Original Article

Di-(*n*-butyl)-phthalate-induced Oxidative Stress and Depression-like Behavior in Mice with or without Ovalbumin Immunization*

ZUO Hao Xiao[1], LI Jin Quan[1], HAN Bing[2], KE Chen Juan[1], LIU Xu Dong[1], ZHANG Yu Chao[1], LI Li[1], and YANG Xu[1,#]

1. Section of Environmental Biomedicine, Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Sciences, Central China Normal University, Wuhan 430079, Hubei, China; 2. Department of Molecular Pharmacology, University Centre for Pharmacy, University of Groningen, Groningen 9713AV, the Netherlands

Abstract

Objective To investigate the relationship between atopic allergy and depression and the role of DBP in the development of depression.

Methods BALB/c mice were randomly divided into eight groups: saline; ovalbumin (OVA)-immunized; saline+DBP (0.45 mg/kg·d); saline+DBP (45 mg/kg·d); DBP (0.45 mg/kg·d) OVA-immunized; DBP (45 mg/kg·d) OVA-immunized; saline+hydrocortisone (30 mg/kg·d); and hydrocortisone (30 mg/kg·d)-exposed OVA-immunized. Behavior (e.g. open-field, tail suspension, and forced swimming tests), viscera coefficients (brain and spleen), oxidative damage [e.g. reactive oxygen species (ROS), malondialdehyde (MDA), and glutathione (GSH)], as well as levels of IgE and IL-4, were then analyzed.

Results In the saline and OVA groups, the degree of depression symptoms in mice increased with increasing DBP concentration. Additionally, the OVA-immunity groups were associated with more serious depressive behavior compared with the same exposure concentration in the saline group. Oxidative damage was associated with a dose-dependent increase in DBP in the different groups. IL-4 and IgE levels were associated with low-dose DBP stimulation, which changed to high-dose inhibition with increasing DBP exposure, possibly due to spleen injury seen at high DBP concentrations.

Conclusion Development of an atopic allergy has the potential to increase the risk of depression in mice, and it seems that DBP helps OVA to exert its effect in our present model. Moreover, the results of our study implicate a certain connection between brain oxidative stress and depression, which deserves a further exploration.

Key words: Di (*n*-butyl) phthalate; Atopic allergy; Depression; Mice; Oxidative stress; Behavioral tests

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#Correspondence should be addressed to YANG Xu, Tel: 86-27-67866997, E-mail: yangxu@mail.ccnu.edu.cn

Biographical note of the first author: ZUO Hao Xiao, female, born in 1989, Master, majoring in biochemistry and molecular biology.

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INTRODUCTION

Depression is a common mental disorder associated with symptoms such as regular negative moods, decreased physical activity, feelings of helplessness and cognitive dysfunction, and can lead to suicide[1]. Approximately one-third of adults in the United States have been classified as severely depressed. The World Health Organization has ranked major depressive disorder (MDD) as one of the heaviest disease burdens to human society, because of its wide geographic distribution and highly debilitating symptoms. The lifetime prevalence rate of MDD in the United States is currently 17%, while the 12-month prevalence rate has been estimated to be 1.7%-8.6%[2]. Several epidemiological studies suggest an association between depression and atopic allergies (i.e. asthma, atopic eczema and allergic rhinitis)[3-8]. However, few animal experiments examining this link have been published.

Humans are routinely 'exposed' to plastics by virtue of their utilization in a multitude of areas. Plastic News reported that, globally, 27.3 billion pounds of flexible packaging was generated in 2004. Phthalates are a group of synthetic chemicals with a wide spectrum of use as plasticizers and solvents in many products. In plastic products, there may be non-bound residual monomers (such as phthalates) that can be released into the environment and create potential health threats[9-10]. Di-(*n*-butyl) phthalate (DBP) ester is one of the most important and widely used industrial additives today because it enhances the flexibility of plastics. Humans and animals can be exposed to these ubiquitous compounds via oral, dermal, inhalational, and iatrogenic routes[11]. DBP has been of significant interest to many disciplines of toxicology since the first reports of its potential to affect human health[12-14]. Recently, it was reported that DBP content in liquor was as 260% high as the national standard. As disclosed by the Taiwan food scandal in 2011, high amount of DBP was found in foods such as beverages, fruit juices, bread, sports drinks, tea, and jam. For a while, food safety and toxicity of DBP attracted considerable public concern.

The prevalence of depression is at a rise and it is highly possible that there is a link between depression and exposure to environmental pollutants. Experimental findings suggest that some small molecular substances in the air act as adjuvants rather than outright act as antigens[15-16]. Researches have shown that phthalate esters may have adjuvant effects on some diseases, including allergic asthma[17]. Therefore, it is possible that a link could exist between DBP exposure and depression and that there is some oxidative damage to the brain and the immune system involved in this pathology[18-22]. Accordingly, this study aimed to elucidate whether an atopic allergy (i.e. allergic inflammation) can mediate DBP-induced depression in mice and, further, what potential mechanism(s) might be involved in a linkage of these two pathologies.

MATERIALS AND METHODS

Animals

BALB/c mice (male, 7-8-wk-old, 22-24 g) were purchased from Hubei Experimental Animal Center (Wuhan, China). All mice were housed in pathogen-free cages in rooms maintained at 20-25 °C with 50%-70% humidity and a 12-h light/dark cycle. All mice were provided *ad libitum* access to commercial diet and filtered water. All protocols used in these studies were approved by the Office of Scientific Research Management of Central China Normal University (March 26, 2010; CCNU-SKY-2010-005). Based on ethical requirements, only 8 mice were used in each treatment group to minimize the number of experimental animals and still ensure statistical power.

Main Reagents and Equipment

DBP (>99%, CAS: 84-74-2), ovalbumin (OVA, CAS: 257-264), and aluminum hydroxide ($Al[OH]_3$, CAS: 21645-51-2), were purchased from Sigma-Aldrich (St. Louis, MO). Tween-80 (CAS: 9005-65-6) was purchased from Amresco (Solon, OH). Hydrocortisone (HC, CAS: 50-23-7) was purchased from Xiandai Hasen Pharmaceutical (Shanghai, China). All other chemicals were of analytical grade and purchased from Sigma, unless specifically noted.

Challenge Protocol

BALB/c mice were randomly allocated into eight groups with eight mice per group, and then received either: (A) saline only; (B) OVA only; (C) saline + 0.45 mg DBP/kg; (D) OVA + 0.45 mg DBP/kg; (E) saline + 45 mg DBP/kg; (F) OVA + 45 mg DBP/kg; (G) saline + 30 mg HC/kg exposure; or, (H) OVA + 30 mg

HC/kg. The protocol used for the various treatments in this study is outlined in Figure 1. In order to simulate the chronic exposure, these doses were selected based on the specific laboratory conditions and previous researches[23-26].

DBP was prepared by dissolving DBP in Tween 80 (at 1:1 ratio) and diluting with sterile saline. Each group was given DBP (or hydrocortisone) intra-gastrically (without anesthesia) daily for 32 days. During this treatment period, the OVA-treated mice (in Groups B, D, F, and H) received OVA by subcutaneous injection of 300 μL of a saline solution containing 50 μg OVA + 1.75 mg aluminum hydroxide (adjuvant) on Days 7, 21, and 28 of the regimen. Thereafter, on Days 33-39 of the experiment, the OVA mice underwent a 30 min aerosol challenge with 1% OVA [50 mg/min OVA; generated via an ultrasonic nebulizer (Yuyue, version 402AI, Danyang, China)]. A similar procedure was performed in the saline-treated hosts (Groups A, C, E, and G), with OVA being replaced by saline at all time points from Day 1-39 of the experiment. The open-field, tail suspension, and forced swimming tests were conducted in the same order as in the overall exposure/treatments protocol, in each case on Days 36, 37, and 39, 1 h after the OVA/saline aerosol challenge. All mice were then euthanized on Day 40 of the experiment to generate tissues/fluids for biochemical analyses. The procedures are outlined in Figure 2 .

Open-field Test (OFT)

On Day 36 of the experimental regimens, an OFT was performed to evaluate mice loco- motor activity, as previously described[27]. The apparatus consisted of a square 40×40 cm base surrounded by a 35-cm wall, with the floor divided into 16 squares. Each mouse was placed individually in the center of the open-field apparatus. Defecation number, distance traveled, and travel trails were recorded[28]. Testing was conducted over a 5 min period and recorded using a video tracking system. Apparatus walls and floors were thoroughly cleaned with 10% ethanol between tests.

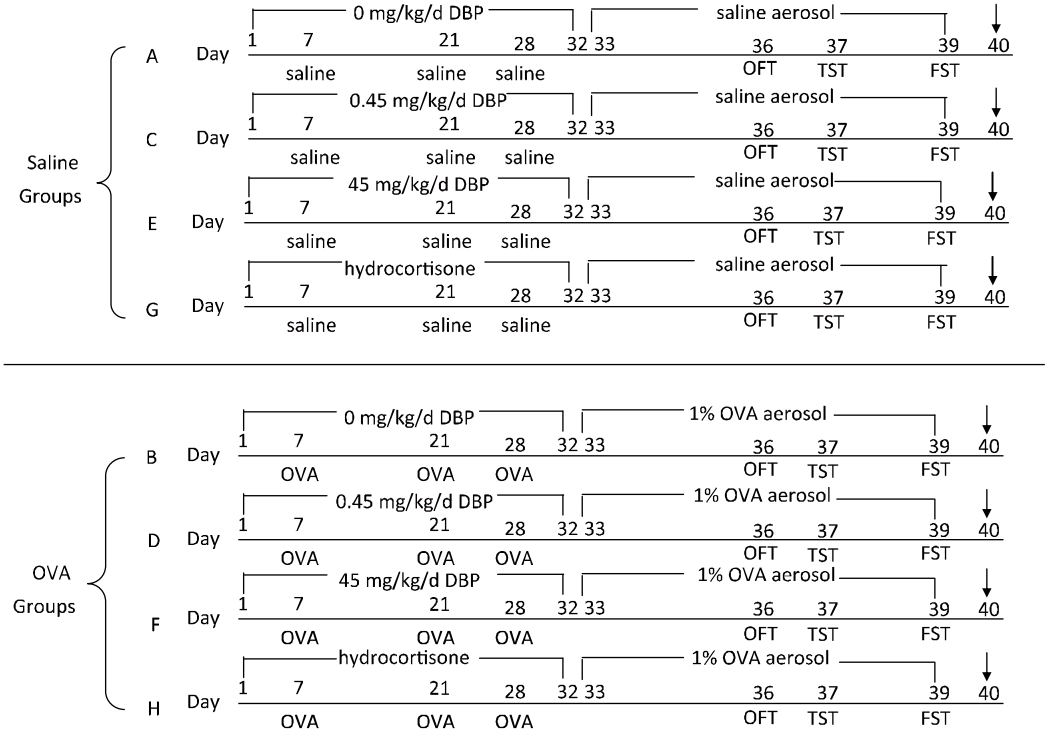

Figure 1. Study protocol. TST: Tail Suspension Test; FST: Forced Swimming Test; OFT: Open Field Test. Groups: (A) Saline-treated + 0 mg DBP/kg exposure; (B) OVA-treated + 0 mg DBP/kg; (C) Saline + 0.45 mg DBP/kg; (D) OVA + 0.45 mg DBP/kg; (E) Saline + 45 mg DBP/kg; (F) OVA + 45 mg DBP/kg; (G) Saline + 30 mg hydrocortisone (HC)/kg exposure; and, (H) OVA + 30 mg HC/kg.

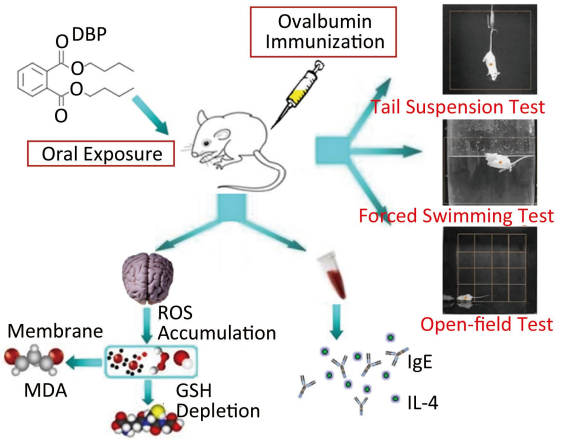

Figure 2. Outline of experiment procedures.

Tail Suspension Test (TST)

On Day 37, mice were subjected to TST in 1 h after the aerosol challenge. The TST was conducted as previously described by Steru et al.[29]. Briefly, mice were individually suspended on the edge of a shelf 58 cm above the floor by adhesive tape, placed about 1 cm from the tip of the tail. Animals were allowed to hang for 6 min and the duration of immobility was recorded during the final 4 min of the test using an Anymaze data collection program (Stoelting, Wood Dale, IL, USA). 'Immobility' was defined as hanging passively and completely motionless.

Forced Swimming Test (FST)

On Day 39 FST was conducted using the method of Porsolt et al.[30], with minor modification. Briefly, mice were individually forced to swim for 6 min (recorded at final 4 min) in a transparent glass cylinder (height: 45 cm, diameter: 20 cm) filled 30 cm high with water (25±0.5 °C). Duration of immobility was defined as time spent floating in water without struggling and making only those movements necessary to keep the head above water.

Serum Sample and Tissue Sample Preparation

Mice were anesthetized intraperitoneally with pentobarbital sodium (100 mg/kg) (Urchen, Shanghai, China). Mice were then euthanized by cervical dislocation and blood was collected and serum was prepared using standard protocols. At necropsy, the brain and spleen of each mouse were removed, trimmed of extraneous tissues, and weighed; the viscera coefficient calculated as 100% x organ weight (g)/body weight (g) was used as an indicator of organ-specific and general toxicity in the mice. Then, each mouse brain was homogenized in ice-cold 0.9% NaCl solution (to produce 1:9 homogenates). The homogenates were then centrifuged at 10 000 rpm for 10 min at 4 °C, and the resultant supernatant was collected and frozen at -70 °C for later analyses.

Reactive Oxygen Species (ROS) Content Assay

Levels of ROS in samples were determined based on the reactions between ROS and by-products of 2', 7'-dichlorofluorescein (DCFH)-DA. After having been transferred into cells, DCFH-DA was cleaved to form DCFH that, in turn, was transformed into highly fluorescent DCF upon reaction with ROS. The DCF was quantified in each sample using a fluorescence monitor (FLx 800 Multi-Detection Microplate Reader, BioTek Instruments, Wisnooski, VT).

In brief, samples were prepared as previously described by Wan et al.[31]. Sample supernatants were harvested and then diluted at 1:100 in 0.9% NaCl (normal saline) solution; 100 µL of this material was then mixed with 100 µL DCFH-DA (10 µmol/L, diluted at 1:1 000 from a stock solution prepared in dimethyl sulfoxide) and placed into the well of a microplate. The reaction mixture was allowed to sit for 10 min in the dark at room temperature (RT) and then analyzed for fluorescence using 485 nm excitation and 520 nm emission settings. The level of ROS was then extrapolated using the relative fluorescence.

Malondialdehyde (MDA) Content Assay

Malondialdehyde (MDA) content in mouse brain homogenates was measured using the method of Draper and Hadley[32]. Briefly, 0.5 mL of each homogenate was mixed with 2 mL of a 0.6% (w/v) thiobarbituric acid (TBA) solution in a glass test tube, placed in a boiling water- bath for 15 min, and then immediately cooled. The mixture was then centrifuged at 10 000 rpm for 10 min and the absorbance of the resultant supernatants was read at 450, 532, and 600 nm in a PowerWave XS Microplate Spectrophotometer (BioTek Instruments). Protein concentration in each brain sample was measured by the method of Lowry[33], using bovine serum albumin as a standard; from this value, the total protein content itself was derived. The level of MDA (C_{MDA}) in each sample was then calculated using the following equation that incorporates the value of the protein content [both total level (mg) and relative

concentration (C_{pro})] in the sample: C_{MDA} (nmol/mgpro)=[6.45x(OD_{532}-OD_{600})-0.56xOD_{450}]/C_{pro}.

Glutathione (GSH) Assay

A total of 200 μL mouse brain homogenate was mixed with 50 μL 10% trichloroacetic acid solution (TCA) to precipitate all proteins present. The sample was then centrifuged at 10 000 rpm (4 °C, 10 min) to produce a clear supernatant. The latter was recovered and then diluted at 1:5 with phosphate-buffered saline (PBS)-EDTA (1 mmol/L) solution, and its pH was adjusted to 7.5 using 1 mol/L NaOH buffer. An aliquot (50 μL) of this sample was then mixed with 150 μL 5, 5'-dithiobis-(2-nitrobenzoic) acid [DTNB (Ellman's Reagent), at 60 μg/mL], transferred into a well in a 96-well plate, and then placed in the dark for 5 min at RT. Thereafter, the absorbance value in each well was measured at 412 nm. The total amount of GSH (μmol/L) in each sample was then estimated using the equation OD_{412}/0.0023, an extrapolation from Beer's Law.

IL-4 and IgE Measures

IL-4 and IgE levels were measured using commercial ELISA kits (IL-4, eBioscience, San Diego, CA, USA; IgE, BioLegend, San Diego, USA) and adhering to manufacturer's instructions. The sensitivities of the kits were 4 and 15 pg/mL, respectively, for IL-4 and IgE.

Statistical Analysis

All data in this paper were expressed as means± SEM. Statistical graphs were generated using Origin 8.0. (OriginLab, Northampton, MA). One-way analysis of variance (ANOVA) and LSD *t*-test were used to determine significance of the differences between groups under SPSS 10.0 (SPSS Inc, Chicago, IL). *P*-values of <0.05 were considered statistically significant.

RESULTS

Body Weight

Table 1 presents absolute body weights (BW, in a 6-wk experimental period) of mice in each group. When compared to those of saline-or OVA-only-treated mice, only weights of mice in the hydrocortisone (HC) groups were significantly (P<0.01) decreased from Week 2 to the end. Figure 3 shows the average final BW of mice in each group at the end of the 6-wk period. All mice, except for those in either HC group, had net increases in BW over this period. With an increase in DBP level, the BW of mice appeared to tend (albeit not significantly) to be lower in both the saline and OVA sets. In contrast, the BW of mice in each HC group was ultimately significantly lower than that of any counterpart in the saline or OVA regimen.

Table 1. Host Body Weights Over Entire Period of Study

Weeks	Weight over the Entire Period of Study (g)													
	Saline control group VS DBP groups							OVA only group VS DBP+ OVA groups						
	Saline	0.45 mg DBP/kg		45 mg DBP/kg		hydrocortisone		OVA	OVA+0.45 mg DBP/kg		OVA+45 mg DBP/kg		hydrocortisone	
	mean	mean	VS saline *P* value	mean	VS saline *P* value	mean	VS saline *P* value	mean	mean	VS OVA *P* value	mean	VS OVA *P* value	mean	VS OVA *P* value
1	25.58	25.32	0.789	24.99	0.447	24.22	0.153	24.99	25.03	0.967	25.51	0.500	24.32	0.403
2	27.42	27.07	0.785	25.86	0.131	23.51	0.001**	26.66	26.56	0.916	26.48	0.832	24.03	0.005$^{\#\#}$
3	28.76	28.19	0.707	26.83	0.105	24.07	0.003**	27.79	27.25	0.610	27.58	0.780	23.95	0.001$^{\#\#}$
4	29.98	28.97	0.550	28.10	0.109	23.93	0.003**	28.86	28.34	0.679	28.51	0.722	23.78	0.002$^{\#\#}$
5	30.42	29.62	0.652	28.35	0.102	24.45	0.000**	29.18	29.54	0.789	29.88	0.471	24.31	0.000$^{\#\#}$
6	30.56	29.88	0.649	28.79	0.096	24.79	0.000**	29.93	30.47	0.676	29.40	0.598	24.95	0.000$^{\#\#}$

Note. $^{**}P$<0.01 *vs.* Group A (Saline-treated + 0 mg DBP/kg exposure); $^{\#\#}P$<0.01 *vs.* Group B (OVA-treated + 0 mg DBP/kg exposure).

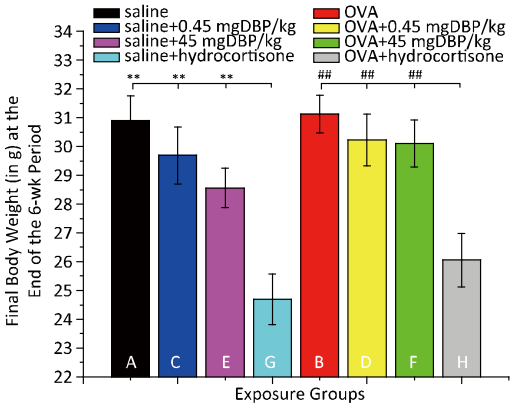

Figure 3. Final body weight (in grams) at the end of 6-wk period. (A) Saline-treated + 0 mg DBP/kg exposure. (B) OVA-treated + 0 mg DBP/kg. (C) Saline + 0.45 mg DBP/kg. (D) OVA + 0.45 mg DBP/kg. (E) Saline + 45 mg DBP/kg. (F) OVA + 45 mg DBP/kg. (G) Saline + 30 mg hydrocortisone (HC)/kg exposure. (H) OVA + 30 mg (HC)/kg. Values were given as $x \pm sx$ (n=7-8). $^{**}P<0.01$ *vs*. saline + 30 mg HC/kg; $^{\#\#}P<0.01$ *vs*. OVA + 30 mg HC/kg.

Behavioral Tests

Figure 4a shows the tail suspension test (TST) results. Time spent immobile was significantly increased (compared to among saline- and OVA-only-treated mice) as a result of DBP exposure in both the saline and OVA groups. When comparing for the contribution from OVA itself, only OVA-treated + 45 mg DBP/kg mice spent significantly longer time immobile than saline-treated + 45 mg DBP/kg counterparts. Further, only mice receiving OVA displayed any dose-related effects from DBP. Lastly, HC significantly affected this parameter more than did the lower dose of DBP (regardless of OVA status) and only compared against mice in the saline-treated + 45 mg DBP/kg group.

Figure 4b illustrates the forced swimming test (FST) results. The time spent immobile in the FST as a result of DBP treatments was dose-related, with significant increases (relative to values seen with OVA-or saline-only-treated mice) only being seen with

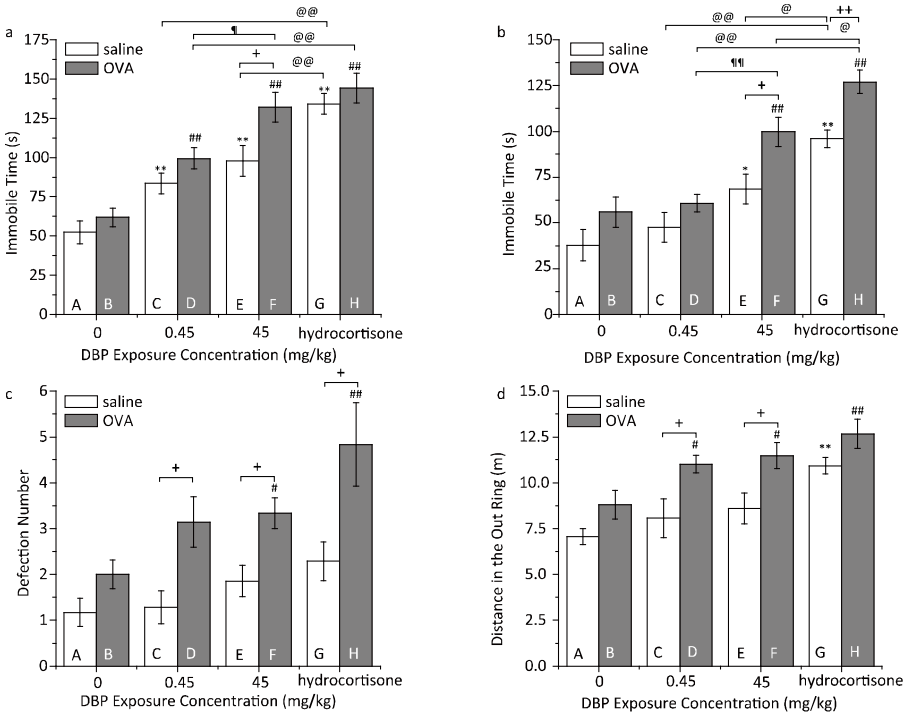

Figure 4. Behavioral analyses. a: Tail Suspension Test (TST); b: Forced Swimming Test (FST); c and d: Open Field Test (OFT); c: defecation number; d: distance in the outer ring. (A) Saline-treated + 0 mg DBP/kg exposure. (B) OVA-treated + 0 mg DBP/kg. (C) Saline + 0.45 mg DBP/kg. (D) OVA + 0.45 mg DBP/kg. (E) Saline + 45 mg DBP/kg. (F) OVA + 45 mg DBP/kg. (G) Saline + 30 mg hydrocortisone (HC)/kg exposure. (H) OVA + 30 mg (HC)/kg. Values were given as $x \pm sx$ (n=6-8). $^{*}P<0.05$, $^{**}P<0.01$ *vs*. Group A; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs*. group B; $^{+}P<0.05$, $^{++}P<0.01$ comparisons against matched counterpart in OVA-treated groups. $^{\P}P < 0.05$, $^{\P\P}P<0.01$ *vs*. 45 mg DBP dose in saline- or OVA-treated groups (can mark C or D *vs*. E and F, respectively). $^{@}P<0.05$, $^{@@}P<0.01$ *vs*. HC-counterpart in saline- or OVA-treated groups (can mark C or E *vs*. G, and D or F *vs*. H).

the higher DBP level. Moreover, in that group, as well as among mice that received HC instead of DBP, the time spent immobile by OVA-treated mice was significantly higher than that by saline counter- parts (45 mg DBP/kg, $P<0.05$; HC, $P<0.01$) at the same exposure concentration. As in the FST, HC significantly affected this parameter more than either DBP dose (regardless of OVA status).

Figure 4c presents mice defecation levels. There was an increase in defecation number in both the saline and OVA groups. The OVA-treated + 45 mg DBP/kg group and the OVA-treated + 30 mg HC/kg group demonstrated a significant increase in defecation number compared with OVA-alone mice. Moreover, defecation number in OVA-treated mice was significantly greater than that in their saline counterparts (0.45 mg DBP/kg, $P<0.05$; 45 mg DBP/kg, $P<0.05$; HC, $P<0.05$) at the same DBP exposure level. However, the defecation number in the saline groups showed a tendency to increase, but this was not significant.

Figure 4d shows the distance mice traveled in the outer ring as a result of DBP exposure. In the OVA-treated group the distance mice traveled was significantly increased (compared to OVA-only-treated group). When considering the contribution from OVA itself, the distance the OVA-treated mice travelled was significantly longer than that their saline counterparts did (0.45 mg DBP/kg, $P<0.05$; 45 mg DBP/kg, $P<0.05$). Moreover, instead of DBP, the use of HC significantly affected this parameter (compared to saline- and OVA-only-treated mice, respectively).

Figure 5 shows mice movement pathways. The movement pathway of the saline controls (Box A) was random in the OFT. As the level of DBP exposure increased, mice tended to 'hide' more often in the outer ring of the field. This pattern was most evident among the mice that received the HC treatment (Boxes G and H). Among the groups that received OVA, the same patterns as seen among the saline-treated groups were evident. However, the effects from the DBP became much more apparent/discernible, especially among the OVA + 45 mg DBP/kg hosts (Box F). Like their HC-treated counterparts, these Box F mice would rarely move towards the central part of the measurement field.

Viscera Coefficients

Figure 6a presents viscera coefficients for the spleens of test mice. In the saline groups, these values tended to increase with increasing DBP dose; saline+45 mg DBP/kg mice showed a significant increase in spleen coefficient compared with values in the saline-only group. However, the opposite trend was noted among groups that had received OVA. The coefficient in the OVA + 45 mg DBP/kg group was extremely lower than that in the OVA-alone group. Among the groups receiving no DBP, or even those that received lower DBP dose, the viscera coefficients were significant greater for OVA-treated as compared to those for saline-treated mice.

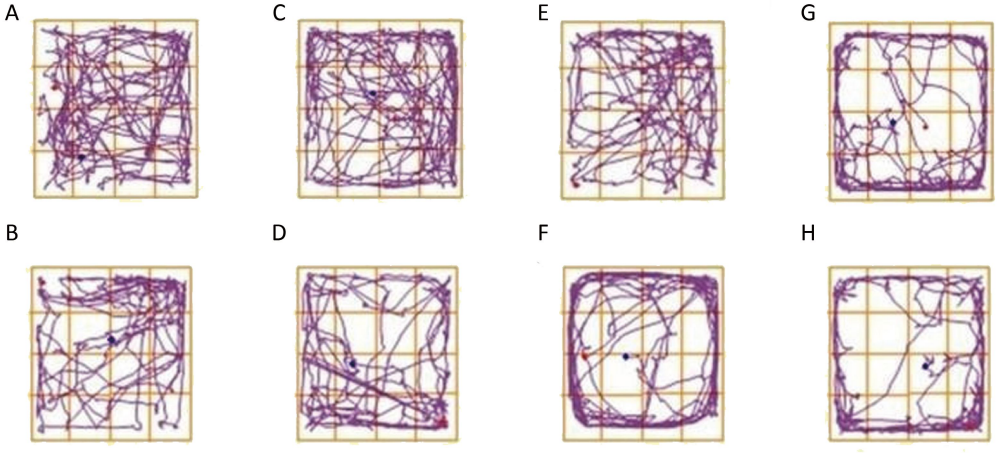

Figure 5. Mice movement pathway in the OFT. (A) Saline-treated + 0 mg DBP/kg exposure. (B) OVA-treated + 0 mg DBP/kg. (C) Saline + 0.45 mg DBP/kg. (D) OVA + 0.45 mg DBP/kg. (E) Saline + 45 mg DBP/kg. (F) OVA + 45 mg DBP/kg. (G) Saline + 30 mg hydrocortisone (HC)/kg exposure. (H) OVA + 30 mg (HC)/kg.

The mice brain examination (Figure 6b) revealed little effect from OVA nor any real impact from the DBP treatments. The brain viscera coefficient showed a decrease trend in the OVA groups compared with the saline counterparts, but no significance.

Oxidative Damage

Figure 7a shows the relative florescence, which reflects the ROS levels in the brains of the treated mice. Among the OVA-treated mice, ROS levels tended to increase with increasing exposure to DBP. In fact, the OVA + 45 mg DBP/kg group demonstrated a significant increase in brain ROS levels compared with those in the OVA-only group. However, this outcome was not significantly different from those groups received the lower DBP dosing. When the OVA contribution to ROS level was assessed, only the OVA + 45 mg DBP/kg group was significantly increased as compared with the saline + 45 mg DBP/kg group. Among all the saline-treated groups, there was a trend to insignificant increase in ROS levels as the DBP concentration exposure increased.

Figure 7b reflects the GSH concentration in brains of test mice. GSH levels were significantly increased as a result of DBP exposure in both the saline and OVA groups. In assessing any OVA role in the outcomes, GSH levels were significantly changed (relative to those in saline + DBP counterparts) at either high or low DBP doses. Moreover, there was a significant increase at the lower DBP dose while a lower level at the higher DBP dose. Only saline-treated mice displayed any concentration-related effects from DBP on GSH values.

Figure 7c presents the MDA content in mice brain data. MDA content was significantly increased in all OVA treatment groups. Further, co-presence of DBP treatment in the OVA groups resulted in significant dose-related increases. Among the saline-treated groups, only the highest DBP concentration resulted in a significant elevation in levels relative to background (i.e. in saline-only mice).

Serum IgE and IL-4 levels

Figure 8a shows test mice serum IgE concentrations. IgE levels in OVA-treated mice were always significantly greater than those in saline counterparts, regardless of DBP status. Among the OVA-treated groups, only the 0.45 mg/kg dose resulted in a significant increase above background (i.e., OVA-only) levels, and IgE level decreased significantly with the increased value of DBP. In contrast, IgE level increased significantly with the increased value of DBP in the saline-treated groups. Similar results were seen for serum IL-4 concentrations (Figure 8b). However, there was a significant decrease at the highest DBP dose in both OVA- and saline-treated groups.

Changes in Indices in the Hydrocortisone Groups

As shown in Figure 9a, ROS levels in group that received 30 mg HC/kg were significantly greater than

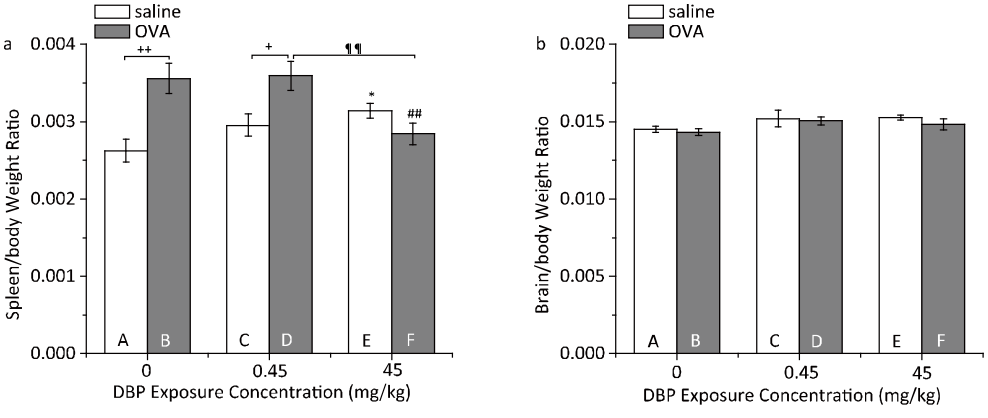

Figure 6. Viscera coefficients. a: Spleen viscera coefficient; b: Brain viscera coefficient. (A) Saline-treated + 0 mg DBP/kg exposure. (B) OVA-treated + 0 mg DBP/kg. (C) Saline + 0.45 mg DBP/kg. (D) OVA + 0.45 mg DBP/kg. (E) Saline + 45 mg DBP/kg. (F) OVA + 45 mg DBP/kg. Values were given as $x \pm sx$ (n=7-8). $^{*}P< 0.05$, $^{**}P<0.01$ *vs*. Group A; $^{\#\#}P<0.01$ *vs*. Group B; $^{+}P<0.05$, $^{++}P<0.01$ comparisons against matched counterpart in OVA-treated groups. $^{¶¶}P<0.01$ *vs*. 45 mg DBP dose in saline- or OVA-treated groups (can mark C or D *vs*. E and F, respectively).

those in groups that received saline-or OVA-treated only. The OVA had little effect on outcomes in groups that either or nor treated with HC. In contrast, while brain GSH levels were significantly increased in the saline + 30 mg HC/kg group compared with that in saline-only hosts, the OVA treatment appeared to have abrogated that effect (Figure 9b). In the case of MDA content (Figure 9c), in mice that received saline and 30 mg HC/kg, there was again a significant increase compared to values seen in mice that received only saline, while mice that received OVA had no impact from HC. However, unlike with the ROS and GSH endpoints, regardless of HC status, the presence of OVA resulted in significantly higher levels of MDA in the brains of mice.

Serum IgE and IL-4 concentrations in mice that received OVA-treated + 30 mg HC/kg were each significantly reduced compared with those in hosts that received OVA only (Figures 9d and 9e). HC had no impact on either parameter among the saline-treated hosts. Interestingly, the presence of OVA resulted in disparate effects on serum IgE and IL-4 levels once HC was introduced into the picture. Regardless of HC status, IgE levels were always significantly greater among OVA-treated mice; on the other hand, while IL-4 levels were greater in OVA-alone *vs*. saline-alone mice, the HC treatment now resulted in a significantly reduced level of cytokine.

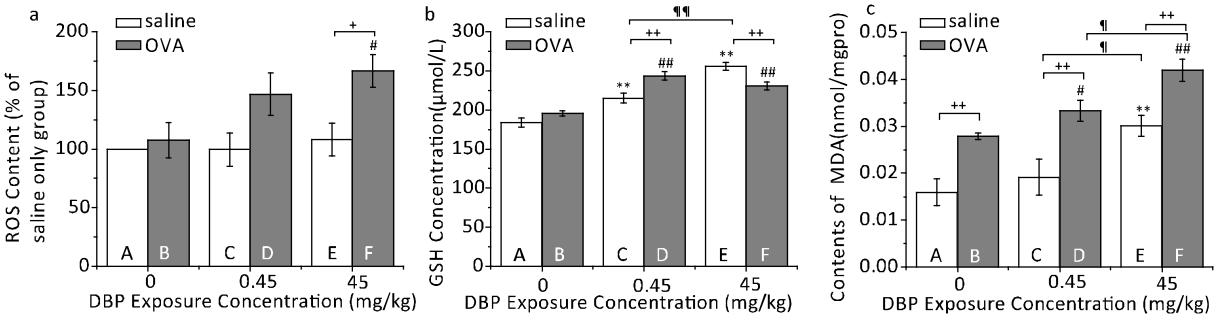

Figure 7. Indices of oxidative damage. a: ROS; b: GSH concentration; c: MDA content. (A) Saline-treated + 0 mg DBP/kg exposure. (B) OVA-treated + 0 mg DBP/kg. (C) Saline + 0.45 mg DBP/kg. (D) OVA + 0.45 mg DBP/kg. (E) Saline + 45 mg DBP/kg. (F) OVA + 45 mg DBP/kg. Values were given as $x \pm sx$ (n=6-8). $^{**}P<0.01$ *vs*. Group A; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs*. Group B; $^{+}P<0.05$, $^{++}P<0.01$ as compared with OVA-treated groups. $^{\P}P<0.05$, $^{\P\P}P<0.01$ *vs*. 45 mg DBP dose in saline- or OVA-treated groups (can mark C or D *vs*. E and F, respectively).

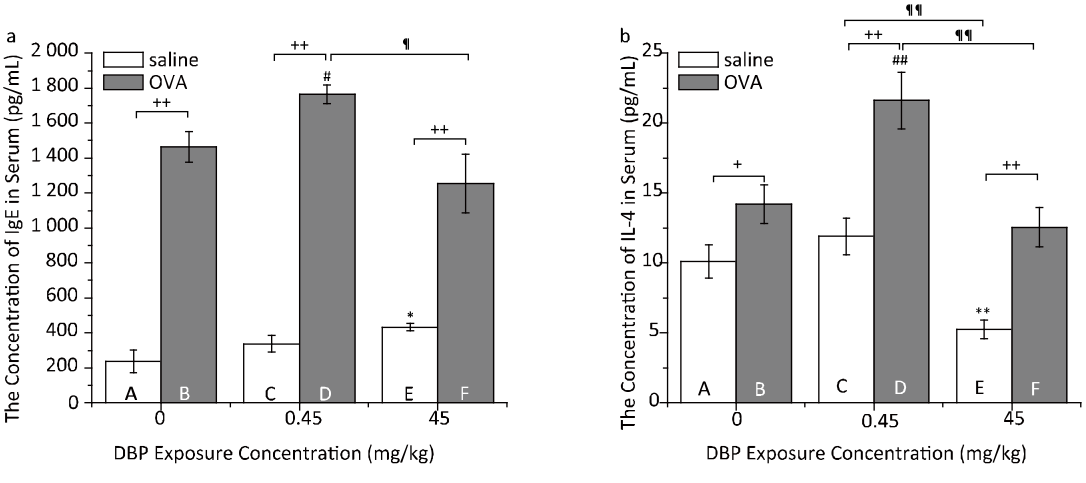

Figure 8. Serum IgE and IL-4 levels. a: IgE; b: IL-4. (A) Saline-treated + 0 mg DBP/kg exposure. (B) OVA-treated + 0 mg DBP/kg. (C) Saline + 0.45 mg DBP/kg. (D) OVA + 0.45 mg DBP/kg. (E) Saline + 45 mg DBP/kg. (F) OVA + 45 mg DBP/kg. Values were given as $x \pm sx$ (n=6-8). $^{*}P<0.05$, $^{**}P<0.01$ *vs*. Group A; $^{\#}P<0.05$ *vs*. Group B; $^{+}P<0.05$, $^{++}P<0.01$ as compared with OVA-treated groups. $^{\P}P<0.05$, $^{\P\P}P<0.01$ *vs*. 45 mg DBP dose in saline- or OVA-treated groups (can mark C or D *vs*. E and F, respectively).

Correlation Analyses

Relationships between extent of oxidative damage and behavioral changes, as well as between any changes in the immunologic and behavioral endpoints assessed here were analyzed. The results indicated associations between behavioral changes and oxidative damage in the brains of the mice (TST and ROS: r=0.390, P<0.05; TST and MDA: r=0.610, P<0.01; FST and MDA: r=0.504, P<0.01).

DISCUSSION

Atopic allergy is a disease with a shift in the balance of T-helper (T_H)-1/T_H2 cells toward a T_H2 cell-dominance. Even now, the number of patients diagnosed as atopic allergy is continually increasing[34-37] and there is likely to be an environmental (i.e., pollution-related) component for this paradigm. Similarly, although depression is traditionally regarded as a psychosocial disorder, some scientists now consider that environmental pollution may play a role in the onset of depression and other neurologic disorders[38]. Therefore, using a BALB/c mice model this study aimed to test a hypothesis that exposure to certain pollutants (DBP specifically) could cause changes in immunomodified (i.e. atopic) mice, and was associated with depression-related endpoints.

The TST, FST, and OFT endpoints are common important tests for assessing depression-related activity in mice[30,39]. In this study, we found that in the saline and OVA (atopic) treated mice, the time spent immobile in the TST and FST increased with the increasing DBP exposure concentrations. Moreover, OVA treated mice kept immobile longer than the saline counterparts at the same DBP exposure levels. To the OFT, depressive symptoms were more obvious in the high DBP exposure dose groups compared with control mice without DBP treatment. The distance traveled in the outer ring by mice in the OVA + 0.45 mg DBP/kg group and OVA + 45 mg DBP/kg group was significantly increased as compared with the OVA-only group. A similar trend was also observed in defecation number and movement pathways. All these findings suggest that allergic mice may be at a greater risk of developing depressive symptoms as compared with non-allergic mice when DBP is present. The behavioral test results

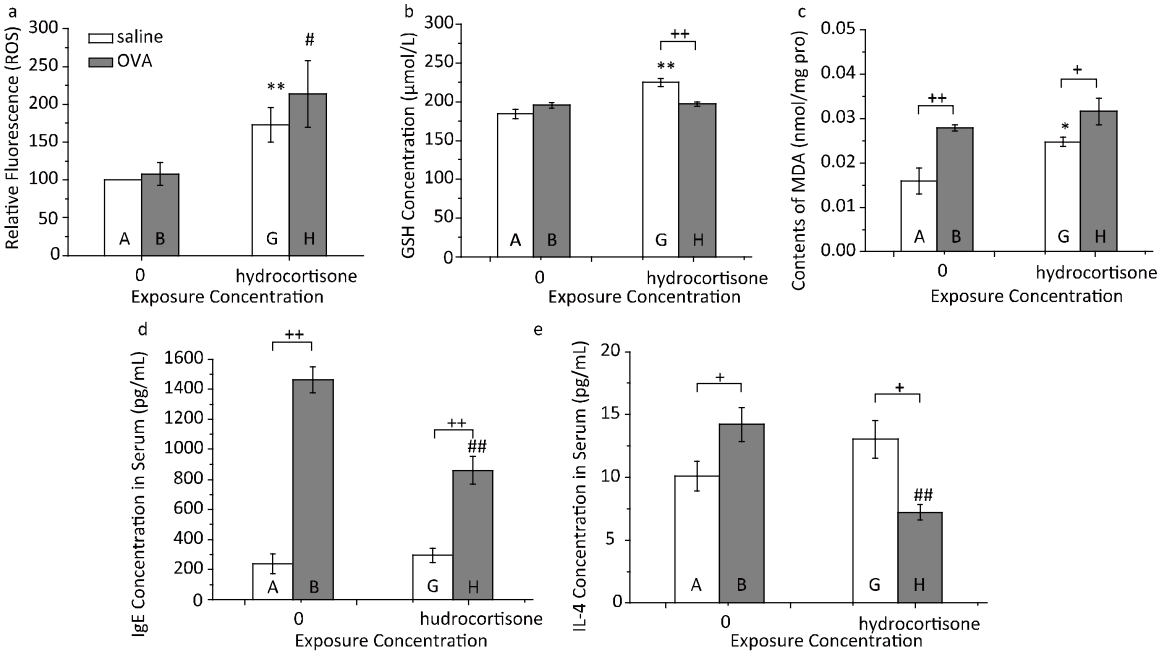

Figure 9. Biochemical indices in hydrocortisone groups. a: ROS; b: GSH; c: MDA; d: serum IgE; e: serum IL-4. (A) Saline-treated + 0 mg DBP/kg exposure. (B) OVA-treated + 0 mg DBP/kg. (G) Saline + 30 mg hydrocortisone (HC)/kg exposure. (H) OVA + 30 mg (HC)/kg. Values were given as $x\pm sx$ (n=6-8). $^{*}P$<0.05, $^{**}P$<0.01 *vs*. Group A; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs*. Group B; ^{+}P<0.05, ^{++}P<0.01 comparisons against matched counterpart in OVA-treated groups.

are consistent with previous epidemiological studies[3-6,40].

In this study, we found a difference in all tests between the OVA-immunized groups as compared with the saline groups. Further, DBP was capable of enhancing this difference and its capability was reflected in the result of behavioral tests (OFT, TST, FST) and assessments of oxidative damage in the brains of the test mice. Previous studies have shown that many low molecular weight agents have adjuvant effects in OVA-induced hyper-responsiveness and inflammation[15,41-42]. Thus, DBP might also have an undefined role in a linkage of allergy and depression-like behavior in mice.

Oxidative stress is the result of an imbalance in the production of anti-oxidants and free radicals[43]. Recently, data have indicated that oxidative stress is involved in the pathology of psychiatric and neurological diseases[44]. Specifically, brain oxidative damage is closely connected with depression[22,45]. Studied have demonstrated that ROS plays a role in the pathophysiology of depression[46-47]. In the present study, the levels of ROS and MDA were associated with DPB exposure dose. Furthermore, oxidative damage in the OVA groups was worse than that in the saline groups at the same DBP exposure concentration. Similar results have been reported in other studies[21,47]. GSH is an important anti-oxidant, which protects cells from ROS and plays a vital role in intracellular ROS removal. Levels of GSH decrease as ROS and MDA levels increase normally. However, in our study GSH level showed a slight rise before decrease. When mice were treated intragastrically with 0.45 mg DBP/kg, brain GSH concentration increased as compared with those treated with 0 mg DBP/kg. The results suggest that the hormesis phenomenon, in which small amounts of free radicals stimulate the organism and induce an increase in antioxidants instead of a decrease, may have played a role in this study[48]. However, after 45 mg DBP/kg administration, a shortage in GSH production became evident because the balance between anti-oxidants and radicals was shifted by an increase in ROS that, in turn, damaged organs in these mice.

Apart from oxidative damage to the brain, certain immune mediators such as cytokines could be additional factors involved in linking allergy and depression[18,20,49-50]. Cytokines have immune modulating effects; most of the physical/ psychological symptoms associated with infection and inflammation can be modulated by cytokines[19]. Serotonin is an important neurotransmitter in the central nervous system, and is closely associated with depression. As noted by Mössner et al.[20], serotonin uptake in B-lymphoblast lines can be affected by IL-4. In our study, IL-4 serum concentration in the OVA groups was significantly higher than that in the saline groups even at the same DBP exposure levels. However, interestingly, there was an increase at 0.45 mg DBP/kg exposure and a decrease at 45 mg DBP/kg exposure in the saline and OVA groups as compared with the values seen for the non-DBP controls (see Figure 8b). Besides, in the 0.45 mg DBP/kg treated groups, it seemed that the relevance of the behavioral analysis and the levels of IgE and IL-4 did exist. However, in the 45 mg DBP/kg treated groups, the spleen was seen to undergo a serious atrophy (compared with the control group), which may be the reason for the deviation of IgE and IL-4 generation and behavioral analyses (see Figure 6a). However, the decrease of IgE and IL-4 had nothing to do with alleviating symptoms of depression. In contrast, our data implicated that the mice depression symptoms was more serious with the increase of DBP exposure concentration. Therefore, we supposed that 45 mg/kg·day might be beyond the relevant regions. In the 0 mg DBP/kg·d exposure groups, the level of IgE and IL-4 in the OVA-treated group increased significantly when compared with saline-treated group. This means the OVA-induced allergy mice model was successful. On the other hand, in the behavioral tests, the degree of depression of the mice in the OVA only-treated group rose slightly compared to the mice in the saline only-treated group, but the difference was not significant. It seems that the association between allergy and depression was not obvious without DBP exposure. This association could be proved in the 0.45 mg DBP/ kg·d and the 45 mg DBP/ kg·d exposure groups. Time spent immobile was significantly increased (compared to the saline-and OVA-only-treated mice) as a result of DBP exposure in saline and OVA groups, especially in the OVA-treated + 45 mg DBP/ kg·d group. All the behavioral test results showed that OVA-treated + 45 mg DBP/kg mice had more serious depression-like behavior than saline-treated + 45 mg DBP/kg counterparts, which means DBP might enhance the connection between OVA-induced allergy and depression.

In the present study, the authors tried to verify an epidemiological hypothesis-using a mouse model-that an environmental pollutant might be

able to strengthen the connection between inflammation and depression. Due to the limited number of mice approved for this study, only a 0.45 and 45 mg DBP/kg dose regimen could be applied as test concentrations. It would be better if a broader range of doses could be used to test, nevertheless, important information was still gleaned from this study. However, a broader range of DBP exposure doses would be desirably used to further verify our hypothesis.

In conclusion, this study has shown that OVA-immunized mice may be at higher risk of developing depressive symptoms. Moreover, when DBP was also present, the degree of depression was higher than that in non-DBP groups, especially among the atopic mice, which was partly proved by the fact that when DBP and OVA were present at the same time, the oxidative stress was higher than either with DBP or OVA alone. In fact, the pathogenesis for depression is so complicated that no conclusion can be drawn so far. Moreover, the results of our study implicate a potential connection between brain oxidative stress and depression. Therefore, further studies on the role of DBP and the mechanisms linking allergy and depression are required.

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CONTRIBUTION ANNOUNCEMENT

ZUO Hao Xiao: Read related references; design the experiment protocol; contact with the laboratory equipments; perform the mice behavioral tests; prepare serum sample and tissue sample; use Origin 8.0 to analyze data; and write the paper.

LI Jin Quan: Design the experiment protocol; look after the animals; give mice DBP (or hydrocortisone) intra-gastrically everyday; watch the mice behavioral video; prepare serum sample and tissue sample; and use SPSS to analyze data.

HAN Bing: Read related references; design the experiment protocol; use Origin 8.0 to analyze data; give a lot of useful suggestions; and write the paper.

KE Chen Juan: Test the mice brain contents of ROS, MDA and GSH; prepare serum sample tissue sample and the whole experiment.

LIU Xu Dong: Do the behavioral tests; prepare serum sample and tissue sample.

ZHANG Yu Chao: Do the behavioral tests; prepare serum sample and tissue sample.

LI Li: Prepare serum sample and tissue sample.

YANG Xu: Design the experiment protocol; analyze data; and write the paper.

Note. The submission has been approved by all of the authors.

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