

Impairment of Di(2-Ethylhexyl) Phthalate on Cellular Immunity in Kunming Mice

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Abstract

Immunity is crucial to the health of animals and it can determine their survival and fitness. Di(2-ethylhexyl) phthalate (DEHP) is widely used as a plasticizer and hence is the most abundant phthalate in the environment. Exposure to DEHP is of great concern for human health. In the present study, we tested the hypothesis that exposure to DEHP would suppress T cell-mediated immunity in mice. Twenty adult male Kunming mice were randomly assigned into the control (n = 10) and the DEHP treatment (n = 10) groups. Both groups have free access to food and water, while the mice in the latter group drank DEHP solution (2000 mg/L) for 42 days. T cell-mediated immunity assessed by phytohaemagglutinin (PHA) response was depressed in the DEHP treated mice compared with the controls, however, wet thymus and spleen mass, white blood cells were not influenced by DEHP treatment. Taken together, different immunological parameters responded differently to DEHP treatment in Kunming mice.

Keywords

Di(2-Ethylhexyl) Phthalate (DEHP), Kunming Mice, Phytohaemagglutinin Response

1. Introduction

The immune system protects animals against environmental pathogens, which is crucial for their health and is important to determine their survival and fitness [1] [2]. However, immune function is influenced by many factors including endocrine disruptors such as di(2-ethylhexyl) phthalate (DEHP) [3]-[6].

DEHP, produced at annual quantities of 2 million tons and widely used in medical devices and plastics, is one

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of the principal phthalates causing human health concerns [7]-[9]. DEHP has endocrine-disrupting property [10]-[12]. Many researchers have focused on the reproductive toxicology of DEHP in rodents [25; 26] and human [13] [14]. The ability of DEHP to impact on immune and allergic responses has been examined [5] [6] [15]. Moreover, some investigators have found that DEHP treatment could inhibit B cell proliferation and reduces the abundance of IgM-secreting cells in cultured immune tissues [16]. However, whether DEHP treatment could suppress cellular immunity still remains unclear.

Phytohaemagglutinin (PHA) response can be used to evaluate mammalian cellular immunity, which is one arm of adaptive immune system and generally responsible for intracellular pathogen control [17] [18]. Immune organs including thymus and spleen are also indicative of immune function [19]-[21]. Thymus is essential for primary T cell development [19], and a larger spleen is representative of stronger immunity [21]. Total white blood cells (or leukocytes, WBC), which are crucial for immune responses against pathogens, are useful to assess the overall health [20].

In the present study, we tested the hypothesis that DEHP would have great influences on immune function in Kunming mice. We expected that immunological parameters including cellular immunity, thymus and spleen mass and white blood cells would be suppressed in DEHP treated mice compared with the controls.

2. Materials and Methods

2.1. Animals and Experimental Design

All animal procedures were licensed under the Institutional Animal Care and Use Committee of Qufu Normal University. Adult male Kunming mice (age: 6 months) used in this study were obtained from the Experiment Animal Center in Jining Medical College of Shandong province. The experiment was carried out from September 6 to October 25 in 2012. The animals were housed individually in plastic cages (30 cm × 15 cm × 20 cm) with sawdust as bedding. The raising conditions are semi-natural in which maximum and minimum ambient temperature and humidity were described in **Figure A** and **Figure B**, and the photoperiod was natural light. Standard rat pellets chow (Animal Breeding Center in Jining Medical College, Jining, China) and water were provided *ad libitum*. After body mass stabilized, 20 mice were randomly divided into the control group (n = 10)

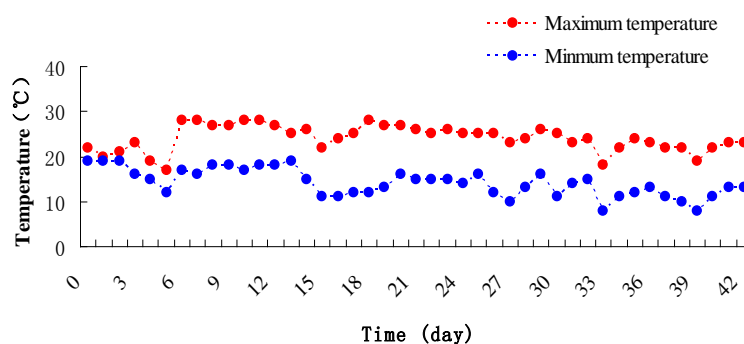


Figure A. The changes of maximum and minimum temperature during the course of the experiment.

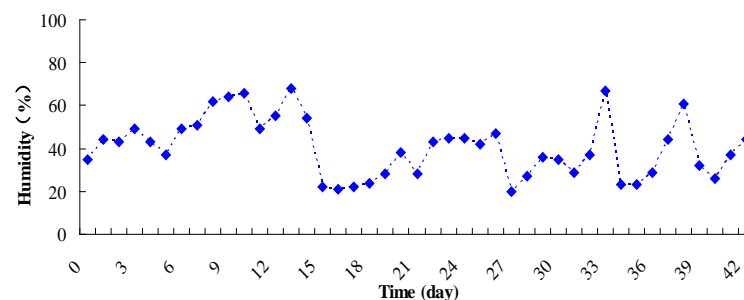


Figure B. The changes of humidity during the course of the experiment.

and the DEHP treated group ($n = 10$) in which each mouse drank DEHP solution (2000 mg/L). The period of the experiment was 42 days. Day 0 and day n represented initial day and n days of treatment, respectively. 4 mice in the DEHP group died on day 7, 20, 34, 40 respectively, and 2 mice in the control group died on day 15, 35 respectively. The data of the six mice were not included in the subsequent statistical analysis.

2.2. Cellular Immunity Assays

PHA response was measured as described previously [18] [22]. Specifically, mice in the control and DEHP groups on day 39 were caught, then we measured their footpad thickness of the left hind foot with a micrometer (Digimatic Indicator ID-C Mitutoyo Absolute cod. 547-301, Japan) to ± 0.01 mm. Immediately thereafter, mice in both groups were injected subcutaneously 0.1 mg of PHA (PHA-P, Sigma L-8754) dissolved in 0.03 mL of sterile saline (pH 7.4) in the middle of the footpad. After 6 h, 24 h, 48 h and 72 h injection, we measured footpad thickness. The PHA response (*i.e.*, cellular immunity) was calculated as the difference between pre- and post-injection measurements divided by initial footpad thickness (PHA response = (post PHA – pre PHA)/pre PHA). Six measures of footpad thickness were taken to obtain the value of each mouse [22].

2.3. Organs

Organs were measured as described previously [22]. In brief, the visceral organs, including heart, thymus, lungs, liver, spleen, kidneys, adrenal glands, testes, epididymis, seminal vesicals and the digestive organs with contents (*i.e.*, stomach, small intestine, caecum and colon) were dissected and weighed (± 1 mg). The stomach, small intestine, caecum and colon were rinsed with saline to eliminate all the gut contents, before being weighed.

2.4. White Blood Cells Assays

At the end of the experiment, after collecting trunk blood, 20 μ L whole blood was diluted immediately in 0.38 mL solution containing 1.5% glacial acetic acid, 1% crystal violet (Sigma) and the leukocytes were counted in an improved Neubauer chamber using microscope. The total number of WBC was determined by counting all leucocytes in the four corner large-squares of the Neubauer chamber, and multiplying the raw data by 5×10^7 to obtain the final values (10^9 cells/L) [23].

2.5. Statistical Analysis

Data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Prior to all statistical analyses, data were examined for normality and homogeneity of variance, using Kolmogorov-Smirnov and Levene tests, respectively. The ratio values such as PHA response were subjected to arcsine transformation. The differences of body mass between the control and DEHP treated groups were analyzed by independent-samples t-test. Group differences in wet organ mass with body mass as the covariate were analyzed by General Linear Model multivariate analysis followed by Bonferroni *post hoc* tests. Group differences in other parameters (PHA response, WBC) were analyzed by independent-samples t-test. Results were expressed as mean \pm SE, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Body Mass

On day 0, body mass between the control and DEHP groups was not different ($t = -0.666$, $df = 12$, $P = 0.518$). There was no difference of body mass between these two groups from day 1 ($t = -0.437$, $df = 12$, $P = 0.670$) to day 42 ($t = -0.064$, $df = 12$, $P = 0.950$) (Figure 1).

3.2. Organs

DEHP treatment decreased the masses of stomach with contents and seminal vesical while increased the mass of colon (Table 1). The masses of thymus, spleen and other organs were all not influenced by DEHP treatment (Table 1).

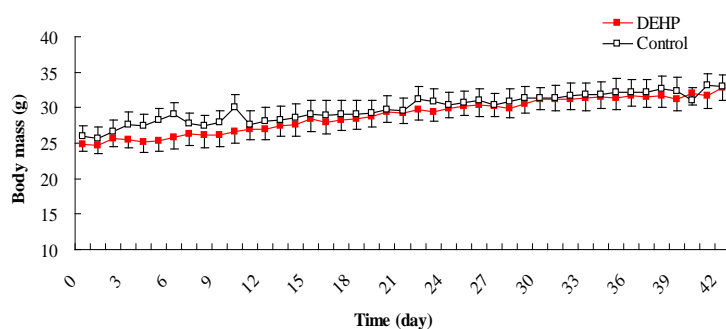


Figure 1. Changes of body mass in mice during DEHP treatment. Values are means \pm SE. Body mass on day 0 between the control and DEHP treatment groups did not differ significantly.

Table 1. Effect of DEHP treatment on wet organ mass in Kunming mice.

Parameters	Control group	DEHP group	Statistical summary	
			$F_{1,10}$	P
Sample size	8	6		
Heart (mg)	153 \pm 6	163 \pm 8	0.826	0.385
Lungs (mg)	334 \pm 63	378 \pm 80	0.185	0.676
Thymus (mg)	43 \pm 10	62 \pm 12	1.678	0.224
Liver (mg)	1912 \pm 91	1829 \pm 115	0.322	0.583
Spleen (mg)	160 \pm 14	125 \pm 18	2.461	0.148
Kidneys (mg)	502 \pm 20	493 \pm 25	0.090	0.771
Adrenal glands (mg)	9 \pm 2	17 \pm 3	4.563	0.058
Stomach with contents (mg)	845 \pm 63 ^a	580 \pm 79 ^b	6.838	0.026
Stomach (mg)	206 \pm 14	233 \pm 17	1.403	0.264
Small intestine with contents (mg)	2608 \pm 261	2419 \pm 330	0.202	0.662
Small intestine (mg)	1170 \pm 109	1295 \pm 138	0.504	0.494
Small intestine length (cm)	69.3 \pm 1.4	64.8 \pm 1.8	3.927	0.076
Caecum with contents (mg)	747 \pm 73	754 \pm 92	0.004	0.953
Caecum (mg)	198 \pm 24	199 \pm 30	0.000	0.989
Caecum length (cm)	3.5 \pm 0.2	3.7 \pm 0.2	0.316	0.586
Colon with contents (mg)	593 \pm 43	585 \pm 54	0.012	0.914
Colon (mg)	271 \pm 11 ^a	316 \pm 14 ^b	6.347	0.030
Colon length (cm)	8.8 \pm 0.5	9.2 \pm 0.6	0.184	0.677
Total digestive tract (mg)	1846 \pm 144	2043 \pm 183	0.717	0.417
Total digestive tract length (cm)	82 \pm 2	78 \pm 2	1.790	0.211
Epididymis (mg)	50 \pm 5	40 \pm 6	1.884	0.200
Testes (mg)	266 \pm 13	248 \pm 16	0.808	0.390
Seminal vesical (mg)	173 \pm 12 ^a	128 \pm 15 ^b	5.602	0.039

Values are means \pm SE. Values for a specific parameter that share different superscripts are significantly different at $P < 0.05$, determined by General Linear Model multivariate analysis followed by Bonferroni *post hoc* tests with body mass as the covariate.

3.3. White Blood Cells

WBC did not differ between the control and DEHP treated mice ($t = 0.342$, $df = 12$, $P = 0.738$) (Figure 2).

3.4. Cellular Immune Response

Compared with the control mice, PHA response in the DEHP treated mice was suppressed significantly after 24 h ($t = -2.822$, $df = 12$, $P = 0.015$), 48 h ($t = -2.859$, $df = 12$, $P = 0.014$) and 72 h ($t = -4.126$, $df = 12$, $P = 0.001$) of PHA injection (Figure 3). Impact of DEHP treatment on PHA response after 6h of postimmunization in DEHP treated mice was near to the significant level in contrast with the control mice ($t = -2.152$, $df = 12$, $P = 0.052$) (Figure 3).

4. Discussion

As expected, cellular immunity was depressed in the DEHP treated mice compared with the control mice. However other immunological parameters such as thymus and spleen mass, white blood cells were not affected by DEHP treatment.

Influences of DEHP on immune responses were carried out in rodents both *in vitro* and *in vivo*. For example, Koike *et al.* (2009) investigated the effects of DEHP on immune cells from atopic prone NC/Nga mice *in vitro*, and found that DEHP enhances bone marrow-derived dendritic cells differentiation and also enhances Th2 response in splenocytes [5]. However, DEHP treatment could inhibit B cell proliferation and reduces the abundance of IgM-secreting cells in cultured immune tissues [16]. Tonk *et al.* (2012) have found the immune system in juvenile and adult wistar rats had different sensitivity to DEHP exposure, in which more immune parameters were affected in juvenile rats compared to adult rats [6]. In our study, cellular immunity was depressed in Kunming mice after DEHP exposure. The disparate results with other researches might be due to the differences in species used, the treatment method and the immune parameters measured [5] [6] [15] [16].

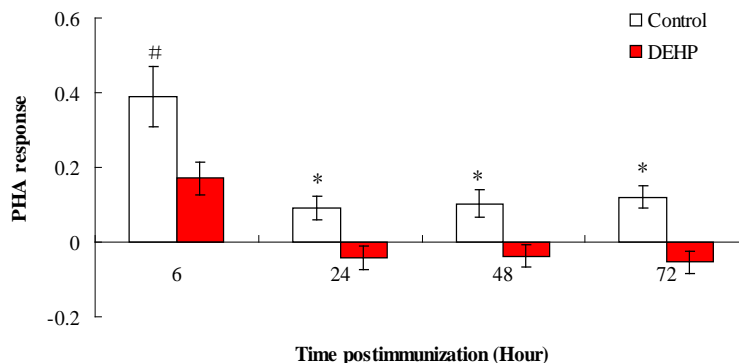


Figure 2. Effect of DEHP treatment on PHA response in mice. Values are means ± SE. PHA response was significantly higher in the control group than in the DEHP treated group. The white column represents the control group and solid column stands for the DEHP treated group. An asterisk (*) indicates statistical differences at $P < 0.05$. A pound (#) indicates nearly approaching the significant level.

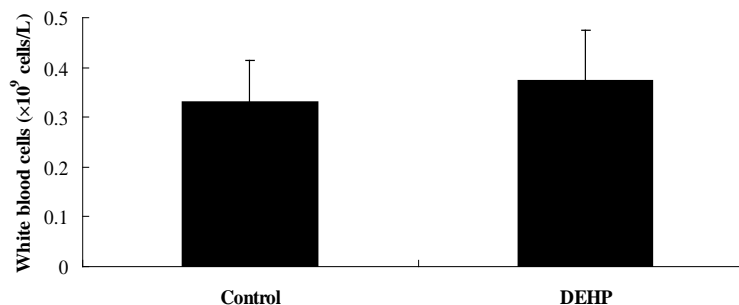


Figure 3. Effect of DEHP treatment on white blood cells in mice. Values are means ± SE. WBC did not differ between the control and the DEHP treated groups.

The major response to stress is the increase of stress hormones [24]. We found that adrenal gland increased nearly significantly after 42 days of DEHP treatment, which might imply the activation of the hypothalamic-pituitary-adrenal (HPA) axis. DEHP treatment might be a stressor to mice, although we did not detect the concentration of stress hormones such as corticosterone. Generally, corticosterone had suppressive effect on immune function. Therefore, the impairment of cellular immunity might be due to the increase of adrenal gland. Future work is required to examine the levels of stress hormones and their relationships with immune function.

In the present study, DEHP treatment decreased seminal vesical mass, which indicated its suppressive effect on reproductive function. This result was consistent with other researches [25] [26]. However, DEHP treatment had no effect on body mass and most organ masses in mice.

5. Conclusion

In conclusion, different immunological indices have different sensitivity to DEHP. DEHP had significant suppressive effect cellular immune response, whereas it did not affect thymus and spleen and white blood cells.

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