represents the mean SEM of six animals. The data was analyzed by one way ANOVA followed by Student's–Newman–Keul's test using SPSS 17.5 version. ''a'' Represents statistical significance between control versus DEHP treatment groups at $P < 0.05$.

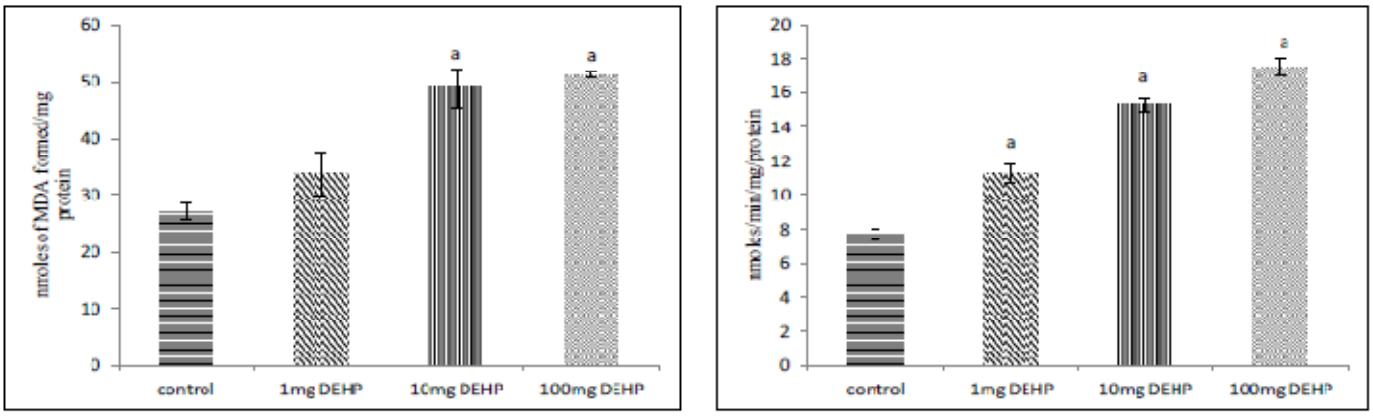
Fig .2. Effect of Lactational exposure to DEHP on histoarchitecture of hippocampus of female pubertal wistar rats





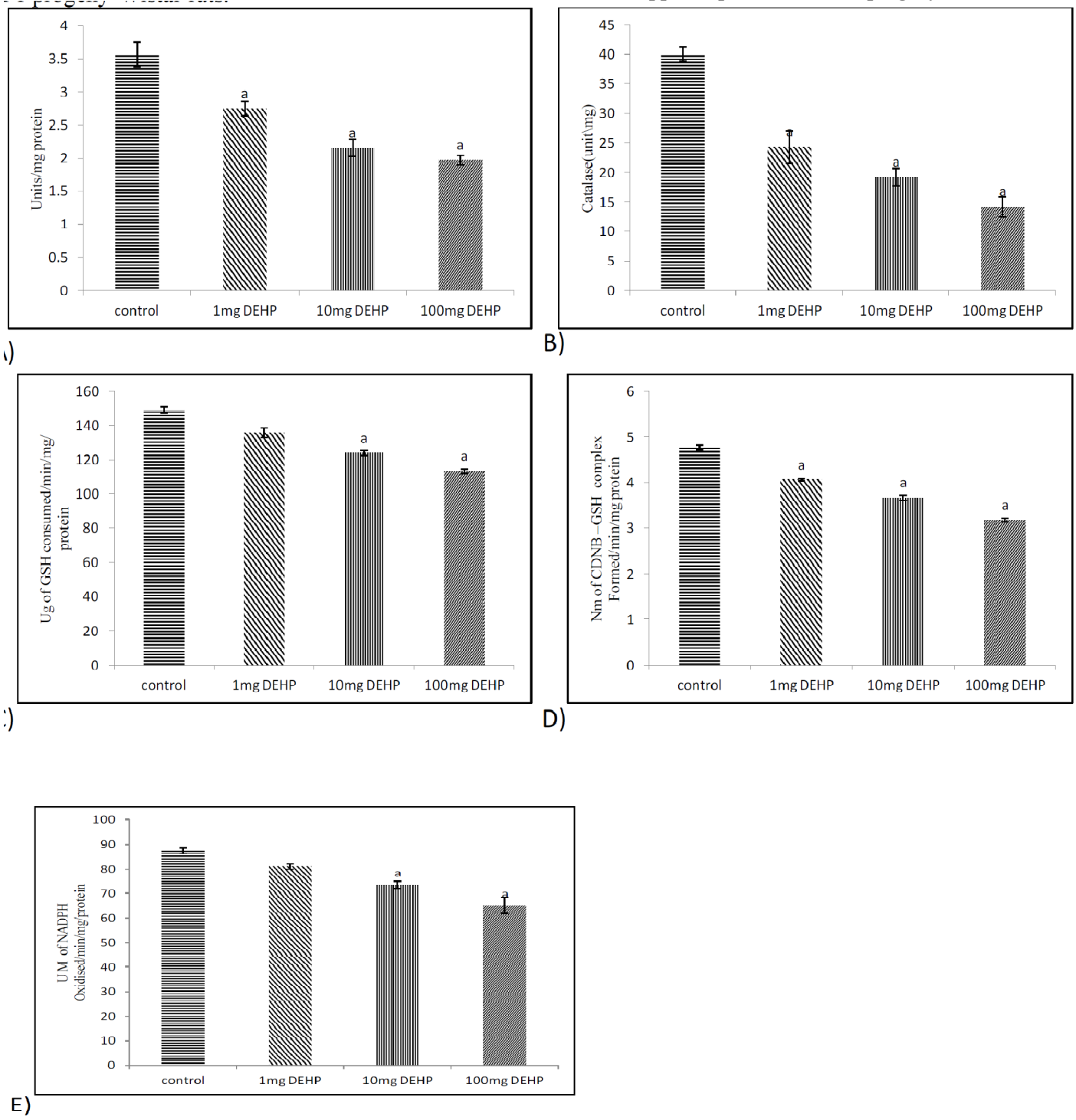
Control (A,B,C), 1mg DEHP treated (D,E,F), 10mg DEHP treated (G,H,I) and 100mg DEHP (J,K,L). The histoarchitecture of hippocampal layer is disturbed in 10 and 100 mg DEHP treated groups as compared to control group. The arrow mark indicates Degenerative neurons in 10 and 100mg groups in CA2 and CA3 regions. CA1, CA2, CA3,CA4-cornu ammonis, Magnification 4X, 10X and 40X (Nikon Eclipse 80i microscopy). SR : stratum radiatum, SO: Startum oriens, H : hilus, SLM : stratum lacunosum-moleculare, DG : dentate gyrus. NPC : normal pyramidal cell, DN: Degenerative neurons.

Fig 3 Effect of Lactational exposure to DEHP on Lipid peroxidation (A) and hydroxyl radical formation (B) In hippocampus of female F1 progenyWistar rats.



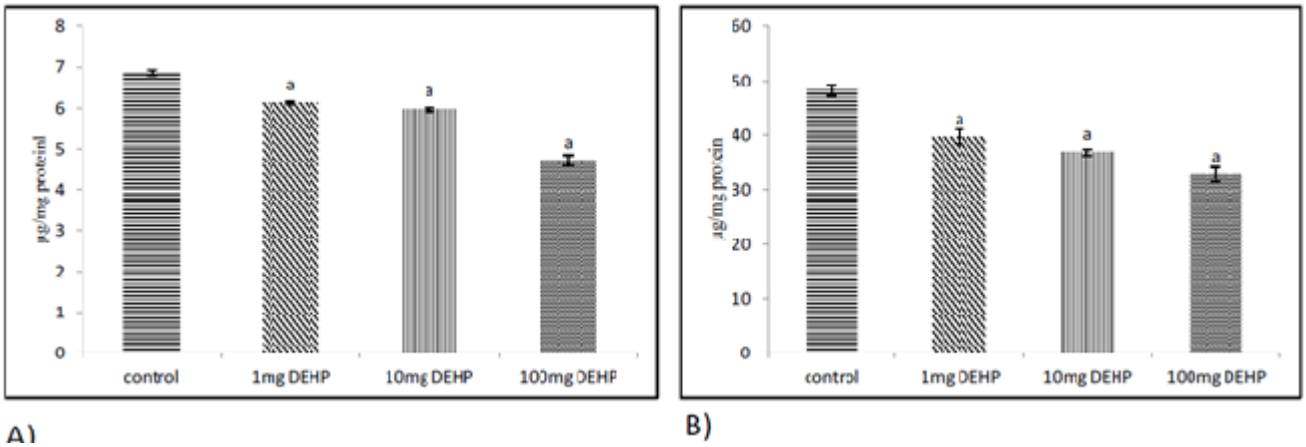
Each bar represents the mean± SEM of Six animals. "a" Represents statistical significance between control versus DEHP treatment groups at P<0.05 level using Student's–Newman–Keuls test.

Fig 4 Effect of Lactational exposure to DEHP on Superoxide dismutase (A), Catalase (B), Glutathione Peroxidase (C), Glutathione-S-Transferase (D) and Glutathione Reductase (E) in the hippocampus of female F1 progeny Wistar rats.



Each bar represents the mean± SEM of six animals. "a" Represents statistical significance between control versus DEHP treatment groups at P<0.05 level using Student's–Newman–Keuls test.

Fig 5 Effect of Lactational exposure to DEHP on Vitamin C (A) and Vitamin E (B) in hippocampus of female F1 progeny Wistar rats.



Each bar represents the mean ±SEM of six animals. "a" Represents statistical significance between control versus DEHP treatment groups at P<0.05 level using Student's–Newman–Keuls test.

Fig 6 Effect of Lactational exposure to DEHP on Na+/K+ ATPase (A), Ca2+ ATPase (B) and Mg2+ ATPase (C) in the hippocampus of female F1 progeny Wistar rats.

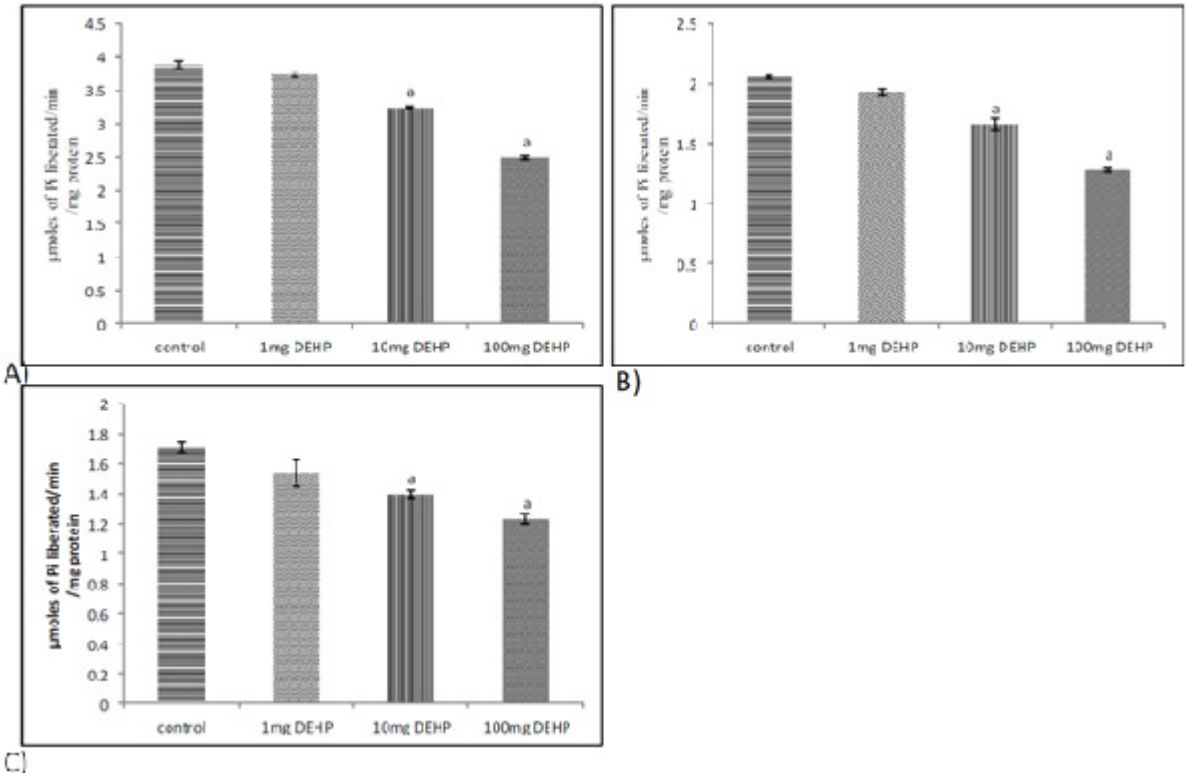
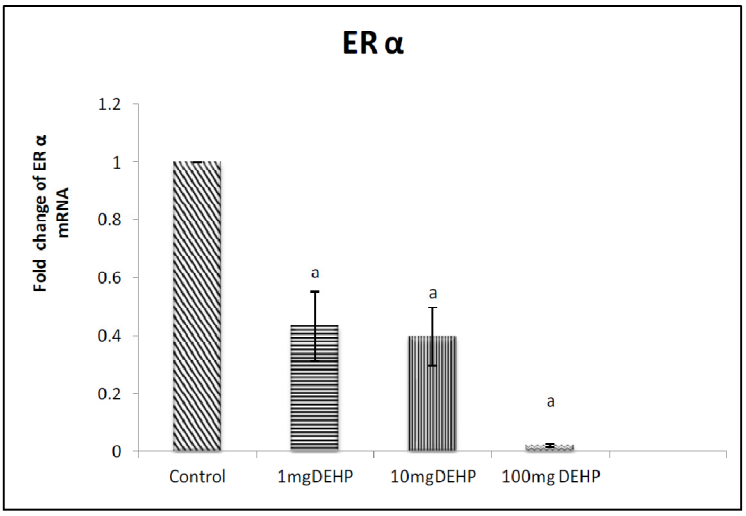
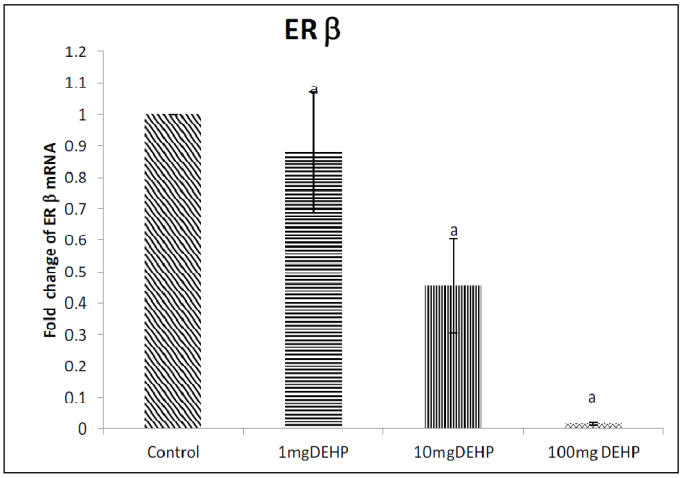


Fig.7. Effect of Lactational exposure of DEHP on ER α mRNA expression in hippocampus of female F1 progeny Wistar rats



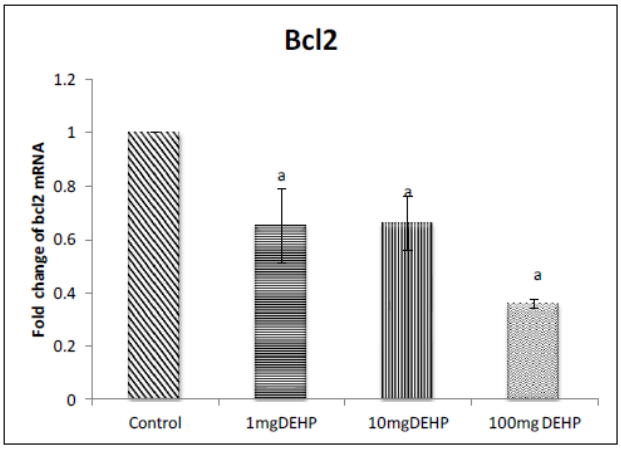
Each bar represents the mean ±SEM of six animals. The data was analyzed by one way ANOVA followed by Student's–Newman–Keul's test using SPSS 17.5 version. ''a'' Represents statistical significance between control versus DEHP treatment groups at P < 0.05.

Fig.8. Effect of Lactational exposure of DEHP on ER β mRNA expression in hippocampus of female F1 progeny Wistar rats



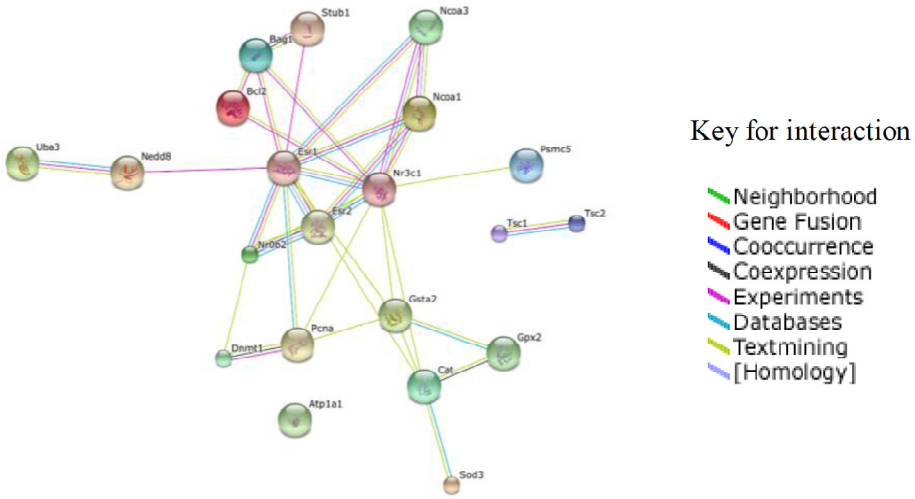
Each bar represents the mean ±SEM of six animals. The data was analyzed by one way ANOVA followed by Student's–Newman–Keul's test using SPSS 17.5 version. ''a'' Represents statistical significance between control versus DEHP treatment groups at P < 0.05.

Fig.9. Effect of Lactational exposure of DEHP on Bcl2 mRNA expression in hippocampus of female F1 progeny Wistar rats



Each bar represents the mean ±SEM of six animals. The data was analyzed by one way ANOVA followed by Student's–Newman–Keul's test using SPSS 17.5 version. ''a'' Represents statistical significance between control versus DEHP treatment groups at P < 0.05.

Fig .10. Protein-protein interaction network of ER α, ER β, Bcl-2 and their downstream interacting partners along with DEHP effector molecules (ROS and ATPase).



The PPI map reveals the different types of interactions between the queried proteins, as specified in the key. This interaction map was constructed using STRING database. It shows that DEHP can directly or indirectly affect many molecules in the hippocampus and thus can cause neurotoxicity.

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