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Diisodecyl phthalate aggravates the formaldehyde-exposure-induced learning and memory impairment in mice

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ABSTRACT

Diisodecyl phthalate (DIDP) is a new type of phthalate used in the coating of pharmaceutical pills and in plastic food wrappers. This research was conducted to investigate whether DIDP could cause learning and memory impairment in mice, using formaldehyde (FA) to construct a positive control. Behavioral analysis showed that oral administration of 15 mg kg^{-1}·d^{-1} DIDP combined with inhalation of 1 mg m^{-3} FA led to learning and memory impairment in mice. Histopathological observations of the brain showed that the pathological alterations in the hippocampi. Detection of testosterone (T) and estradiol (E2) levels in the brain and serum showed that E2 levels were associated with learning and memory disorders. Reactive oxygen species (ROS), reduced glutathione (GSH), malondialdehyde (MDA), and 8-hydroxy-2-deoxyguanosine (8-OH-dG) revealed the increased oxidative stress levels. Detection of caspase-3, NF-κB, the phosphorylated cAMP response-element binding protein (p-CREB) and the brain derived neurotrophic factor (BDNF) showed that the protective effect mediated by BDNF, is reduced. However, some of these effects were blocked by the administration of Vitmin E (VitE, 100 mg kg^{-1}·d^{-1}) or 17β-estradiol (17β-E2, 100 μg kg^{-1}). These data suggest that DIDP may aggravate the FA-exposure-induced learning and memory impairment in mice, and that 17β-E2 could be utilized to avoid these adverse effects.

1. Introduction

Phthalates (PAEs) are the most widely used artificial plasticizers, while diisodecyl phthalate (DIDP) is a new type of PAE used in the production of plastic to increase flexibility, and is even used in the coatings of pharmaceutical pills and in plastic food wrappers (Ejaredar et al., 2015). There has been recent concern in both the USA and the European Union regarding DIDP toxicity and bioaccumulation. The European Union has set a maximum specific migration limit for the sum of DIDP and diisononyl phthalate (DINP) from food contact materials of 9 mg kg^{-1} food (Cao, 2010). Plasticizers, including DIDP, were detected over the limit for the first time in food products in Taiwan in 2011 (Yang et al., 2013). Subsequently, the Department of Health of the HKSAR Government found that the plasticizer component in GlaxoSmithKline's antibiotic “Antibacterial” (Augmentin) made in France, is twice the specified upper limit for plasticizers in food contact materials in Europe.

The toxicity of DIDP in humans is not comprehensively understood nor entirely clear. Like other phthalates, DIDP is not covalently bound to plastics, and can therefore be easily emitted into the environment and subsequently make its way into the human body (Halden, 2010). Evidence from animal studies suggest that DIDP at dietary levels ranged from 0.02 to 0.8% (or approximately 15–600 mg kg^{-1}·d^{-1}) for 10 weeks increased the liver weights and histopathologic changes (Hushka et al., 2001; European Food Safety Authority (EFSA), 2008). Several studies have suggested that there is a possible link between exposure to phthalates and negative behavioral effects (Min et al., 2014; Ma et al., 2015; Tang et al., 2015). Studies have confirmed that exposure to di (2-

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ethylhexyl) phthalate (DEHP) and benzyl butyl phthalate (BBP) can impair the learning and memory abilities of mice (Min et al., 2014; Tang et al., 2015), and that DINP can induce cognitive deficits and anxiety in mice (Ma et al., 2015). It is not known whether DIDP can have these same effects.

Formaldehyde (FA) is the simplest of the aldehydes found in nature, and has become the main pollutant in indoor air. It is harmful to human health and is known to be carcinogenic. In view of its widespread use, toxicity, and volatility, FA has been implicated as being an important agent responsible for the development of neurocognitive disorders. Increasing evidence shows that prolonged exposure to FA is significantly associated with symptoms such as depression, memory decline and emotional instability (Songur et al., 2010). The results from animal experiments have shown that FA exposure can induce learning and memory disabilities (Lu et al., 2010; Tong et al., 2013).

DIDP and FA are widely present in the general environment. It is possible for humans to ingest DIDP (the main route is oral) and also to be exposed to FA (the primary route is inhalation). Children are usually the most likely to be exposed to DIDP and FA (Le et al., 2011), and are at greater risk from exposure to these chemicals than adults, since their brains are still in the development stage, and their central nervous systems are more vulnerable to toxic chemicals (Weiss, 2000).

Although the estrogenic effect of DIDP itself is not as strong as that of DEHP and DINP, it is still possibly enter the brain through the blood-brain barrier because of its fat-solubility, and affect the estrogen levels (Joensen et al., 2012). Estradiol (E2) is the primary and the most active estrogen in the brain (Toran-Allerand, 2005). An increasing amount of data suggest a neuroprotective role for 17β-Estradiol (17β-E2) (Kim et al., 2016; Zhu et al., 2017), whereby it rapidly increases the dendritic spine density of pyramidal cells in the hippocampus and consolidates hippocampal memory (Frick, 2015). It is unclear whether DIDP exposure interferes with the E2-mediated neuroprotective effects on learning and memory. This is a scientific question worthy of our study. In addition, the brain is another important target of oxidative stress because it produces reactive oxygen species (ROS) in the metabolic process. Indeed, both oral exposure to DIDP and inhalation of FA result in oxidative stress responses (Ferguson et al., 2011; Shen et al., 2016; Wei et al., 2017). Tang et al. (2015) have shown that the antioxidant vitamin E (VitE) plays a protective role by down regulating oxidative stress induced by DEHP.

Based on the above evidence, we hypothesized that: (1) A decreased E2 level induced by DIDP in the brain may affect the learning and memory abilities of mice; (2) DIDP may also cause learning and memory impairment through oxidative stress in FA-exposed mice. Thus, DIDP may aggravate the FA-induced learning and memory disabilities in mice by both oxidative stress and abnormal E2 levels in the brain (Graphical abstract).

In this study 3-week-old Kunming (KM) mice were exposed to gaseous FA to build a model of learning and memory impairment. We used the model mice as the positive control, and these were also orally exposed to DIDP at the same time for 3 weeks with pathway blocker 17β-E2 or VitE as a protective agent, to explore the oxidative damage to the brain caused by combined exposure to FA and DIDP, and whether the combined exposure has a synergistic effect to induce more severe learning and memory impairment.

2. Materials and methods

2.1. Animal care

Specific pathogen-free male KM mice (3-week-old, 15 ± 2 g weight) were purchased from the Experimental Animal Center of Hubei Province (Wuhan, China) and housed under standard laboratory conditions (temperature, 20–25 °C; humidity, 50–70%; and 12 h day/night cycles). The mice were given a standard chow diet and water ad libitum, except during exposure periods. All the mice spent one week in this environment before initiation of the study. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Office of Scientific Research Management of Hubei University of Science and Technology (Xianning, China) with a Certificate approval ID: HBUST-IACUC-2018-001.

2.2. Reagents and kits

DIDP (> 99%, CAS: 26761-40-0), FA (10%, CAS: 50-00-0), 17β-E2 (98%, CAS: 79037-37-9), Vit E (≥99%, CAS: 59-02-9), 2′,7′-dichlorodihydrofluorescein (DCFH-DA), and 2-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tween 80 (CAS: 9005-65-6) was obtained from Amresco (Solon, OH, USA). The mouse kit for reduced glutathione (GSH) was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse ELISA kits for 8-hydroxy deoxy guanosine (8-OH-dG), cysteine-aspartic acid protease 3 (caspase-3), nuclear factor κB (NF-κB), phosphorylated cAMP response element binding protein (p-CREB), brain derived neurotrophic factor (BDNF) were obtained from eBioscience (San Diego, CA, USA). Mouse kits for T and E2 were obtained from Beijing North Biotechnology Institute (Beijing, China). All other chemicals were of the highest grade available commercially, or as indicated.

2.3. Experimental protocol

Ninety KM mice were randomly divided into nine groups: (A) negative control (saline) group; (B) 0.15 mg kg^{-1}·d^{-1} DIDP group; (C) 1.5 mg kg^{-1}·d^{-1} DIDP group; (D) 15 mg kg^{-1}·d^{-1} DIDP group; (E) 150 mg kg^{-1}·d^{-1} DIDP group; (F) 1 mg m^{-3} FA group; (G) 1 mg m^{-3} FA + 15 mg kg^{-1}·d^{-1} DIDP group; (H) 1 mg m^{-3} FA + 15 mg kg^{-1}·d^{-1} DIDP + 100 mg kg^{-1}·d^{-1} VitE group, (I) 1 mg m^{-3} FA + 15 mg kg^{-1}·d^{-1} DIDP + 100 μg kg^{-1}·d^{-1} 17β-E2 group. The mice received repeated exposure over a period of 3 weeks. The water maze experiment started on the 13th day of exposure as shown in Fig. 1A. The brain tissue of each mouse was taken for tissue sectioning and the preparation of an homogenate. The homogenate was then used to determine the levels of ROS, GSH, malondialdehyde (MDA), 8-OH-dG, caspase-3, NF-κB, p-CREB, and BDNF.

2.4. Chemical exposure

According to the official recommendation of EFSA (2008), the daily intake limit of DIDP for normal adults is 0.15 mg kg^{-1}·d^{-1}. Based on this, we chose DIDP concentrations of 0.15, 1.5, 15, 150 mg kg^{-1}·d^{-1} for our experiment. DIDP and Tween 80 were mixed according to the volume ratio of 1:1. The corresponding groups were given a daily gavage according to body weight of 0.01 mL g^{-1} for 21 days. The concentration of VitE administered was chosen to be 100 mg kg^{-1} according to Yousef et al. (2006). VitE and Tween 80 were mixed to a 1:1 vol ratio. The solution was prepared with sterilized normal saline and administered by daily gavage at the rate of 0.01 mL g^{-1}. The concentration of 17β-E2 was set to 100 μg kg^{-1} according to the experiment of Zhang et al. (2012), in which Zhang et al. (2012) provided the evidence that 17β-E2 protects against neuronal apoptosis. 17β-E2 was dissolved in DMSO with a concentration of 272 μg/100 μL. 0.01 mL g^{-1} body weight was administered three times a week. The concentration of Tween 80 used in our experiments has been shown in previous pharmacological experiments *in vivo* to be inert (Wu et al., 2017) and has no toxic side effects on the organism, therefore the negative control group was given only saline.

According to previous studies by Wei et al. (2017), 1 mg m^{-3} FA can cause a decline in the learning and memory abilities in mice. On this basis, the concentration of FA was set to be 1 mg m^{-3}. The mice were placed in a glass tank connected to a small intelligent environmental climate chamber (transformed from an 8.4 L aerated dryer) that

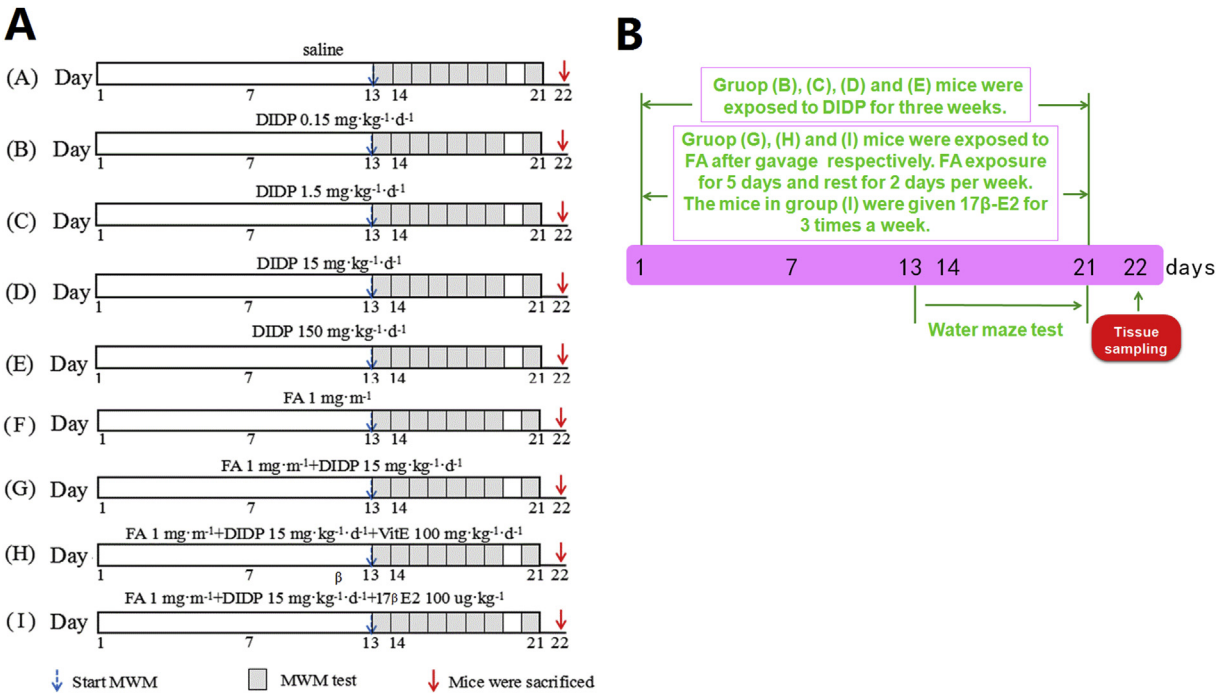


Fig. 1. Experimental protocol. A. Experiment grouping; B. Experimental schedule.

continuously and steadily produced gaseous FA with a concentration of 1 mg m^{-3}. The humidity in the tank was 45% ± 5% and the air flow rate was 2 L min^{-1}. According to occupational work times (work for 5 days, 8 h a day and rest for 2 days per week), mice in model groups were exposed to gaseous FA for 5 days per week for 8 h per day, and lasted 3 weeks. Neither food nor water was provided during the period of FA inhalation.

DIDP and VitE were administered via gavage at a regular time every day, after which all the mice in the (F), (G), (H) and (I) groups were exposed to FA. The (A) ~ (E) groups were subjected to the water maze test after gavage, while the (F), (G), (H) and (I) groups were subjected to the water maze test after FA exposure. The experimental schedule is shown in Fig. 1B.

2.5. Morris water maze (MWM)

MWM is mainly used in behavioral neuroscience to study spatial learning and memory abilities of test rodents. The experimental device is shown in Fig. 2A. The circular pool is divided into four quadrants I, II, III and IV. A black circular escape platform is placed at a fixed position 1 cm below the water surface. The water was kept at a temperature of 24 ± 2 °C.

The training included releasing each mouse facing the pool wall from a fixed position in the I, II, and III quadrants, and allow them to find the fixed escape platform. A camera connected to a computer, tracks the swimming mouse from the time it enters the water for 60 s. If, within the 60 s tracking period, the mouse locates the platform, the time to find the platform is called the escape latency. If the platform could not be found within 60 s, the escape latency is recorded as 60 s, the experimenter then guides the mouse to the escape platform where it remains for 15 s. This training is given daily over a 7-day training period, and is known as the navigation test, and is used to evaluate mouse learning abilities.

On the 8th day of the MWM experiment, the mice were kept away from the water maze, giving them time to forget the location of the platform. On the 9th day, the platform was removed from the pool for the spatial probe test. The mice were put into the pool in same manner as before, to test their memory. The camera device recorded the swimming trajectories of the mice, and the amount of swimming time spent in the target quadrant. The experimental data were analyzed using AnyMaze software.

2.6. Histological preparation of brain tissue

On the 22nd day, the mice were sacrificed by cervical dislocation. The brains were removed and fixed in 4% paraformaldehyde for 24 h. After routinely embedded in paraffin, serial sections of samples were made vertically from central region by using rotary microtome (RM2245, Leica, Germany) at 10 μm thickness. Sections were attached onto the glass slide on its exact position and dried in the 42 °C slide warmer for 6 h, and then deparaffinized by xylene. And they were stained by hematoxylin-eosin and Nissl separately. The stained hippocampal regions were examined under a light microscope (BX51, Olympus, Japan).

2.7. Tissue sample preparation

The collected brains were weighed in a fully automatic electronic balance. The tissue was placed in 10 mL g^{-1} ice-cold 1 × phosphate-buffered saline (PBS, pH = 7.5) and homogenized using a glass homogenizer. Homogenate was centrifuged at 9800 g for 10 min at 4 °C. Supernatants were collected and frozen at −70 °C until needed. The protein concentration of the supernatant was determined using a Folin assay.

2.8. Detection of T and E2 levels in brain tissue and serum

Radioimmunoassay was used to determine T and E2 levels in the brain tissue and serum. The procedure was carried out strictly according to manufacturer's instructions. The sensitivity of T by this assay is 10 pg ml^{-1} and its concentration detection range is from 10 to 800 pg ml^{-1}. The sensitivity of E2 by this assay is 1.0 pg ml^{-1} and its concentration detection range is from 10 to 200 pg ml^{-1}.

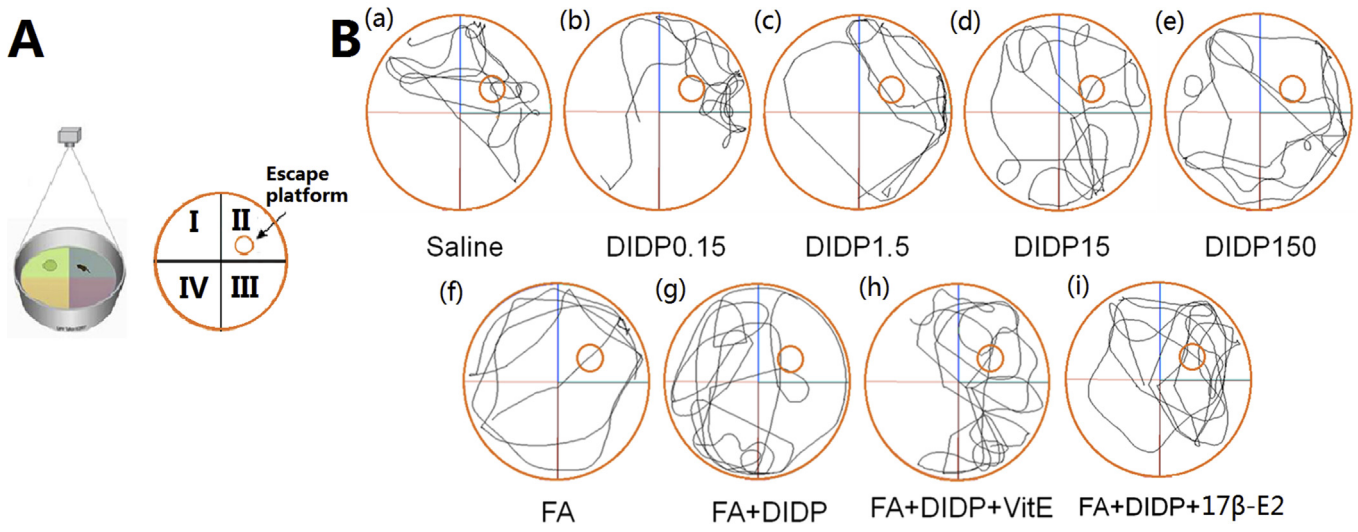


Fig. 2. A.Water maze showing the division into quadrants; B. The swimming trajectories of the different exposure groups. (a) negative control (saline) group; (b) 0.15 mg $kg^{-1}·d^{-1}$ DIDP group; (d) 1.5 mg $kg^{-1}·d^{-1}$ DIDP group; (d) 15 mg $kg^{-1}·d^{-1}$ DIDP group; (e) 150 mg $kg^{-1}·d^{-1}$ DIDP group; (f) positive control (1 mg m^{-3} FA) group; (g) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP group; (h) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP + 100 mg $kg^{-1}·d^{-1}$ VitE group, (i) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP + 100 μg $kg^{-1}·d^{-1}$ 17β-E2 group.

2.9. Oxidative stress assay

ROS levels in the brain supernatant were determined by DCFH-DA fluorescent assay (Lebel et al., 1992). The supernatant was diluted 50-fold with PBS (pH = 7.5), then 100 μL of the dilution was transferred into a 96-well microplate, and 100 μL of 10 μmol L^{-1} DCFH-DA fluorescent dye was added. This was incubated in the dark at 37 °C for 30 min. Fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a fluorescence reader (Hide Chameleon V, Hidex, Finland).

The GSH concentration in the brain supernatant was measured using a GSH kit. Samples were analyzed using a microplate reader to measure absorbance at 405 nm, performed in strict accordance with the kit manufacturer's instructions. GSH levels were calculated according to the formula: GSH (μmol·g^{-1} prot) = [(measure OD_{405} - blank OD_{405})/(standard OD_{405} - blank OD_{405})] × standard concentration × sample dilution factor/homogenate protein concentration.

MDA concentration in the brain supernatant was determined using the thiobarbituric acid (TBA) method (Draper and Hadley, 1990). A 0.5 mL sample was added to 2 mL of 0.6% TBA solution and allowed to react in boiling water for 15 min. After cooling with cold water, the mixtures were centrifuged at 9800 g for 10 min at 4 °C, and the supernatant collected to measure absorbance at 532, 600 and 450 nm. MDA levels were obtained according to the formula: MDA (μmol·g^{-1} prot) = [6.45(OD_{532}-OD_{600}) - 0.56 × OD_{450}]/homogenate protein concentration.

The 8-OH-dG concentration in the brain supernatant was measured using an ELISA kit in strict accordance with the manufacturer's instructions, absorbance was measured at 450 nm to calculate the content of 8-OH-dG. The sensitivity by this assay is 1.0 ng mL^{-1}.

2.10. Analysis of NF-κB and caspase-3 content

NF-κB and caspase-3 concentrations were measured using ELISA kits according to the manufacturer's instructions. The sensitivities of the ELISA kits are 1.0 pg mL^{-1} for NF-κB and 1.0 pmol L^{-1} for caspase-3.

2.11. Analysis of BDNF and p-CREB content

BDNF and p-CREB concentrations in the brain were measured using ELISA kits according to the manufacturer's instructions. The sensitivities of the ELISA kits were 1.0 pg mL^{-1} for BDNF and 0.1 ng mL^{-1} for p-CREB.

2.12. Statistical analysis

Statistical analyses and chart generation were carried out using GraphPad Prism 5.0 software (OriginLab, Berkeley, CA, USA). A post hoc test was used to compare the mean of the two groups, and the SNK-q test was performed to do a pairwise comparison between groups of samples after a one-way ANOVA test. A P-value of < 0.05 was regarded as significant.

3. Results

DIDP treatment and co-exposure with FA had negative effects on the behavior of mice in the MWM test.

3.1. Spatial probe test

The spatial probe test showed that different groups of mice exhibited different spatial memory abilities, these results are shown in Fig. 2B. The swimming trajectories of the negative control group mice were concentrated in the quadrant where the escape platform had been (the II quadrant). However, the swimming trajectories of the DIDP15, DIDP150, FA and the FA + DIDP groups were scattered and disorderly. The swimming trajectories of the FA + DIDP + VitE group and the FA + DIDP + 17β-E2 group were concentrated more in the target quadrant than those of the FA + DIDP group.

3.2. Navigation test

The escape latency of the mice decreased over time for each group, with the control group showing the most significant decrease, and the DIDP150 group and the FA + DIDP group showing the least decrease in escape latency (Fig. 3A).

On the last day of the MWM test, the mice in each of the exposure groups spent less time in the target quarter than the negative control group ($P_{trend} < 0.01$) (Fig. 3B). Of the exposure groups, the DIDP15 and the DIDP150 groups showed a significant difference ($p < 0.05$) compared with the negative control group. The FA and FA + DIDP groups had an extremely significant difference ($p < 0.01$) compared with the

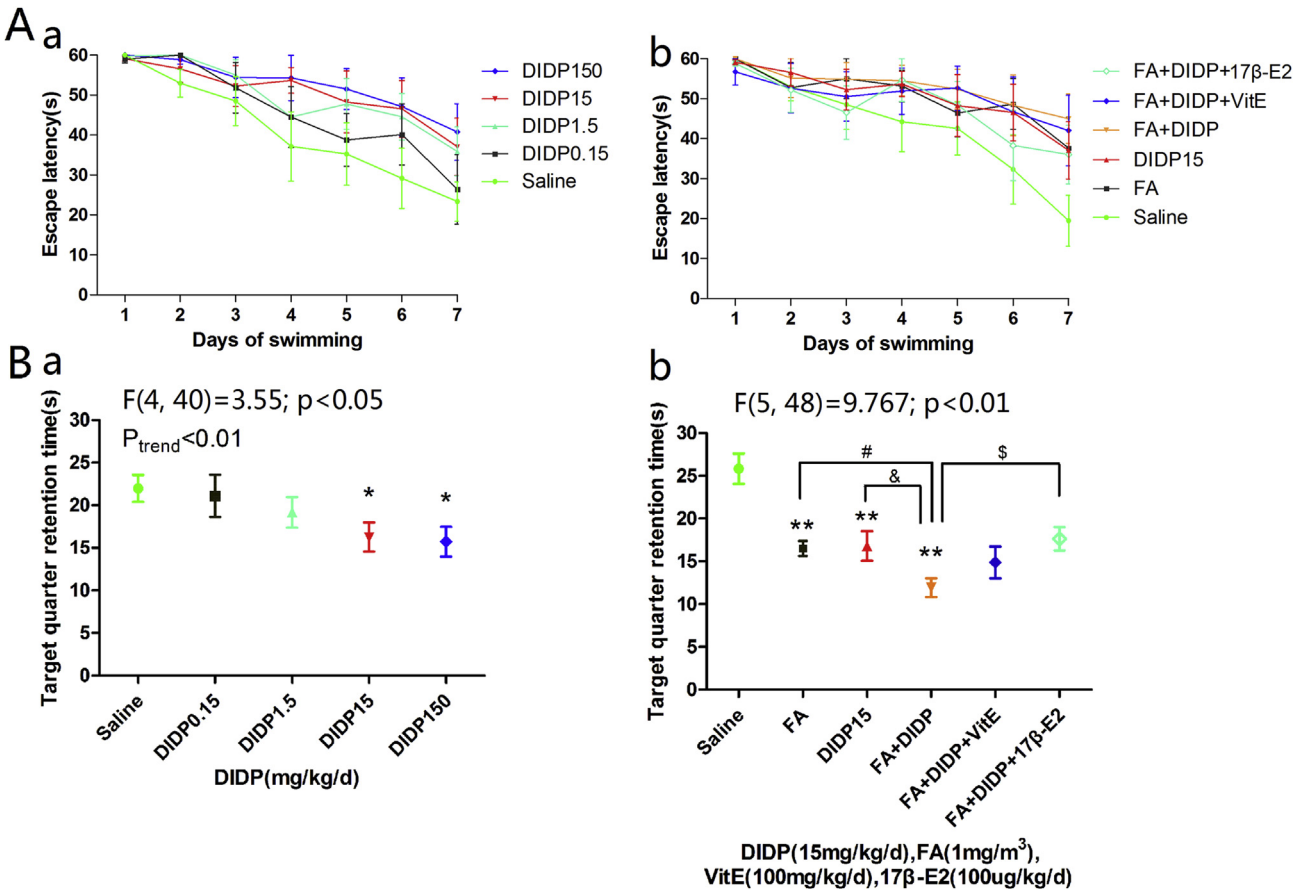


Fig. 3. A.The average escape latency (s) from the MWM test (n = 9). **a.** saline, 0.15 mg $kg^{-1}·d^{-1}$ DIDP group, 1.5 mg $kg^{-1}·d^{-1}$ DIDP group, 15 mg $kg^{-1}·d^{-1}$ DIDP group, 150 mg $kg^{-1}·d^{-1}$ DIDP group. **b.** saline, 1 mg m^{-3} FA group, 15 mg $kg^{-1}·d^{-1}$ DIDP group, 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP group, 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP + 100 mg $kg^{-1}·d^{-1}$ VitE group, 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP + 100 μg $kg^{-1}·d^{-1}$ 17β-E2 group. B. The target quarter retention time of the MWM test (n = 9). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with saline group; $^{\#}p < 0.05$, FA + DIDP group compared with FA group; $^{\&}p < 0.05$, FA + DIDP group compared with DIDP 15 group; $^{\$}p < 0.05$, FA + DIDP +17β-E2 group compared with FA + DIDP group.

negative control group. The FA + DIDP group had a shorter target quadrant retention time than the FA group, and the FA + DIDP + 17β-E2 group had a longer retention time in the target quadrant than the FA + DIDP group ($p < 0.05$).

3.3. DIDP treatment and co-exposure with FA were responsible for histopathological changes in the observed brain sections

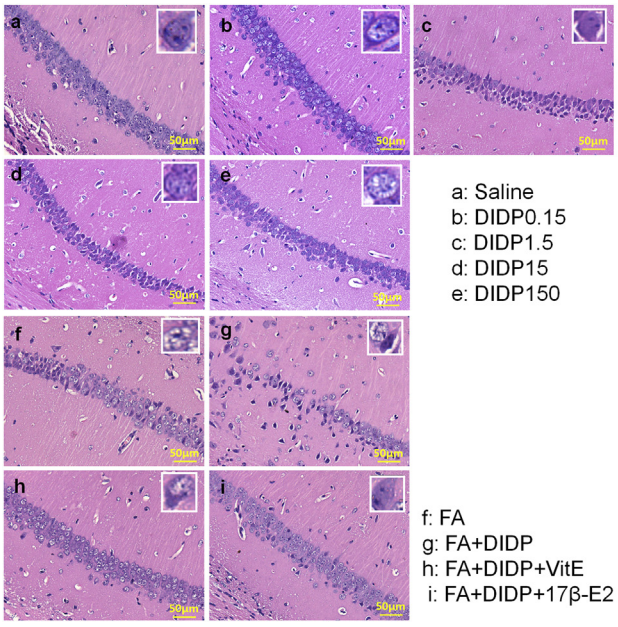
H&E staining (Fig. 4) showed that the hippocampal CA1 pyramidal neurons of the negative control group were arranged normally. With increasing DIDP concentrations, the damage to the pyramidal neurons in the hippocampal CA1 region was gradually made worse, showing loose and disordered arrangements, and swelling deformations. Fig. 4 also showed that the hippocampal CA1 region of the FA + DIDP group exhibited more obviously loose and scattered arrangements than that of the FA and DIDP15 groups. We also observed that a large number of neurons disappeared in the CA1 region of the FA + DIDP group, while some of the neurons were pyknotic or vacuolated. Although the cells in the FA + DIDP + VitE and FA + DIDP + 17β-E2 groups were damaged, they were still arranged more regularly with a thicker cell layer than in the FA + DIDP group.

Nissl staining (Fig. 5) showed that the hippocampal CA1 pyramidal neurons of the negative control group were clear, with small cell bodies, long axis dendrites, uniform cytoplasm, clear nucleoli, and abundant Nissl bodies. With increasing DIDP exposure concentrations, the pyramidal neurons in the hippocampal CA1 region showed loss of Nissl substance and swelling deformations. Partial pyramidal neurons in the DIDP15 and DIDP150 groups were deeply stained and shrunken. Fig. 5 also showed that a large number of neurons in the CA1 region of the FA + DIDP group exhibited cytoplasmic pyknosis and deep staining, with some neurons showing vacuolated lesions, and a decrease in Nissl substance. Comparing the FA + DIDP + VitE and FA + DIDP + 17β-E2 groups with the FA + DIDP group, we saw that the neuron morphology was regular, and the reduction of Nissl substance was relieved.

3.4. DIDP treatment and co-exposure with FA broke the balance between T and E2 levels in the brain and serum

Fig. 6A shows that the level of T in serum decreased with an increase in DIDP concentration, while the E2 level increased ($P_{trend} < 0.01$). The T and E2 levels in serum in the FA + DIDP group were significantly lower than those in the FA and DIDP15 groups. However, the E2 levels in serum in the FA + DIDP group were significantly higher than those in the FA group and the DIDP15 group ($p < 0.05$). Fig. 6B showed that the level of T in the brain decreased with increasing DIDP concentration, and the E2 level decreased ($P_{trend} < 0.01$). The T the E2 levels in the brain homogenate in the FA + DIDP group were significantly lower than those in the FA and DIDP15 groups ($p < 0.01$).

The levels of T in the brain and serum in the FA + DIDP + VitE and FA + DIDP + 17β-E2 groups were higher than those in the FA + DIDP group (Fig. 6A(b, d)). And the E2 level in the brain in the FA + DIDP + VitE and FA + DIDP + 17β-E2 groups were higher than those in the FA + DIDP group. The E2 levels in serum showed an



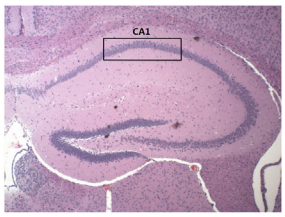


Fig. 4. H&E staining of the hippocampal CA1 region in the different groups (40 ×). (a) negative control (saline) group; (b) 0.15 mg $kg^{-1}·d^{-1}$ DIDP group; (d) 1.5 mg $kg^{-1}·d^{-1}$ DIDP group; (d) 15 mg $kg^{-1}·d^{-1}$ DIDP group; (e) 150 mg $kg^{-1}·d^{-1}$ DIDP group; (f) positive control (1 mg m^{-3} FA) group; (g) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP group; (h) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP + 100 mg $kg^{-1}·d^{-1}$ VitE group, (i) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP + 100 μg $kg^{-1}·d^{-1}$ 17β-E2 group.

opposite trend (Fig. 6B (b, d)).

3.5. DIDP treatment and co-exposure with FA increased the oxidative stress levels in the brain

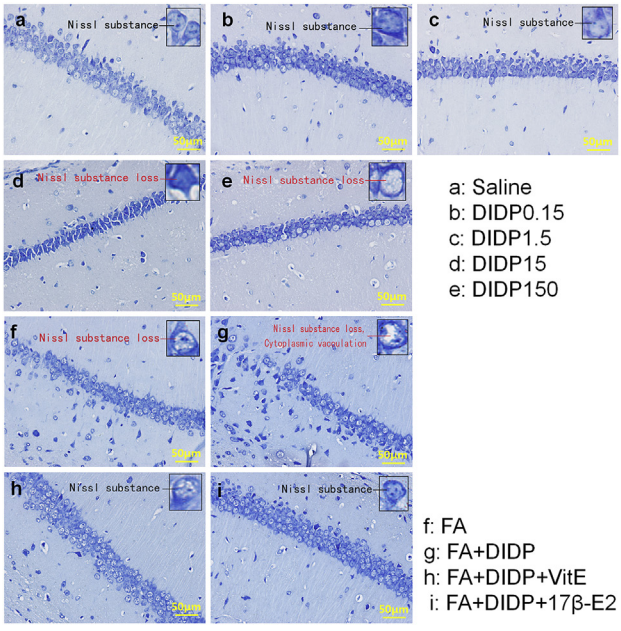
Fig. 7 showed that the content of ROS, MDA and 8-OHdG in the mouse brain homogenate increased with increasing DIDP concentrations, while the GSH content decreased ($P_{trend} < 0.01$). The levels of ROS, MDA and 8-OHdG in the FA + DIDP group were significantly higher than those in the FA and the DIDP15 groups. The levels of ROS, MDA and 8-OHdG in the FA + DIDP + VitE and FA + DIDP + 17β-E2 groups were lower than those in the FA + DIDP group, while the GSH levels moved in an opposite direction to the other three indicators.

3.6. DIDP treatment and co-exposure with FA affect the expression level of NF-κB and Casp-3 in the brain

Fig. 8A shows that NF-κB and Casp-3 levels increase in the mouse brain homogenate with increasing DIDP exposure concentrations. Compared with the FA group and the DIDP15 group, the NF-κB and Casp-3 levels in the FA + DIDP group increased significantly, while these levels in the FA + DIDP + VitE and FA + DIDP + 17β-E2 groups decreased significantly when compared with the FA + DIDP group ($p < 0.05$).

3.7. DIDP treatment and co-exposure with FA decreases BDNF mediated protection in the brain

Fig. 8B shows that the BDNF and p-CREB levels decreased in the mouse brain homogenate with increasing DIDP exposure concentrations. Levels of BDNF and p-CREB in the FA + DIDP group decreased



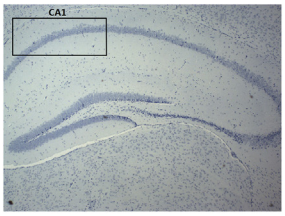


Fig. 5. Nissl staining of the hippocampal CA1 region in different groups (40 ×). (a) negative control (saline) group; (b) 0.15 mg $kg^{-1}·d^{-1}$ DIDP group; (d) 1.5 mg $kg^{-1}·d^{-1}$ DIDP group; (d) 15 mg $kg^{-1}·d^{-1}$ DIDP group; (e) 150 mg $kg^{-1}·d^{-1}$ DIDP group; (f) positive control (1 mg m^{-3} FA) group; (g) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP group; (h) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP + 100 mg $kg^{-1}·d^{-1}$ VitE group, (i) 1 mg m^{-3} FA + 15 mg $kg^{-1}·d^{-1}$ DIDP + 100 μg $kg^{-1}·d^{-1}$ 17β-E2 group.

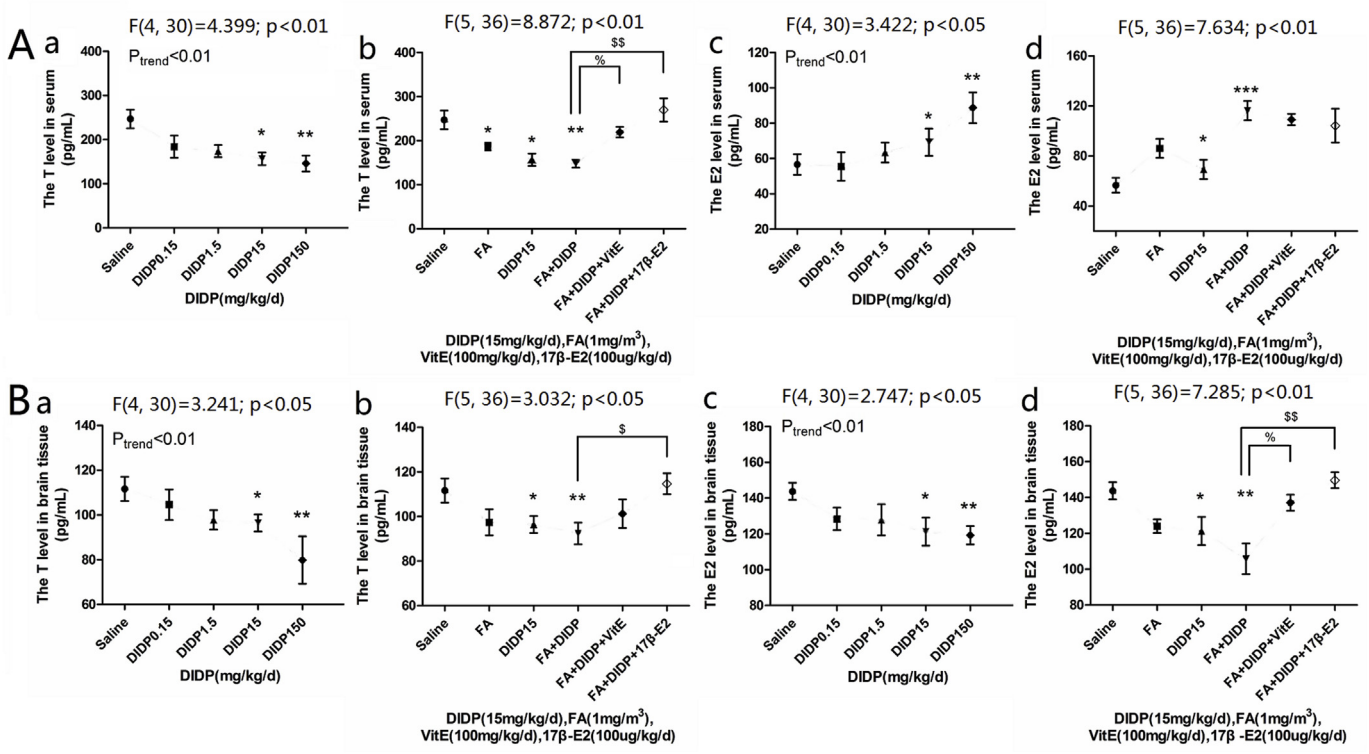


Fig. 6. A. The T and E2 levels in the serum in different groups (n = 7). B. The T and E2 levels in the brain in different groups (n = 7). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with saline group; $^{\#}p < 0.05$, FA + DIDP group compared with FA group; $^{\&}p < 0.05$, FA + DIDP group compared with DIDP 15 group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, FA + DIDP +17β-E2 group compared with FA + DIDP group.

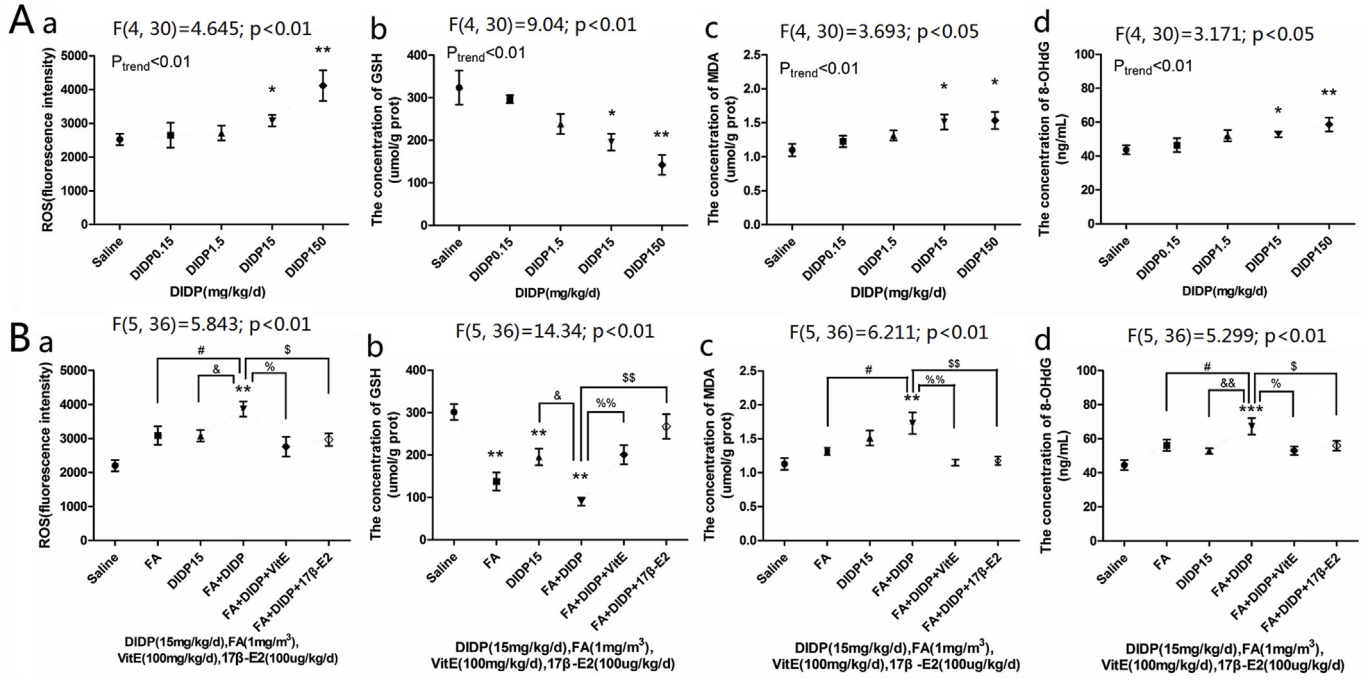


Fig. 7. Oxidative stress levels in the brain of different groups (n = 7). A. ROS fluorescence; B. GSH concentrations; C. MDA concentrations; D. 8-OHdG levels. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with saline group; $^{\#}p < 0.05$, FA + DIDP group compared with FA group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, FA + DIDP group compared with DIDP 15 group; $^{\%}p < 0.05$, $^{\%\%}p < 0.01$, FA + DIDP + VitE group compared with FA + DIDP group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, FA + DIDP +17β-E2 group compared with FA + DIDP group.

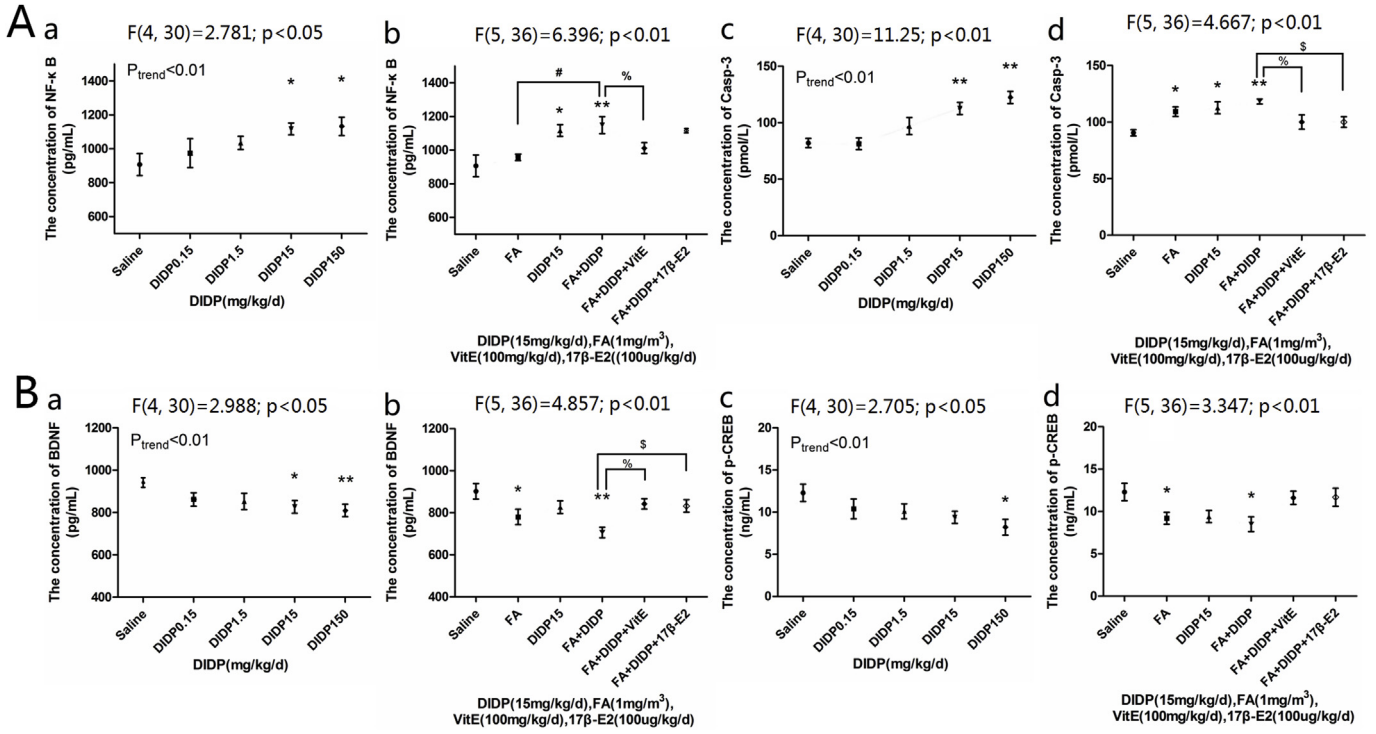


Fig. 8. A. NF-κB and Casp-3 contents in the brain in the different groups (n = 7). B. BDNF and p-CREB levels in the brain in different groups (n = 7). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with saline group; $^{\#}p < 0.05$, FA + DIDP group compared with FA group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, FA + DIDP group compared with DIDP 15 group; $^{\%}p < 0.05$, FA + DIDP + VitE group compared with FA + DIDP group. $^{\$}p < 0.05$, FA + DIDP +17β-E2 group compared with FA + DIDP group.

compared with the FA and DIDP15 groups. The levels in the FA + DIDP + VitE and FA + DIDP + 17β-E2 groups increased compared with the FA + DIDP group.

4. Discussion

DIDP has been listed since 2007 under Proposition 65 as a substance known to the state of California to induce reproductive toxicity. Previous studies have determined that DIDP may be only slightly toxic for the liver (EFSA, 2008). However, some recent studies have reported that a similar compound, DINP, can cause hippocampal damage, and can significantly impair the learning and memory abilities of mice (Ma et al., 2015). Our study reported that DIDP at a high dose (150 mg kg^{-1}·d^{-1}) has negative effects on learning and memory in mice, while DIDP at a low dose (15 mg kg^{-1}·d^{-1}) when combined with FA exposure, still poses a health risk, resulting in learning and memory impairment and damage to the hippocampi of mice. Furthermore, by adding the blocking agent VitE and 17β-E2, we found that the learning and memory abilities of mice in the FA + DIDP15 + VitE and FA + DIDP15 + 17β-E2 groups increased correspondingly to some extent, suggesting that the adverse effects of DIDP15 and FA were associated with abnormal estradiol levels and oxidative stress in the brain.

Pathway one: DIDP aggravates the FA-caused learning and memory impairment by changing estradiol levels in the brain, and the neuroprotective effects of 17β-E2.

The MWM is an ideal experimental model used to investigate the learning and memory abilities of treated mice. It has been proved to be a robust and reliable test strongly correlated with learning and memory (Vorhees and Williams, 2006). FA is known to cause learning and memory impairment (Liu et al., 2008). Therefore, we used FA to construct a positive control in order to explore whether DIDP combined with FA exposure may result in more severe learning and memory impairment. The results showed that the FA + DIDP group had a longer average escape latency in the navigation test than either the FA or the DIDP15 groups. In the spatial probe test on the 9th day, the FA + DIDP group spent less time in the target quadrant, with a trajectory showing less purpose, indicating that DIDP aggravates the learning and memory impairment caused by FA.

The hippocampus plays a pivotal role in learning and memory (Buzsáki and Moser, 2013). Pathological observations show that the hippocampal CA1 region of the FA + DIDP group was more seriously damaged than that of the FA and DIDP15 groups. Moreover, the loss of Nissl substance in the pyramidal cells of the hippocampal CA1 region will affect the synthesis of neurotransmitters and subsequently affect the signaling between neurons (Bernert et al., 2003).

The abnormal estrogen level may be responsible for the adverse effects of FA + DIDP exposure. The results showed that the E2 levels in serum increased significantly in the FA + DIDP group compared with the control group, while the E2 levels in the brain showed a significant downward trend in the FA + DIDP group. Meanwhile, the levels of T in the FA + DIDP group decreased significantly, either in the serum or in the brain homogenate. As a substrate, the concentration of T is closely related to the synthesis of endogenous estrogen in the brain. Studies have shown that exposure to PAEs can reduce the level of T (Parks et al., 2000; Howdeshell et al., 2008). Parks et al. (2000) showed that exposure to DEHP reduced serum T levels in both male fetal and neonatal rats. Howdeshell et al. (2008) reported that exposure to different types of PAEs reduced T production by dose accumulation. These findings are supported by epidemiological studies conducted by Pan et al. (2006).

Compared with the FA + DIDP group, the FA + DIDP + 17β-E2 group had a shorter escape latency and spent more time in the target quadrant, with a swimming trajectory concentrated more in the target quadrant. The FA + DIDP + 17β-E2 group mice swam more purposefully, indicating that learning and memory had improved. These findings are consistent with those of Englerchiurazzi et al. (2012). Although there is still damage in the FA + DIDP + 17β-E2 groups, the degree of damage was less than that seen in the FA + DIDP group, implying that the addition of 17β-E2 has a certain protective effect against

FA + DIDP.

Some recent research suggest that E2 including 17β-E2 plays a nutritional and protective actions in the brain and cognitive function (Arevalo et al., 2014; Villa et al., 2016; Zhu et al., 2017). The neuroprotective effects of E2 in the brain may be mediated by three pathways (McCARTHY, 2008), as follows: First, both *in vitro* and *in vivo* studies have shown that 17β-E2 (Chakrabarti et al., 2016; Kim et al., 2016) is a natural antioxidant, which can reduce the ROS level in the organism and can significantly improve the cognitive function of the brain. Second, 17β-E2 can also protect the neurons mediated by BDNF, enhances synaptic plasticity, neurite growth, hippocampal neurogenesis, and long-term potentiation (Numakawa et al., 2010; Petrovska et al., 2012). Another study indicates that E2 increase BDNF levels in the hippocampus (Luine and Frankfurt, 2013). Third, E2 exerts biological effects by binding to specific estrogen receptors in cells. Studies have shown that estrogen receptor β (ER-β) is closely related to learning and memory function (Rissman et al., 2002). Liu et al. (2008) showed that the activation of ER-β can improve the pathological morphology of hippocampal neurons, increase the dendritic branches of hippocampal neurons, regulate hippocampal synaptic plasticity and improve hippocampal dependent cognition.

Pathway two: DIDP aggravates the FA-exposed learning and memory disabilities by up-regulating oxidative stress in the brain, and the antioxidant properties of VitE.

Oral exposure to DIDP or inhaled FA can cause oxidative stress in mice (Shen et al., 2016; Wei et al., 2017). Oxidative damage is also one of the known causes of brain damage (Ma et al., 2015). Oxidative stress refers to the imbalance between oxidation and antioxidation in the body, leading to accumulation of ROS in the body, and causing damage to cells and tissues. Oxidative stress states may cause oxidative damage to macromolecules, membranes and DNA (Kevin et al., 2004). An increase in free radicals initiates a lipid peroxidation process in an organism. MDA is one of the final products of peroxidation in cells, and is commonly known as a marker of oxidative stress (Draper and Hadley, 1990). 8-OHdG is the basic by-product of ROS oxidation of DNA, and is an important biomarker for evaluating DNA oxidative damage (Collins et al., 1996). GSH is an important reductant and free radical scavenger *in vivo*, and is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals. In our study, the levels of ROS, MDA, and 8-OHdG in the DIDP15, DIDP150, FA and FA + DIDP groups were higher than those in the control group, and the GSH content was lower than that in the control group. Compared with the FA + DIDP group, the levels of ROS, MDA and 8-OHdG decreased, and GSH content increased in the FA + DIDP + VitE group ($p < 0.05$, $p < 0.01$). This indicates that DIDP exposure leads to an increase in oxidative stress in the brain, and that VitE reduces the oxidative stress level due to its antioxidant effect. This is consistent with the results reported by Shen et al. (2016), that 200 mg kg^{-1}·d^{-1} DIDP increased ROS levels and decreased GSH content in the ear tissues of mice.

When the concentration of DIDP was 0.15 or 1.5 mg kg^{-1}·d^{-1}, the oxidative stress indicators we looked at were not significantly different from the control group. When the concentration of DIDP was as high as 15 or 150 mg kg^{-1}·d^{-1}, high levels of oxidative stress were observed, including inflammation and even apoptosis, which was significantly different from the control group. $P_{trend} < 0.01$ showed that there was a dose-dependent relationship between the degree of injury and the DIDP exposure concentration.

Sustained oxidative stress can induce an inflammatory response and activate inflammatory factors (Reuter et al., 2010). NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, etc. NF-κB plays a key role in regulating the immune response to infection, and has also been implicated in processes of synaptic plasticity and memory (Shankar et al., 2012). Further escalation will trigger disturbances in mitochondrial function, resulting in cell apoptosis or necrosis. Caspases are crucial mediators of programmed cell death (apoptosis). Caspase-3 is a frequently activated death protease, and is required for some typical apoptotic markers (Porter and Jänicke, 1999). Our results, showing that the levels of NF-κB and caspase-3 in the DIDP15, DIDP150 and FA + DIDP groups were higher than those in the control group, is statistically significant, indicating inflammation and apoptosis in the brain of mice. It is possible that DIDP caused ROS accumulation and increases in oxidative stress, induced inflammation and apoptosis in the brain of mice, and thereby affected the function of the hippocampus.

The CREB facilitates learning and memory in the frontal cortex, hippocampus, and amygdale, and its phosphorylation plays a noteworthy role in neuroprotection. Research conducted by Min et al. (2014) proved that BBP exposure impaired the learning and memory abilities of mice, and that the levels of CREB phosphorylation decreased after BBP exposure. We also detected that the levels of p-CREB in the brains of the DIDP150, FA, FA + DIDP groups decrease significantly, comapred with that of the negative control group. In addition, BDNF is a target involved in estrogen regulation of hippocampal memory consolidation (Pluchino et al., 2013; Bekinschtein et al., 2014). The results showed that the levels of BDNF in the brains of the DIDP15, DIDP150, FA and FA + DIDP group were significantly different from that of the negative control group.

VitE with antioxidant properties has a protective role against the adverse effects (Srinivasan et al., 2011). Its main biological function is to act as a fat-soluble antioxidant has been postulated. In this role, VitE as a radical scavenger, delivering a hydrogen atom to free radicals. Meanwhile, VitE is incorporated into cell membranes, which are therefore protected from oxidative damage (Galli et al., 2016). As our results showed that, the learning and memory abilities of the mice in the VitE blocking group were improved, and the degree of cell damage in brain was mitigated. Accordingly, VitE can affect against these adverse changes caused by FA + DIDP through down-regulation of the oxidative stress, which then reduces the occurrence of downstream events such as inflammation and apoptosis after FA + DIDP exposure.

Although the testing method of MDA in this study is insufficient and even further studies are needed to explore the potential mechanisms underlying the FA and DIDP co-exposure effects, our results still suggested that DIDP, at high dose 150 mg kg^{-1}·d^{-1}, adversely affects the learning and memory abilities of mice. And it is also shown that oxidative stress and/or abnormal estradiol level in the brain may be one of the reasons that DIDP can aggravate the FA-exposure-impaired learning and memory in mice, and that 17β-E2 could be utilized to avoid these adverse effects.

5. Conclusions

This study provides a new finding that low doses of DIDP do have less adverse effects on learning and memory in mice than do high concentrations of DIDP. It also demonstrated that DIDP combined with FA exposure, will result in greater harm. This makes it clear that more attention should be paid to the influence of DIDP in the indoor environment, especially for those children who may also be exposed to indoor FA.

Conflict of interest statement

Nothing declared.

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Transparency document

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