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Neurobehavioral changes induced by di(2-ethylhexyl) phthalate and the protective effects of vitamin E in Kunming mice

Jiaqi Tang[1#], Ye Yuan[1#], Chenxi Wei[1], Xiaomei Liao[1], Eewa Nanberg[2], Yinping Zhang[3], Carl-Gustaf Bornehag[2]*, Xu Yang[1]*

1. *Section of Environmental Biomedicine, Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Science, Central China Normal University, Wuhan, China*
2. *Department of Health Sciences, Karlstad University, SE-651 88 Karlstad, Sweden*
3. *Department of Building Science, Tsinghua University, Beijing, China*

[#] *These two authors contributed equally to this work*

[*]*Corresponding authors: Xu Yang, Professor, College of Life Science, Central China Normal University, Wuhan, China; Email, yangxu@mail.ccnu.edu.cn; Tel,+86-13871361954; and Carl-Gustaf Bornehag, Professor, Department of Health Sciences, Karlstad University, SE-651 88 Karlstad, Sweden; Email, carl-gustaf.bornehag@kau.se; Tel, +46-547002540.*

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ABSTRACT

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer commonly used in PVC that may leach into the environment, and has been shown to adversely affect the health of humans and animals. We undertook a study to ascertain the neurotoxicity of DEHP in Kunming mice. This study included three rounds of testing. In the first round, Kunming mice were exposed to different concentrations of DEHP (0, 5, 50, 500 mg/kg.day) after which their cognitive ability was assessed using the Morris water maze (MWM) test. The reactive oxygen species (ROS) content in tissue and the malondialdehyde (MDA) content of brains were also measured. In the second round, vitamin E (50 mg/kg.day) was given daily as an anti-oxidant *via* the intragastric route. Cognitive deficits and locomotor activity, as well as ROS and MDA contents were tested employing the same methods. In the third round, the depressive mood of mice after DEHP exposure (500 mg/kg.day) was measured using the open field test, the tail suspension test, and the forced swim test. The main findings of this study include: (1) A statistical association exists between DEHP oral exposure and spatial learning (DEHP 500mg/kg.day) and memory (DEHP 50mg/kg.day) dysfunction as ascertained by the MWM test of Kunming mice. (2) A statistical association was also found between DEHP oral exposure (50 and 500mg/kg.day) and oxidative stress (ROS and MDA) of mouse brain tissue. (3) Co-administration of vitamin E (50mg/kg.day) diminishes the elevation of ROS and MDA induced by DEHP (50mg/kg.day) from significant levels to not significant levels. (4) Co-administration of vitamin E (50mg/kg.day) protects against mouse memory dysfunction induced by DEHP (50mg/kg.day) from being significant to being not significant. (5) In the 5mg/kg.day DEHP exposure groups, oxidative stress in brain tissue, and neurobehavioral changes were not found. (6) High dose DEHP exposure (500mg/kg.day) may induce behavioral despair in mice.

Conclusions: These data suggest that DEHP is neurotoxic with regard to cognitive ability, and locomotor activity.

Keywords: Di(2-ethylhexyl) phthalate; vitamin E; neurobehavioral changes; cognitive activity; locomotor activity; protective effect

INTRODUCTION

Di(2-ethylhexyl) phthalate (DEHP) is a man-made chemical widely used in industry and commerce. This man-made chemical has a wide spectrum of industrial applications, and ultimately appears in a wide range of consumer products, as well as in food processing and in medical applications (Swan, 2008). DEHP is an endocrine disruptor and has been shown to disrupt reproductive tract development in male rodents in an anti-androgenic manner (Parks et al., 2000). In addition, a number of non-reproductive endpoints have been reported in literature, often at high doses, including hepatocellular carcinoma, anovulation and decreased fetal growth (Hauser and Calafat, 2005).

DEHP is primarily used as a plasticizer in the manufacture of polyvinyl chloride (PVC), which is used extensively in consumer products, flooring and wall coverings, food and water contact applications, and medical devices (ATSDR, 2002). DEHP has been measured in residential indoor environments in both house dust and indoor air (Rudel et al., 2003), and has also been measured in foods, milk and drinking water (Wormuth et al., 2006). Humans are exposed to DEHP by multiple routes. Exposures can be oral: DEHP contaminated food, water and other liquids and in children through mouthing of toys and teethers. Exposure can also be via inhalation: DEHP volatilizes from PVC, nail polish and hair spray. Medical transfusion (haemodialysis, neonatal transfusion and parenteral nutrition) via DEHP-containing tubing and products is an important exposure route for DEHP, because via this route the DEHP exposure dose can reach very high levels (FDA, 2001). The Centers for Disease Control and Prevention (CDC) has published data on levels of phthalate metabolites in a large population-based sample of the US population over 6 years of age. These data demonstrate the ubiquitous nature of phthalates and variations in concentration by ethnicity, sex, and age (CDC, 2003; CDC, 2005). The typical human exposure level to DEHP ranges from 3 to 30 μg/kg.day (Doull et al. 1999). This dose can be exceeded under specific medical transfusions, reaching 1.5 mg/kg.day for haemodialysis patients Loff et al. 2000) and 10 to 20 mg/kg.day during neonatal transfusion or parenteral nutrition (Kavlock et al. 2006).

Since 2000, a number of epidemiological studies have found a series of health problems that are related to environmental DEHP exposure (Swan, 2008). In addition, experimental results from some related toxicological studies also support these findings (Bornehag, 2009). However, studies relating to DEHP-induced neurobehavioral, adverse effects are rare, with only one epidemiological study (Cho et al, 2010) and two animal toxicology studies (Tanaka, 2002; Tanaka 2005) being found in the literature. The epidemiological study found a negative association between DEHP exposure and the Wechsler Intelligence Scale for Children vocabulary score; and the toxicology studies found that DEHP exposure at high doses (0.03 and 0.09 % of diet) produced adverse effects in the neurobehavioural parameters in mice. However, the molecular mechanism of these neurobehavioural changes is still unclear.

Fortunately, scientific research related to the topic of DEHP-induced neurobehavioral adverse effects has made significant progress. (1) Adverse neurobehavioral changes may be caused by oxidative stress of brain tissue, especially hippocampus tissue (Murakami, 2005; Li et al, 2011; Liu et al, 2014); (2) Oxidative stress of body tissue and cells may be derived from environmental DEHP exposure (Kasahara, 2002; Ghosh et al, 2010; Chen et al, 2012). Therefore, following the logic of syllogism we suspect that oxidative stress/damage may be the key event, via which DEHP exposure may cause damage to brain (hippocampus) tissue, and this in turn causes the neurobehavioral adverse effects. Vitamin E (Vit E or Ve) is a common fat-soluble antioxidant (Traber, 2006). Antioxidants protect cells from the oxidative damaging effects of free radicals, which are molecules that contain an unshared electron. Unshared electrons are highly energetic and react rapidly with oxygen to form reactive oxygen species (ROS) (Verhagen et al, 2006). As a medicine, Vit E may help prevent or delay the chronic diseases associated with oxidative stress, for example heart disease (Stampfer et al, 1993) and cognitive decline (Sano et al, 1997). As a molecular biological reagent, it may help us to make sure that a certain pathological change is mediated by oxidative stress/damage (Alzoubi et al, 2012). In such studies, Vit E is often used in 50~100 mg/kg.day dose ranges (Rocksen et al, 2003).

Hypotheses of the current study: (1) DEHP exposure at environmental levels may cause neurobehavioral changes to the body; (2) if vitamin E can relieve these pathological changes, then

the oxidative stress induced by DEHP exposure may be one of the pathological mechanisms for these neurobehavioral changes.

MATERIALS AND METHODS

All protocols were approved by the Office of Scientific Research Management of Central China Normal University (8 November 2011; CCNU-SKY-2011-008), which is in Wuhan, China.

Animals

Male Kunming mice (7–8 weeks of age; 25–28 g) were purchased from the Hubei Experimental Animal Center (Wuhan, China). All mice were group housed in pathogen-free cages (5 mice to a cage) in rooms maintained at 20–25°C with 50–70% humidity and a 12-h light–dark cycle. The cages we used were independent ventilation cages (IVC); each cage was equipped with a separate air inlet and outlet system. All mice were provided *ad libitum* access to a commercial diet and filtered water. The food for the animals was also purchased from the Hubei Experimental Animal Center (Wuhan, China).

Main Reagents and Equipment

DEHP (>99%; CAS: 117-81-7) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tween-80 (CAS: 9005-65-6) was obtained from Amresco (Solon, OH, USA). Hydrocortisone (HC, CAS: 50-23-7) was obtained from Xiandai Hasen Pharmaceuticals (Shanghai, China). All other chemicals were of analytical grade and purchased from Sigma-Aldrich unless stated otherwise.

The equipment and biomarkers used in our study are shown in Table 1.

Table 1.

Experimental Design and Animal Exposure

The study comprised three rounds of testing, as described below.

Figure 1.

First round of testing

The aims of the first round were to ascertain: (i) if DEHP exposure affects the cognitive ability of mice; (ii) if DEHP exposure can cause oxidative stress in the mouse brain; and (iii) to determine what the appropriate dose of DEHP should be for the second round. Thirty-six mice were randomly assigned to one of 4 dosage groups: vehicle control (0.9% NaCL/ Tween 80 1:1), and 5, 50, or 500 mg/kg/day in a dosing volume of 10 mL/kg body weight. (These DEHP exposure doses have been used in previous studies, and were considered reasonable (Schmidt, 2012; Erkekoglu et al, 2010; Loff et al, 2000)). Animals received daily intragastric intubations via a metal gastric tube for 10 consecutive days (once a day between 7:00-8:30 am).

Second round of testing

The aims of the second round were to investigate if vitamin E can protect: (i) brain tissue from DEHP-induced oxidative stress; and (ii) the cognitive ability of mice from DEHP-induced weakening. Thirty-six mice were randomly assigned to one of 4 dosage groups: vehicle control (0.9% NaCL/ Tween 80 1:1), DEHP (50 mg/kg/day); combined exposure (DEHP 50 mg/kg/day + vit. E 50 mg/kg/day); and vit. E control. Vit. E at 50 mg/kg/day is considered a good dosage choice for these types of studies (Rocksen, 2003). Animals received daily intragastric intubations (DEHP and/or vit. E) via a metal gastric tube for 10 consecutive days (once a day between 7:00-8:30 am).

Third round of testing

The aim of the third round was to investigate if DEHP exposure may reduce locomotor activity ability in mice. Thirty mice were randomly assigned to one of 3 dosage groups: vehicle control (0.9% NaCL/ Tween 80 1:1), DEHP (500 mg/kg/day) and depression-like symptom control (positive control, hydrocortisone 50 mg/kg/day for 10days *via* intraperitoneal injection (Gregus et al, 2005)). All animals received daily intragastric intubations via a metal gastric tube for 10 consecutive days (once a day between 7:00-8:30 am).

Morris Water Maze (MWM)

The MWM test was designed to investigate the spatial cognitive abilities of laboratory mice. Rodents were placed in a circular, featureless pool of cool opaque water, where they had to swim until they discovered the escape platform (which was invisible and beneath the water surface). Mice were allowed to rest on the platform before returning to the water for another attempt. After several days of training, the rodents learned to swim directly onto the platform, presumably by using spatial cues from the room as a reference (Lu Z, 2008). The biomarkers for neurobehavioral changes are "escape latency" (for learning) and "swimming time in the northeast quadrant (probe test)" (for memory) as measured by the Morris water maze test. The groups were subjected to the MWM to ascertain their learning ability from the 1st to the 7th day, to measure memory ability by the search-to-platform on the 10th day, and then to determine the level of oxidative stress on the 11th day.

The training began 4 h after the daily exposure of the mice. The mice were placed into the pool in the same order: SW quadrant, SE quadrant, NE quadrant, and then NW quadrant. Each mouse had three trials per day. The inter-trial period was ≤60 s. All trials had a maximum duration of 60 s, and the mice remained on the platform for 60 s at the end of each trial on the first day. Escape latency (time required to find the platform) was measured. If the animals did not find the platform within 60 s, they were gently pushed to the platform and allowed to stay there for 5 s, and the latency was recorded as 60 s. The mice were subjected to 7 days of training, on the 8th and 9th day the MWM was not used, and then on the 10th day, it was used again. The platform on the 10th day was taken away from the pool, and then each animal was released from the S point for the probe test. A pickup camera recorded their tracks for 60 s.

After the MWM test the whole brains were collected and the necropsy started 1 hr later. The whole process must be completed within 1 hr. Animals were kept in a holding facility and individually taken for necropsy in a separate room.

Measurements of Oxidative Stress in Brain Tissue

Preparation of tissue samples

Mice were killed by cervical dislocation. Brain tissue was collected, weighed in the completely automatic electronic balance, and homogenized in ice-cold 0.9% NaCl to produce 1:9 (weight/ volume; g/ml) homogenates. Homogenates were centrifuged at 5000 rpm (100×g) for 10 min at 4°C. The supernatant was collected and frozen at –20°C for assessment of the levels of reactive oxygen species (ROS) and malondialdehyde (MDA).

ROS content assay

Levels of ROS in the samples were determined based on the reactions between ROS and the byproducts of 2',7'-dichlorofluorescein (DCFH)-DA (Wu et al, 2013; Bejma et al, 1999). After transfer into cells, DCFH-DA is cleaved to form DCFH, which in turn is transformed into highly fluorescent DCF upon reaction with ROS. DCF was quantified in each sample using a fluorescence monitor (FLx 800 Multi-Detection Microplate Reader; BioTek Instruments, Wisnooski, VT, USA). At first, 2 μL of sample solution was removed to a test tube, and 198 μL of phosphate-buffered saline (PBS) at pH 7.5 was added. Then, 100 μL of the sample solution was removed to a 96-well microplate, and 100 μL of DCFH-DA fluorescent dye added, diluted 1000-fold by PBS (pH = 7.5). The level of ROS in the supernatant was detected using a fluorescent microplate spectrophotometer at an excitation wavelength of 485 nm and emission wavelength of 525 nm.

MDA content assay

MDA content in mouse brain homogenates was measured using the Draper and Hadley method (Draper, 1990). Briefly, 0.5 mL of each homogenate was mixed with 2 mL of a 0.6% (*w/v*) thiobarbituric acid solution in a glass test tube, placed in a boiling water bath for 15 min, and cooled immediately. The mixture was then centrifuged at 10,000 ×g for 10 min and the absorbance of the resultant supernatants read at 450, 532, and 600 nm in a PowerWave XS Microplate Spectrophotometer (BioTek Instruments). Total protein for each sample was determined by the Lowry method (Lowry et al, 1951). The level of MDA (C_{MDA}) in each sample was calculated using the following equation (with total protein), which incorporates the value of protein content (both total level [in mg] and corresponding concentration [C_{pro}]) in the sample:

$$C_{MDA}\ (\text{nmol/mgpro}) = (6.45 \times [OD_{532} - OD_{600}] - 0.56 \times OD_{450})/C_{pro}.$$

Experiments for Locomotor activity

These experimental groups were subjected to the open field test (OFT) on the 7th day, Tail suspension test (TST) on the 8th day, and the forced swim test (FST) on the 10th day. All of these behavioral tests were administered from 11:00 to 12:30.

Open field test (OFT)

The OFT is an animal test which may be used to assay anxiety-like behaviors (Prut et al, 2003) in rodents in scientific research. The apparatus used consisted of a square base (40 × 40 cm) surrounded by a 35-cm wall, with the floor divided into 16 squares. The "border" is defined as the 12 outer periphery squares and the "center" as the 4 central squares. Each mouse was placed individually in the center of the open-field apparatus. Testing was conducted over 5 min (300 s) and recorded using a video tracking system. The walls and floors of the apparatus were cleaned thoroughly with 10% ethanol between tests. Time spent in the border, and time spent in the center, were the endpoints collected.

Tail suspension test (TST) and forced swim test (FST)

The TST (Steru et al, 1985/Nature Neuroscience Methods) is also an experiment used to assay anxiety-like behaviors in rodents. Changes in immobility time indicate changes in mood. It is widely used to detect depressive behavior induced by stress and the potential antidepressant effects of drugs. Briefly, mice were suspended individually on the edge of a shelf 58 cm above the floor by adhesive tape placed 1 cm from the tip of the tail. Mice were allowed to hang for 6 min and the duration of immobility during the final 4 min (240s) was recorded using a video tracking system. "Immobility" was defined as hanging passively and completely motionless (depression-like symptom), and was automatically judged using the computer software ANY-Maze^{TM} (Stoeling Co. USA).

The FST (Petit-Demouliere.et al, 2005 / Nature Neuroscience Methods) is a test used to measure the depressive mood induced by stress and the effect of antidepressant drugs on the behavior of laboratory animals (typically rats or mice). FST was conducted using the method of Porsolt et al. (Porsolt et al, 1977), with minor modification. Briefly, mice were forced individually to

swim for 6 min (with the final 4 min being recorded) in a transparent glass cylinder (height, 45 cm; diameter, 20 cm) filled 30 cm high with water (22 ± 0.5°C). "Immobility" was defined as the time spent floating in water without struggling and making only those movements necessary to keep the head above water, and automatically judged by the computer software ANY-Maze^{TM} (Stoeling Co. USA).

Immobility has been described as a symptom of "behavioral despair", and both tests have been suggested as animal models of human depression (although this view is somewhat controversial). Nonetheless, these tests can be used to predict depressant-like symptoms and antidepressant-like activity (Steru et al, 1985/Nature Neuroscience Methods; Petit-Demouliere.et al, 2005 / Nature Neuroscience Methods).

Statistical Analyses

Data are means ± SEM. Statistical graphs were generated using Origin 6.0 (OriginLab, Northampton, MA, USA), which was also used for slope analyses in Figure 1A and Figure 2A. Statistical analyses were carried out using SPSS version 13.0 (SPSS, Chicago, IL, USA). A repeated measures ANOVA followed by a Tukey's post-hoc test (Tukey test) was used for WMWT escape latency analyses; and all other data were analyzed by a One-way ANOVA followed by a Tukey test. A p-value of ≤ 0.05 was considered significant.

RESULTS

The first round of testing

In the MWM, the hidden-platform acquisition test is applicable for investigating the spatial learning abilities of laboratory animals. Figures 2A and 2B show the escape latency of different DEHP groups for all 7 days. Mice in each experimental group showed a clear decrease in escape latency over the 7 days of training. The latency of the control group showed the fastest decrease (fastest learning) (slope=-5.79 s/d, $p<0.01$), whereas that of the 500 mg/kg/day group showed the slowest decrease (slowest learning) (slope=-124 s/d, $p<0.05$).

Figure 2.

The probe test can be used to evaluate the spatial memory of laboratory animals. The swimming time in the NE quadrant for the four DEHP exposure groups are shown in Figure 2C. Mice in the control group spent more time in the NE quadrant (where the platform had been); the other groups with DEHP exposure spent less time in the NE quadrant. The swimming pathway on the 10th day is shown in Figure 2D. The swimming pathway of the control group was purposeful and orderly with a focus on the NE quadrant. The swimming pathway of mice in the 500 mg/kg/day group was irregular and without purpose.

The levels of ROS and MDA after administration of DEHP are shown in Figures 3A and 3B. Compared with the control group, a dramatic and dose-dependent elevation of ROS and MDA content was observed in each group. It was significantly increased in the 50 and 500 mg/kg/day groups ($p<0.05$ or $p<0.01$).

Figure 3.

The second round of testing

Figure 4.

Figures 4A and 4B show that the latency of the mice that were exposed to DEHP had the slowest decrease (slowest learning, slope=-4.30 s/d, $p<0.01$), and that the group with vitamin E (slope= -5.80 s/d, $p<0.01$) was similar to the control group (faster learning, slope=-5.70 s/d, $p<0.01$). Figure 4C shows that compared with the control group, mice in the DEHP exposure group spent less time ($p<0.01$) in the NE quadrant (in which the escape platform was located).

Figure 5.

Figures 5A and 5B show that ROS and MDA contents were significantly increased in the DEHP-only group ($p<0.05$, $p<0.01$), but not so in the vitamin E group. It is interesting that the

DEHP+Vit E combined exposure group had a significant difference compared to the DEHP and control groups (#: $p<0.05$, $p<0.05$). This difference may indicate that vitamin E played a protective role on the brain tissue against oxidative stress.

The third round of testing

The results of the OFT are shown in Figure 6. Mice in the control group spent more time in the center area, while the DEHP (500 mg/kg/day) group was similar to the hydrocortisone group. However, the differences among the groups in the OFT were not significant ($p>0.05$, $p>0.05$).

Figure 6.

In the TST (Figure 7A) and the FST (Figure 7B), the immobility time (seconds) showed significant increases in the DEHP group compared with the control group ($p<0.01$ and $p<0.05$); Similar immobility times were observed in the DEHP and hydrocortisone dose groups.

Figure 7.

DISCUSSION

The main findings of this study include: (1) A statistical association exists between DEHP oral exposure and spatial learning (DEHP 500mg/kg.day) and memory dysfunction (DEHP 50mg/kg.day) as determined by the MWM test of Kunming mice. (2) A statistical association is also found between DEHP oral exposure (at 50 and 500mg/kg.day levels) and oxidative stress (ROS and MDA) of mouse brain tissue. (3) Co-administration of vitamin E (50mg/kg.day) diminishes the elevation of ROS and MDA induced by DEHP (50mg/kg.day) from significant to not significant. (4) Co-administration of vitamin E (50mg/kg.day) can protect mouse memory from dysfunction induced by DEHP (50mg/kg.day), from weakly significant ($p=0.044$) to not significant. (5) Oxidative stress in brain tissue, and neurobehavioral changes are not found in the 5mg/kg.day DEHP exposure groups. (6) High dose DEHP exposure (500mg/kg.day) may induce behavioral despair in mice.

Studies on DEHP-induced neurobehavioral adverse effects are rare, we found only three papers in the literature. The two animal experimental studies suggest that higher levels of DEHP (0.03 and 0.09 % of diet) may have adverse effects as shown by neurobehavioral tests on mice (surface righting, negative geotaxis, cliff avoidance, swimming behavior and olfactory orientation) (Tanaka, 2002; Tanaka 2005). The epidemiological study (Cho et al, 2010) found a negative association between DEHP exposure and the score obtained from the Wechsler Intelligence Scale for Children vocabulary test. Unfortunately the DEHP exposure level was assessed by measuring the level of urine MEHP, which is the main metabolite of DEHP. Our study has three main contributions in this area: (1) A lower DEHP exposure level (50mg/kg.day) may induce adverse effects as demonstrated in the neurobehavioral test (probe test); (2) Oxidative stress may be one of the biological mechanisms for mediating the DEHP-induced neurobehavioral adverse effects; and (3) Antioxidants, such as vitamin E, may protect nerve cells from these adverse effects.

There is an association between learning and memory. Learning is the integration of all types of reactions, and memory is the storage of this integration. The MWM has been used widely in neuroscience, neurobehavior, and neuropharmacology studies of laboratory animals owing to the simplicity of the device and the procedures (D'Hooge et al, 2001).

Oxidative stress has been defined as a “disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage” (Sies, 1991). The brain consumes a large quantity of oxygen, making it particularly susceptible to oxidative stress (Andersen, 2004). ROS (which are generated excessively under oxidative stress) reversibly or irreversibly damage nucleic acids, proteins, free amino acids, lipids, lipoproteins, carbohydrates, and macromolecules in connective tissue (Voitkun et al, 1999). Excess production of ROS in the brain has been implicated as a factor underlying the etiology of DEHP-induced neurotoxicity.

Studies have shown that ROS production is responsible for oxidative stress, and that an increase in ROS induces the death of dopaminergic cells. In the present study, we found that ROS generation in the brains of mice in the DEHP-treatment groups increased significantly compared with that seen in the control group. Excess generation of ROS can damage a wide variety of cellular constituents

(DNA, RNA, proteins, sugars, and lipids), thereby compromising cell viability. Typically, lipid peroxidation is the primary result of oxidative stress, and correlated effects on MDA levels are also observed. MDA is a metabolite of the lipid peroxidation of membranes.

Vitamin E is the primary fat-soluble, chain-breaking, antioxidant-protecting, lipid bilayer (Traber, 2006). Administering vitamin E to rats caused a reduction in the serum level of MDA (a marker of lipid peroxidation). It has been reported that antioxidants such as vitamin E can improve Alzheimer's disease as well as chronic intermittent hypoxia caused by cognitive impairment and learning disabilities (Joseph et al, 1992).

The present study showed that DEHP increased MDA content in the brain. These results suggest that DEHP can induce excessive generation of ROS and could reinforce lipid peroxidation in the brain, thereby affecting oxidation–anti-oxidation homeostasis. We also showed that vitamin E protected the mouse brain from oxidative damage induced by DEHP, but the evidence was not strong enough to show vitamin E protected a mouse's cognitive ability ($p>0.05$)

In the OFT, mice exposed to DEHP spent a lot of time in the border area compared with control mice, but this difference was not significant. In the FST and TST, the immobility time was significantly increased in the DEHP group ($p<0.05$); the results and behavior of the DEHP treated mice were similar to the mice in the hydrocortisone group. This result suggests that mice exposed to DEHP may have a greater risk of developing depression-like symptoms than normal mice. The results of the behavior tests were consistent with those of previous studies (Boberg et al, 2011).

The DEHP exposure doses (5, 50, and 500 mg/kg.day) in this animal study were higher than typical human exposure levels of 3–30μg/kg.day (Doull et al. 1999), however the dose of 5 mg/kg.day coincide with the exposure levels from specific medical transfusions 1.5 mg/kg.day to 20 mg/kg.day (Loff et al. 2000; Kavlock et al. 2006). For testing the non-reproductive endpoints of DEHP exposure, high exposure doses such as 500 mg/kg.day (Schmidt, 2012) and 0.03 and 0.09 % of daily diet (Tanaka, 2002; Tanaka 2005) have been reported in the literature.

Limitations of this study. Our study did not include an analysis of the MWM pathway for additional semi-quantitative evaluation. For example, (1) both 60-sec probe trial and 30-sec epoch,

(2) swimming speed, and (3) swimming distance. Further studies by our group will take these analyses into account.

In conclusion, the MWM demonstrated the toxic effects of DEHP on cognition, and the TST and FST demonstrated the risk of development of depression-like symptom caused by exposure to DEHP. These results also support the notion that oxidative stress may be one of the mechanisms contributing to injury of the CNS. Long term exposure experiments are needed to investigate the role of oxidative stress in DEHP-induced neurotoxicity, and in particular the related mechanism.

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Figure Legends

Figure 1. Schematic diagram of the experimental design and animal exposure

Figure 2. Results of neurobehavioral tests in the first round of testing. (A) Escape latency of different DEHP groups on different days (Slope analyses were taken by Origin 6.0). (B) Average escape latency of the four DEHP exposure groups for the 7 days (Repeated measures ANOVA: $F_{(3, 20)}$=5.629, p=0.006; Tukey's post-hoc test: **: p<0.01 compared with the control group; $^{\#\#}$: p<0.01 compared with the 500mg/kg.d group). (C) Swimming time in the northeast (NE) quadrant (One-way ANOVA: $F_{(3, 20)}$ =3.982, p=0.022; Tukey's post-hoc test: *: p<0.05 compared with the control group). (D) The typical swimming pathway records of the four DEHP exposure groups by probe test with a Morris Water Maze on the 10th day.

Figure 3. Results of oxidative stress in mouse brains in the first round of testing. (A) ROS content in the brain tissue of the four DEHP exposure groups (One-way ANOVA: $F_{(3, 20)}$ =6.399, p=0.003; Tukey's post-hoc test: *: p<0.05, **: p<0.01 compared with the control group). (B) MDA content in the brain tissue of the four DEHP exposure groups (One-way ANOVA: $F_{(3, 20)}$ =4.501, p=0.014; Tukey's post-hoc test: *: p<0.05 compared with the control group).

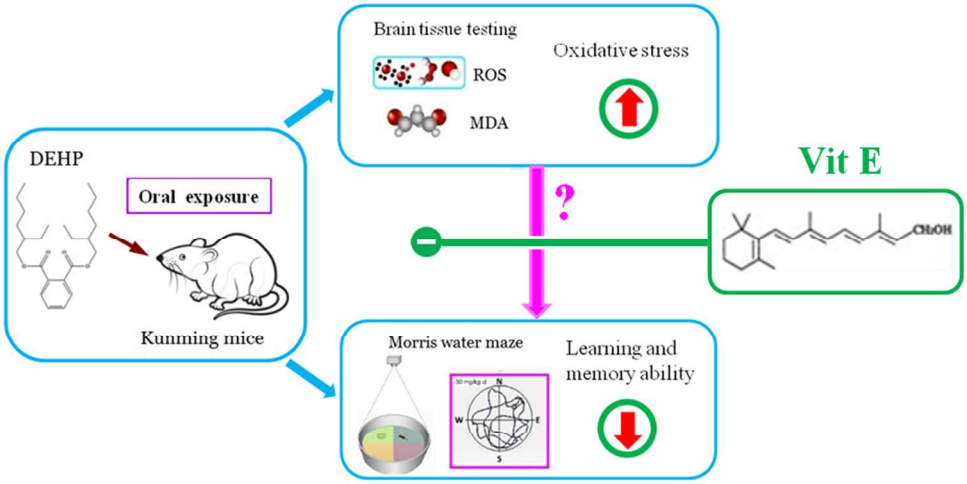
Figure 4. Results of neurobehavioral tests in the second round of testing. (A) Escape latency of the four experimental groups on different days (Slope analyses were taken by Origin 6.0). (B) Average escape latency of the four experimental groups for the 7 days (Repeated measures oneway ANOVA:

$F_{(3, 20)}$=0.432, p=0.732). (C) Swimming time in the northeast (NE) quadrant (oneway ANOVA: $F_{(3, 20)}$ =4.702, p=0.012; Tukey's post-hoc test: *: p<0.05 compared with the control group; Tukey's post-hoc test: #: p<0.05 compared with the Vit E group). (D) The typical swimming pathway records of four experimental groups by probe test with a Morris Water Maze on the 10th day.

Figure 5. Results of oxidative stress of mouse brains in the second round of testing. (A) ROS content in the brain tissue of the four experimental groups (oneway ANOVA: $F_{(3, 20)}$ =3.267, p=0.043; Tukey's post-hoc test: *: p<0.05 compared with the control group). (B) MDA content in the brain tissue of the four experimental groups (oneway ANOVA: $F_{(3, 20)}$=6.720, p=0.003; Tukey's post-hoc test: **: p<0.01 compared with the control group; Tukey's post-hoc test: $^{\#\#}$: p<0.01 compared with the Vit E group).

Figure 6. Results of neurobehavioral OFT in the third round of testing. (A) The typical running pathway record of the three experimental groups using an OFT device. (B) Time in border field (One-way ANOVA: $F_{(2, 26)}$ = 0.900, p=0.419). (C) Time in centre field (One-way ANOVA: $F_{(2, 26)}$ =0.900, p=0.419).

Figure 7. Results of neurobehavioral TST and FST tests in the third Round of testing. (A) Immobility time in the TST (One-way ANOVA: $F_{(2, 23)}$=6.735, p=0.004; Tukey's post-hoc test: *: p<0.05, **: p<0.01 compared with the control group). (B) Immobility time in the FST (One-way ANOVA: $F_{(2, 23)}$ =9.639, p=0.001; Tukey's post-hoc test: *: p<0.05, **: p<0.01 compared with the control group).



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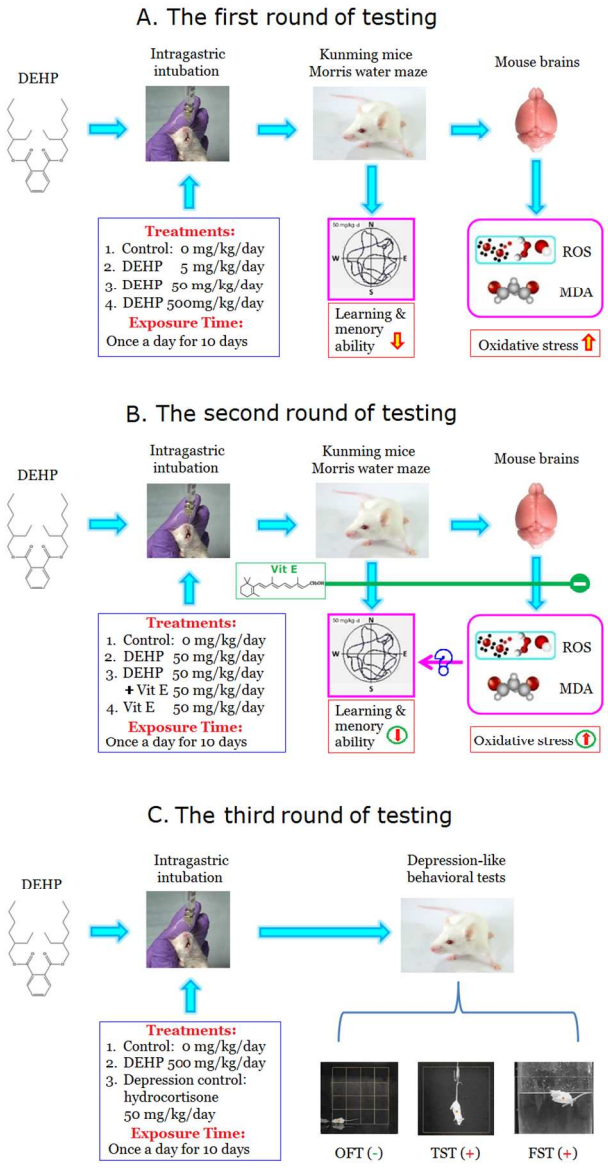

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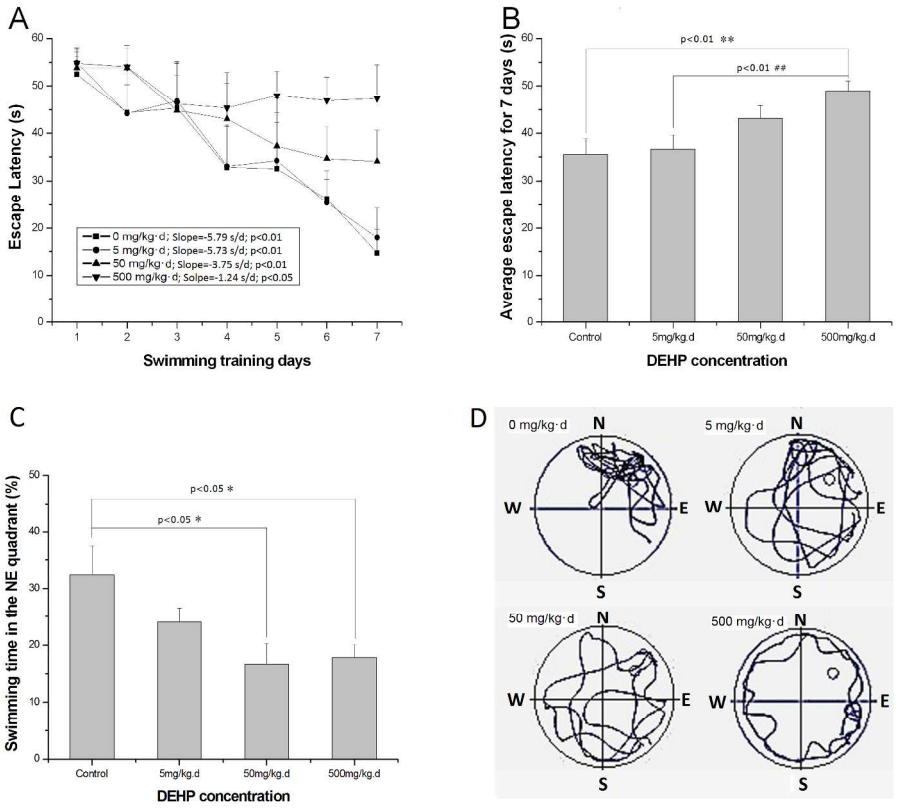

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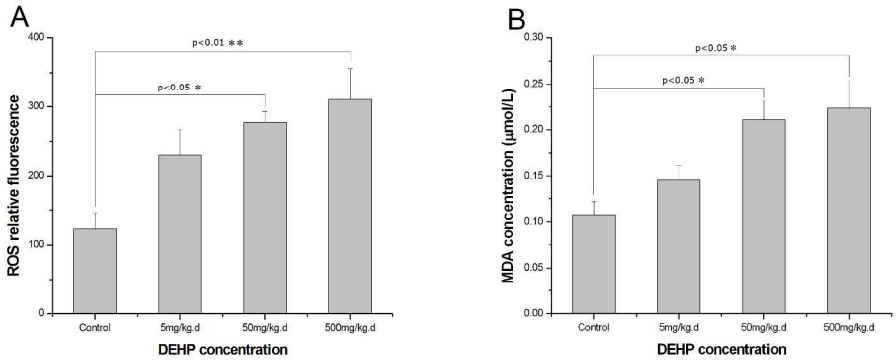

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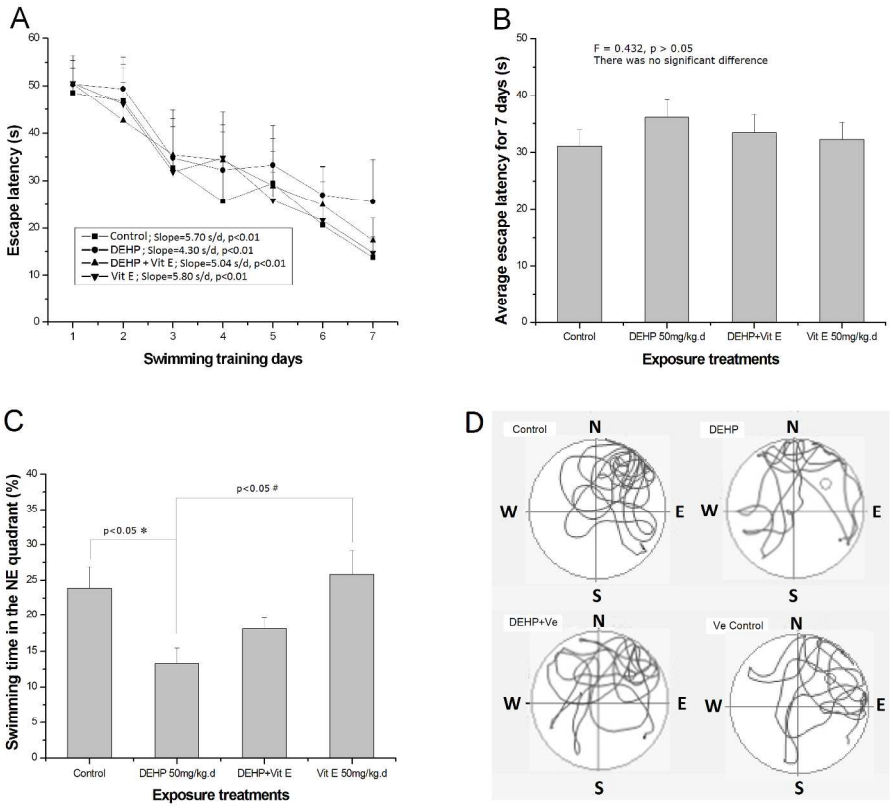

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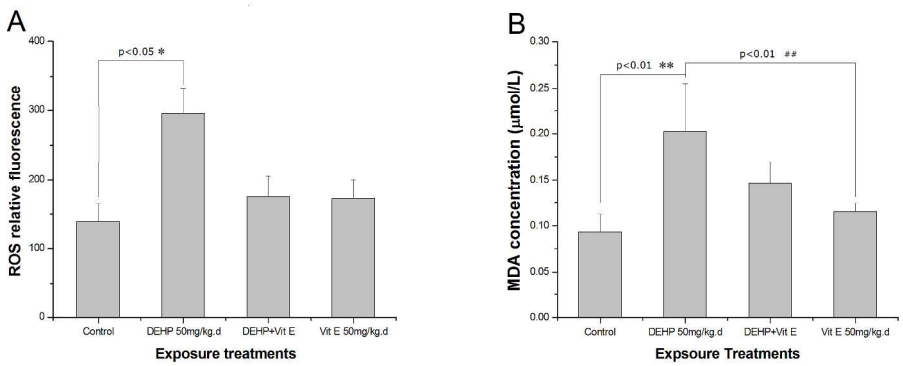

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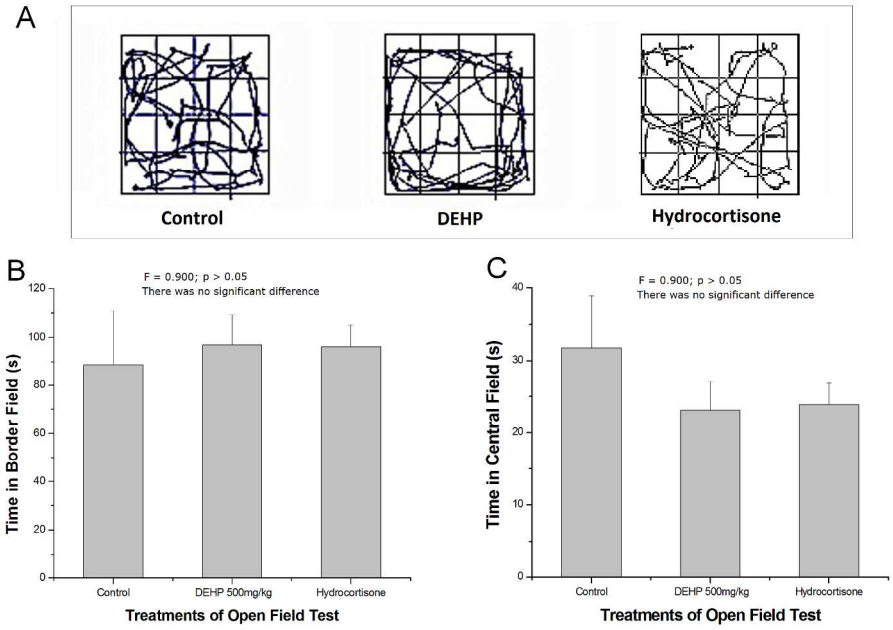

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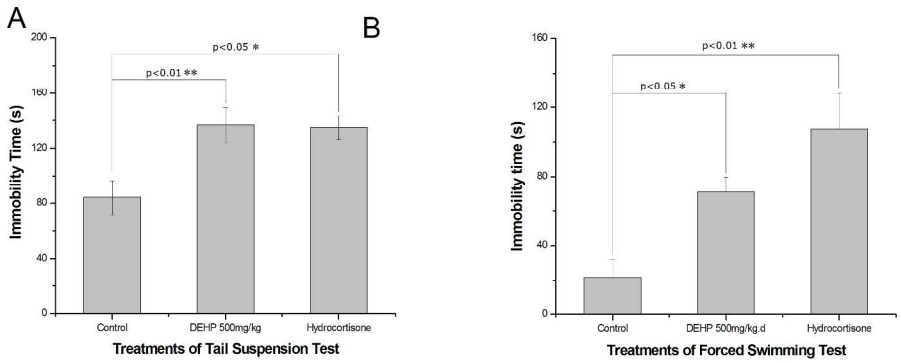

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