



# Combined effects of obesity and di-(2-ethylhexyl) phthalate on testosterone levels and kisspeptin/GPR54 expression in hypothalamus and testes of male mice

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## Abstract

**Background:** This study evaluated whether obese male mice exposed to di-(2-ethylhexyl) phthalate (DEHP) showed synergistic effects on testosterone levels and the potential underlying mechanism.

**Methods:** Forty-eight male mice were assigned to six groups for 12-week treatments as follows: normal, DEHP100, diet-induced obesity (DIO), DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300. Serum hormone levels, including testosterone (T), luteinizing hormone (LH), and leptin, were detected by ELISA. The levels of Ob-R, kisspeptin, and GPR54 protein expression in hypothalamus and testicular tissues were measured by western blot.

**Results:** There were significantly lower levels of serum T and LH, higher levels of serum leptin and Ob-R, and kisspeptin and GPR54 protein expression were reduced in hypothalamus and testicular tissues in the DIO and DEHP groups compared with controls. Moreover, serum T and leptin levels were more severe in the combined DIO and DEHP exposure group than in the single exposure groups. Serum LH levels and GPR54 expression in the testis were significantly decreased in DIO + DEHP300 mice compared with DIO mice ( $p < 0.05$ ).

**Conclusion:** Obesity- and DEHP-only exposure had adverse effects on testosterone levels in mice, which may be due to high leptin levels and decreased Ob-R, kisspeptin, and GPR54 expression. Obesity combined with DEHP exposure had an additive adverse effect on testosterone levels in mice. One of the potential mechanisms is higher leptin levels and decreased GPR54 expression in the testes.

**Keywords:** Diethylhexyl phthalate; Enzyme-linked immunosorbent assay; Obesity

## 1. INTRODUCTION

The prevalence of obesity has become a worldwide health burden.<sup>1</sup> In 2016, the World Health Organization reported that 39% of adults were overweight, among which 13% were obese.<sup>2</sup> Thus, obesity may affect >650 million adults.<sup>2</sup> Obesity is a risk factor for many diseases, including cardiovascular disease, sleep apnea, osteoarthritis, and some cancers.<sup>3-8</sup>

Recently, it has been determined that there is an association between obesity and male reproduction based on epidemiological surveys and animal experiments.<sup>1,9-11</sup> Endocrine-disrupting chemicals (EDCs) are exogenous substances that have recently been the focus of several studies because of their ability to change

endocrine function. Di-(2-ethylhexyl) phthalate (DEHP) is a common EDC that can damage male reproductive organs and function.<sup>12</sup> In China, DEHP is ubiquitous because it is found in many daily products, such as food containers and plastic bottles and bags.<sup>13</sup> Therefore, obese people are also exposed to DEHP. Interestingly, there is a similar mechanism underlying obesity and DEHP on male reproductive dysfunction, as evidenced by endocrine changes *in vivo*.<sup>14-16</sup> However, whether there is a synergistic effect of obesity combined with DEHP exposure on reproductive dysfunction has not been tested *in vivo*.

In our previous study, we found that there is enhanced damage on male reproduction from obesity and DEHP exposure.<sup>17</sup> The mechanistic analysis suggested that the key reason was reduced testosterone levels. We also found that reduced testosterone levels may be due to oxidative stress or high leptin levels. However, the exact molecular mechanism was not determined. In this study, we first tried to ascertain whether the possible mechanism might involve high leptin levels leading to decreased testosterone levels in obese mice with DEHP exposure.

Testosterone regulates male reproductive development and function.<sup>18</sup> Testosterone levels are regulated by the hypothalamic-pituitary-gonadal (HPG) axis, in which gonadotropin-releasing hormone (GnRH) plays an important role in controlling testosterone levels.<sup>19</sup> First, GnRH secretion increases in the hypothalamus. Second, secretion of luteinizing hormone

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(LH) and follicle-stimulating hormone (FSH) increases in the pituitary gland. Finally, testosterone levels are regulated when LH and FSH combine with their respective receptors in testicular tissues. GnRH secretion is influenced by a number of biomolecules, among which leptin is the key factor.<sup>20,21</sup> Guzmán et al<sup>22</sup> found that leptin stimulates GnRH secretion and then promotes reproductive function. Thus, GnRH may play a key role in how leptin influences testosterone levels.

It has been reported that the leptin receptor OB-R, which belongs to the I cytokine receptor family but shows biological effects when bound to leptin, is not found on all GnRH neurons; thus, leptin may regulate GnRH indirectly.<sup>23–26</sup> We found that kisspeptin and the kisspeptin receptor GPR54 may mediate signaling between leptin and GnRH.<sup>17,23–26</sup> The kisspeptin/GPR54 axis has been reported to regulate puberty and reproductive function.<sup>27</sup> In our previous study, high leptin levels were found in obese DEHP-exposed mice.<sup>17</sup> However, the questions of whether DEHP dose affects kisspeptin/GPR54 expression and if the kisspeptin/GPR54 axis plays a key role in controlling GnRH secretion and testosterone levels in obese DEHP-exposed mice remain unanswered. In recent studies, leptin, kisspeptin/GPR54, and GnRH were detected in testis.<sup>28,29</sup> In addition, our unpublished results have demonstrated that testosterone levels are regulated by kisspeptin/GPR54 as well as GnRH in the testes of obese mice. Studies have not reported whether there are changes in leptin, kisspeptin/GPR54, or GnRH in obese DEHP-exposed mice. We hypothesized that leptin, kisspeptin/GPR54, and GnRH may play an abnormal indirect role at the hypothalamus level and/or a direct role at the testes level to regulate testosterone levels in obese DEHP-exposed mice.

Therefore, in this study, we explored the possible mechanisms of high leptin levels and decreased testosterone levels by detecting changes in OB-R, kisspeptin/GPR54, and GnRH in hypothalamus and testes tissues from mice with obesity alone, DEHP alone and combined obesity and DEHP exposure.

## 2. METHODS

### 2.1. Animals

All experimental procedures were conducted in conformity with the institutional guidelines for the care and use of laboratory animals at China Medical University (Shenyang, China) (CMU2019186). All experimental procedures conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985). All mice were obtained from the Experimental Animal Center of China Medical University.

In total, 112 C57BL/6J male mice (5 weeks old) were used in this study. Mice were maintained in a controlled temperature of 25°C ± 2°C and humidity of 55% ± 10%. There was a 12-hour light/dark cycle in the mice house. Food and water were provided ad libitum.

### 2.2. Diet

The 112 mice were first randomly assigned to two groups, with 16 fed a normal diet and the other 96 fed a high-fat diet. The normal laboratory diet was from China Medical University, and the high-fat diet was homemade. In the normal and high-fat diets, 10% and 45% of total calories were from fat, respectively. The composition of the high-fat diet was 73% standard chow, 20% lard, 7% casein, and trace amounts of multiple vitamins.<sup>11</sup> Detailed methods can be found in our prior study.<sup>11</sup>

### 2.3. The definition of obesity

In total, 96 mice were given the high-fat diet. According to Levin's method, after 8 weeks, the 32 mice in the upper tertile

of body weight gain were defined as high-fat diet-induced obesity (DIO) mice.<sup>30</sup> These 32 DIO mice were then randomly assigned to the following four groups: obesity alone (one group, as an obesity control), and obesity plus DEHP exposure (three groups). Each group contained eight mice, and the remaining 64 mice given the high-fat diet were removed from the experiment.

### 2.4. DEHP exposure

To evaluate the combine effect of obesity and DEHP, 24 DIO mice were given an oral gavage of DEHP once per day starting from the ninth week to the end of the study. The DEHP exposure levels were 30 mg/kg (low dose exposure), 100 mg/kg (middle dose exposure), and 300 mg/kg (high dose exposure). We also established an only DEHP exposure group as the DEHP-exposed control. The mice in the DEHP (100 mg/kg) only exposure group were given normal diet.

### 2.5. Group methods

The mice were divided into six groups (n = 8, per group). To more easily describe the results, the group were named as listed in Table 1.

### 2.6. Experimental procedures, tissue processing, and assays

All 112 mice were fed a normal diet for 1 week so that they could acclimate to the new environment. Then all mice were assigned into six groups: normal group, DEHP100 group, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 group (n = 8 per group).

In the end of the 12th week, all animals were euthanized after ether inhalation anesthesia. We collected blood samples from the vena cava, and then the hypothalamus and testicles were immediately removed. The epididymal fat and retroperitoneal fat were removed and weighed. Hypothalami and testicles were frozen at -80°C. Serum was separated from blood samples. Hypothalami and testicles were used for protein expression studies, while serum samples were used for hormones measurements, including testosterone, LH, and leptin. Each sample was tested in duplicate (Supplementary material, <http://links.lww.com/JCMA/A56>).

### 2.7. Hormone detection

Testosterone, LH, and leptin were measured by ELISA. We performed all ELISA procedures according to the kit instructions. The detection limit of the testosterone kit (Enzo Life Science, Inc., Farmingdale, NY, USA) was 7.81 pg/mL. The detection limit of the LH kit (Boster Biological Technology Co., Ltd., Wuhan, China) was 62.5 pg/mL. The detection limit of the leptin kit (Merck Millipore, Darmstadt, Germany) was 0.23 ng/mL.<sup>11</sup>

### 2.8. Western blotting

Western blotting was performed as described in a prior study.<sup>17</sup> Hypothalami and testicles were washed twice in ice-cold phosphate-buffered saline (PBS), and then dissociated in radioimmunoprecipitation assay (RIPA) buffer. The samples were then centrifuged. 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 50 µg of total protein from each sample, which was then transferred to membranes. After blocking, membranes were incubated with anti-Leptin-R antibody (AF497, diluted 1:1000; R&D Systems, Minneapolis, MN, USA), anti-kisspeptin antibody (ab19028, diluted 1:100; Abcam, Cambridge, UK), and anti-GPR54 antibody (abs136643, diluted 1:100; Absin, Shanghai, China). Membranes were then washed and incubated with secondary antibodies (diluted 1:5000; ZSGB-BIO, Beijing, China).

**Table 1****Treatment groups in this study**

Group name	Exposure	Diet
Normal group	No	12 wk normal diet
DEHP100 group	DEHP (100 mg/kg body weight) for 4 wk from the ninth week	12 wk normal diet
DIO group	Obesity	12 wk high-fat diet
DIO + DEHP30 group	Obesity + DEHP (30 mg/kg body weight) for 4 wk from the ninth week.	12 wk high-fat diet
DIO + DEHP100 group	Obesity + DEHP (100 mg/kg body weight) for 4 wk from the ninth week	12 wk high-fat diet
DIO + DEHP300 group	Obesity + DEHP (300 mg/kg body weight) for 4 wk from the ninth week	12 wk high-fat diet

DEHP = di-(2-ethylhexyl) phthalate; DIO = diet-induced obesity.

Detection was performed by chemiluminescence using ECL solution (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was tested at least in triplicate.

### 2.9. Statistical analysis

Measurement data are described as mean  $\pm$  SD. One-way analysis of variance (ANOVA) was used to compare differences among the six groups, followed by the LSD test. The significance level was set at 0.05,  $\alpha = 0.05$ .

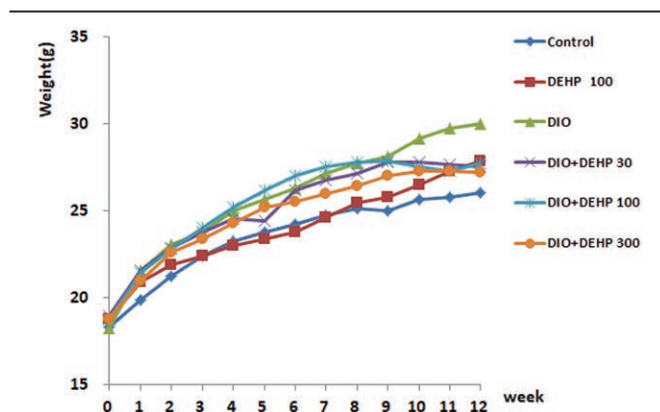
## 3. RESULTS

### 3.1. Body weight changes in the six experimental groups of mice

As shown in Fig. 1, at 12 weeks, the highest weights were recorded in the DIO group ( $30.0 \pm 2.4$ g), which was significantly higher than the other groups ( $p < 0.05$ ). Compared with control mice ( $26.0 \pm 2.1$ g), the weights of the DEHP100 mice ( $27.9 \pm 1.4$ g) were increased. The weights of DIO + DEHP100 mice ( $27.7 \pm 1.1$ g) were higher than control mice ( $p < 0.05$ ). No significant difference in weight was found between the DIO + DEHP30 ( $27.6 \pm 1.3$ g), DIO + DEHP300 ( $27.2 \pm 1.1$ g), and control mice ( $p > 0.05$ ).

### 3.2. The absolute and relative weights of epididymal and retroperitoneal fat

The absolute epididymal fat (Absolute Epi Fat) and retroperitoneal fat (Absolute Ret Fat) weights in DEHP100, DIO, DIO + DEHP30, and DIO + DEHP100 mice were significantly higher than those in control mice ( $p < 0.05$ ). Absolute Epi Fat weights in DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 mice were significantly lower than those in DIO mice ( $p < 0.05$ ).



**Fig. 1** Body weight in the six experimental groups of mice.

Compared with DIO + DEHP30 mice, Absolute Epi Fat and Absolute Ret Fat weights in DIO + DEHP300 mice were significantly decreased ( $p < 0.05$ ) (Table 2).

The relative epididymal (Relative Epi Fat) and retroperitoneal fat (Relative Ret Fat) weights in DIO and DIO + DEHP30 mice were significantly higher than those in control mice ( $p < 0.05$ ). But the Relative Epi Fat and Relative Ret Fat in DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 mice were significantly lower than those in DIO mice ( $p < 0.05$ ). Compared with DIO + DEHP30 mice, the Relative Epi and Relative Ret Fat weights were decreased significantly in DIO + DEHP300 mice ( $p < 0.05$ ) (Table 2).

### 3.3. Testosterone levels

At 12 weeks, a significant decrease in fasting testosterone levels was found in DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 mice compared with control mice ( $p < 0.05$ ) (Fig. 2). A significant decrease in testosterone levels was found in DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 mice compared with DIO mice ( $p < 0.05$ ). A significant decrease in fasting testosterone was also found in the DIO + DEHP100 group compared with the DEHP100 group ( $p < 0.05$ ).

### 3.4. LH levels

At 12 weeks, compared with control mice, fasting LH levels in DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 mice were significantly decreased ( $p < 0.05$ ). LH levels in DIO + DEHP300 mice were significantly decreased compared with DIO mice ( $p < 0.05$ ). Furthermore, LH levels in DIO + DEHP300 mice were significantly lower than those in DIO + DEHP30 mice ( $p < 0.05$ ) (Fig. 3).

### 3.5. Leptin levels

At 12 weeks, compared with control mice, fasting leptin levels in DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 mice were significantly increased ( $p < 0.05$ ). Leptin levels in DIO + DEHP100 and DIO + DEHP300 mice were significantly higher than those in DIO mice ( $p < 0.05$ ). Furthermore, leptin levels in DIO + DEHP100 and DIO + DEHP300 mice were significantly higher than those in DIO + DEHP30 mice ( $p < 0.05$ ) (Fig. 4). Compared with DEHP100 mice, leptin levels in DIO + DEHP100 mice were significantly increased ( $p < 0.05$ ).

### 3.6. Ob-R, kisspeptin, and GPR54 protein expression in the hypothalamus

Compared with the control group, Ob-R expression in the DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups was decreased ( $p < 0.05$ ). Ob-R protein levels in DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 mice were significantly reduced to 67%, 55%, 65%, 61%, and 55% of that in control mice, respectively ( $p < 0.05$ ) (Fig. 5).

**Table 2****The absolute and relative weights of epididymal and retroperitoneal fat after 12 wk (mean  $\pm$  SD)**

Group	n	Absolute Epi Fat, g	Relative Epi Fat, g/100 g	Absolute Ret Fat, g	Relative Ret Fat, g/100 g
Control	8	0.26 $\pm$ 0.10	1.24 $\pm$ 0.42	0.06 $\pm$ 0.02	0.27 $\pm$ 0.09
DEHP100	8	0.45 $\pm$ 0.23 <sup>a</sup>	1.81 $\pm$ 0.92	0.13 $\pm$ 0.04 <sup>a</sup>	0.52 $\pm$ 0.16 <sup>a</sup>
DIO	8	0.92 $\pm$ 0.33 <sup>a</sup>	3.13 $\pm$ 0.90 <sup>a</sup>	0.27 $\pm$ 0.12 <sup>a</sup>	0.94 $\pm$ 0.42 <sup>a</sup>
DIO + DEHP30	8	0.65 $\pm$ 0.08 <sup>ab</sup>	2.47 $\pm$ 0.27 <sup>ab</sup>	0.19 $\pm$ 0.04 <sup>ab</sup>	0.71 $\pm$ 0.16 <sup>ab</sup>
DIO + DEHP100	8	0.52 $\pm$ 0.14 <sup>ab</sup>	1.94 $\pm$ 0.51 <sup>ab</sup>	0.12 $\pm$ 0.04 <sup>abc</sup>	0.46 $\pm$ 0.17 <sup>bc</sup>
DIO + DEHP300	8	0.45 $\pm$ 0.07 <sup>bc</sup>	1.72 $\pm$ 0.27 <sup>bc</sup>	0.12 $\pm$ 0.03 <sup>abc</sup>	0.46 $\pm$ 0.12 <sup>bc</sup>

Data are presented as mean  $\pm$  SD. Epi = epididymal; Ret = retroperitoneal; Relative Epi Fat weight = epididymal fat weight  $\div$  body weight  $\times$  100; Relative Ret Fat weight = retroperitoneal fat weight  $\div$  body weight  $\times$  100. DEHP = di-(2-ethylhexyl) phthalate; DIO = diet-induced obesity.

<sup>a</sup>Statistically significant difference ( $p < 0.05$ ) compared with the control group.

<sup>b</sup>Statistically significant difference ( $p < 0.05$ ) compared with the DIO group.

<sup>c</sup>Statistically significant difference ( $p < 0.05$ ) compared with the DIO + DEHP30 group.

Compared with the control group, kisspeptin expression in the DEHP100, DIO, DIO + DEHP100, and DIO + DEHP300 groups was decreased ( $p < 0.05$ ). Kisspeptin expression in DIO + DEHP300 mice was significantly lower than that in DIO + DEHP30 mice ( $p < 0.05$ ); however, kisspeptin expression in DIO + DEHP30 mice was significantly higher than that in DIO mice ( $p < 0.05$ ) (Fig. 5).

Compared with the control group, GPR54 expression in the DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups was decreased. GPR54 protein levels in the DIO and DIO + DEHP300 groups were significantly reduced to 67% and 62% of that in controls, respectively ( $p < 0.05$ ) (Fig. 5).

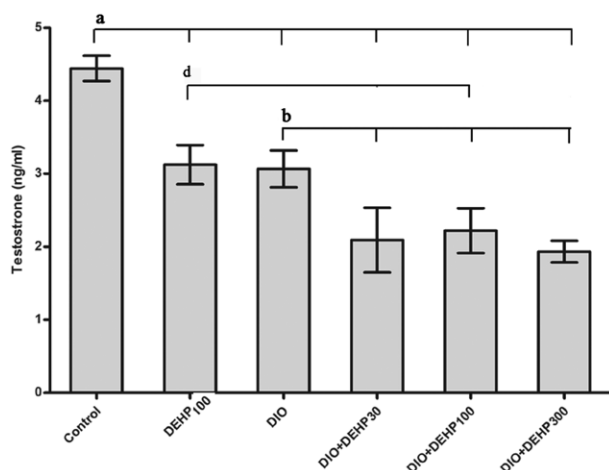
### 3.7. Ob-R, kisspeptin, and GPR54 protein expression in the testis

Compared with the control group, Ob-R expression in the DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups was decreased. Ob-R protein levels in the DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups were significantly reduced to 64%, 62%, 68%, 59%, and 45% of that in the control group ( $p < 0.05$ ).

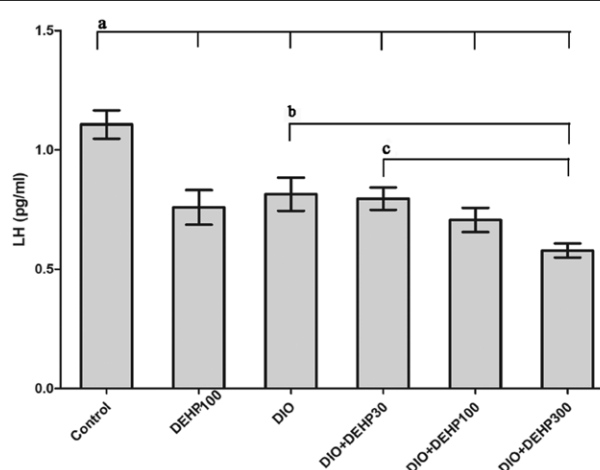
Ob-R expression in DIO + DEHP300 mice was significantly lower than that in DIO + DEHP30 mice ( $p < 0.05$ ) (Fig. 6).

Compared with the control group, kisspeptin expression in the DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups was decreased. Kisspeptin protein levels in the DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups were significantly reduced to 42%, 70%, 70%, and 36% of that in controls ( $p < 0.05$ ). Furthermore, kisspeptin expression in DIO + DEHP300 mice was significantly lower than that in DIO + DEHP30 mice ( $p < 0.05$ ) (Fig. 6).

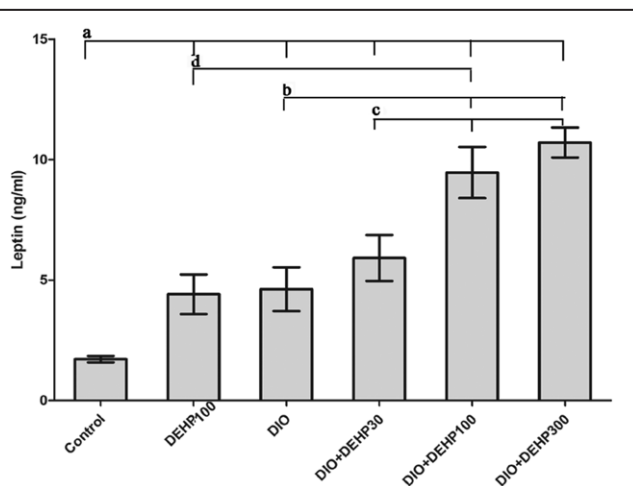
Compared with the control group, GPR54 expression in the DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups was decreased. GPR54 protein levels in the DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups were significantly reduced to 66%, 56%, 59%, and 44% of that in controls ( $p < 0.05$ ). Furthermore, compared with the DIO group, GPR54 expression in DIO + DEHP300 mice was significantly decreased ( $p < 0.05$ ) (Fig. 6). Compared with the DEHP100 group, GPR54 expression in the DIO + DEHP100 group was significantly decreased ( $p < 0.05$ ).



**Fig. 2** Testosterone level in the six experimental groups of mice. The bar graphs show the testosterone level in the control, DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. Each bar represents mean  $\pm$  SE.  $n = 6$ . (a)  $p < 0.05$  denote statistically significant compared with control group; (b)  $p < 0.05$  denote statistically significant compared with DIO group; (d)  $p < 0.05$  denote statistically significant compared with DEHP100 group. DEHP = di-(2-ethylhexyl) phthalate; DIO = diet-induced obesity.



**Fig. 3** LH levels in the six experimental groups of mice. The bar graphs show the LH level in the control, DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. Each bar represents mean  $\pm$  SE.  $n = 6$ . (a)  $p < 0.05$  denote statistically significant compared with control group; (b)  $p < 0.05$  denote statistically significant compared with DIO group; (c)  $p < 0.05$  denote statistically significant compared with DIO + DEHP30 group. DEHP = di-(2-ethylhexyl) phthalate; DIO = diet-induced obesity; LH = luteinizing hormone.



**Fig. 4** Leptin levels in the six experimental groups of mice. The bar graphs show the leptin level in the control, DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. Each bar represents mean  $\pm$  SE.  $n = 6$ . (a)  $p < 0.05$  denote statistically significant compared with control group; (b)  $p < 0.05$  denote statistically significant compared with DIO group; (c)  $p < 0.05$  denote statistically significant compared with DIO + DEHP30 group; (d)  $p < 0.05$  denote statistically significant compared with DEHP100 group. DEHP = di-(2-ethylhexyl) phthalate; DIO = diet-induced obesity.

#### 4. DISCUSSION

Obesity combined with DEHP exposure is common in the lives of many people. Furthermore, the doses of DEHP exposure are low throughout daily activities. When we designed the exposure doses in this study, we referenced other studies.<sup>31,32</sup> And our previous study used the same DEHP doses and found that DEHP increased obesity-induced damage to the male reproductive system in mice.<sup>17</sup> However, the exact mechanism of how obesity and DEHP combined to affect the male reproductive system was unclear. According to our prior study, the obesity model was established within 8 weeks, so DEHP exposure started was from the ninth week.<sup>17</sup>

In this study, the fat weights of DEHP and DIO mice were higher than those of control mice. As other studies have reported, DEHP exposure increased the body fat of mice.<sup>33,34</sup> High-fat diet also increased the body fat of mice.<sup>11,35</sup> We investigated whether there was a possible combinatorial effect of fat from high-fat diet and DEHP combine exposure mice, but the fat weights of DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 mice were lower than those of DIO mice. Thus, we conclude that the underlying reasons for fat accumulation from high-fat diet and DEHP were different. First, the former is the fat increase caused by the high energy from the high-fat diet, while the latter is due to an endocrine disorder, which resulted in a lower fat weight in obese mice with DEHP exposure than DIO mice. Second, there may be a toxicity effect of DEHP, especially in the middle and high dose groups. DEHP exposure may also affect visceral organs; thus, weight loss compared with DIO mice may be due to the toxicity of DEHP.<sup>36</sup> The exact reasons should be determined in a corollary study.

In our previous work, male obesity and DEHP were shown to have a combined effect on plasma testosterone levels.<sup>17</sup> Testosterone is a sex hormone that plays an important role in male reproduction. Studies have found that low testosterone levels may contribute to male obesity and hypogonadism.<sup>37,38</sup> From this study, compared with the control group, significantly lower serum testosterone levels were found in DEHP100, DIO, DIO + DEHP30, DIO + DEHP 100, and DIO + DEHP300 mice. Moreover, compared with DEHP and DIO mice, lower serum

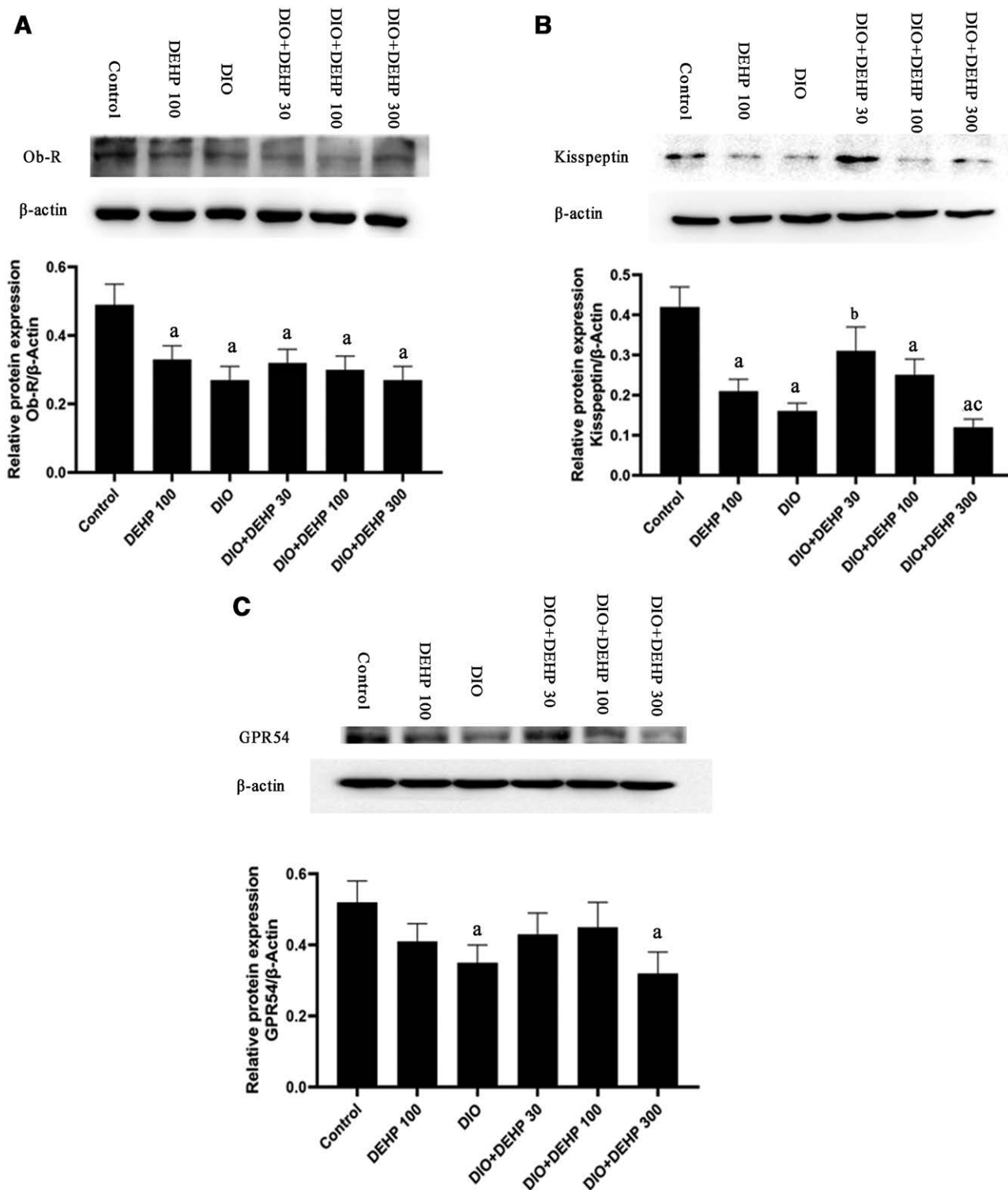
testosterone levels were found in DIO + DEHP mice; there was no significant difference in serum testosterone levels among mice exposed to the three DEHP doses. Therefore, low-dose DEHP should be a cause of concern, as it is widely available in people. Similar to our prior study, obesity alone, DEHP alone, and obesity combined with DEHP exposure reduced testosterone levels, which may be the main reason for damage to male reproduction in obese, DEHP-exposed, and obese mice combined with DEHP exposure.<sup>17</sup>

Next, we wanted to address why there were the lowest testosterone levels in obese mice with DEHP exposure. Mechanistically, testosterone can be regulated by the HPG axis, in which the role of GnRH is important.<sup>19</sup> GnRH pulsatile secretion promotes LH and FSH release, which then increases testosterone secretion in the testis.<sup>19</sup> In this study we determined LH levels as a surrogate for serum GnRH levels.<sup>39</sup> Compared with control mice, the fasting LH levels in DEHP100, DIO, and obese mice with DEHP exposure were significantly decreased. LH levels in obese mice with high-level DEHP exposure were significantly lower than obese mice. Furthermore, LH levels in DIO mice combined with high-level DEHP exposure were lower than in DIO mice combined with low-level DEHP exposure. Overall, low testosterone levels may be due to reduced GnRH levels in the obesity alone and DEHP alone groups; second, we consider that there was an additive effect on reducing GnRH levels that was attributable to the combined effect of obesity and DEHP, which resulted in a low testosterone level in DIO + DEHP mice.

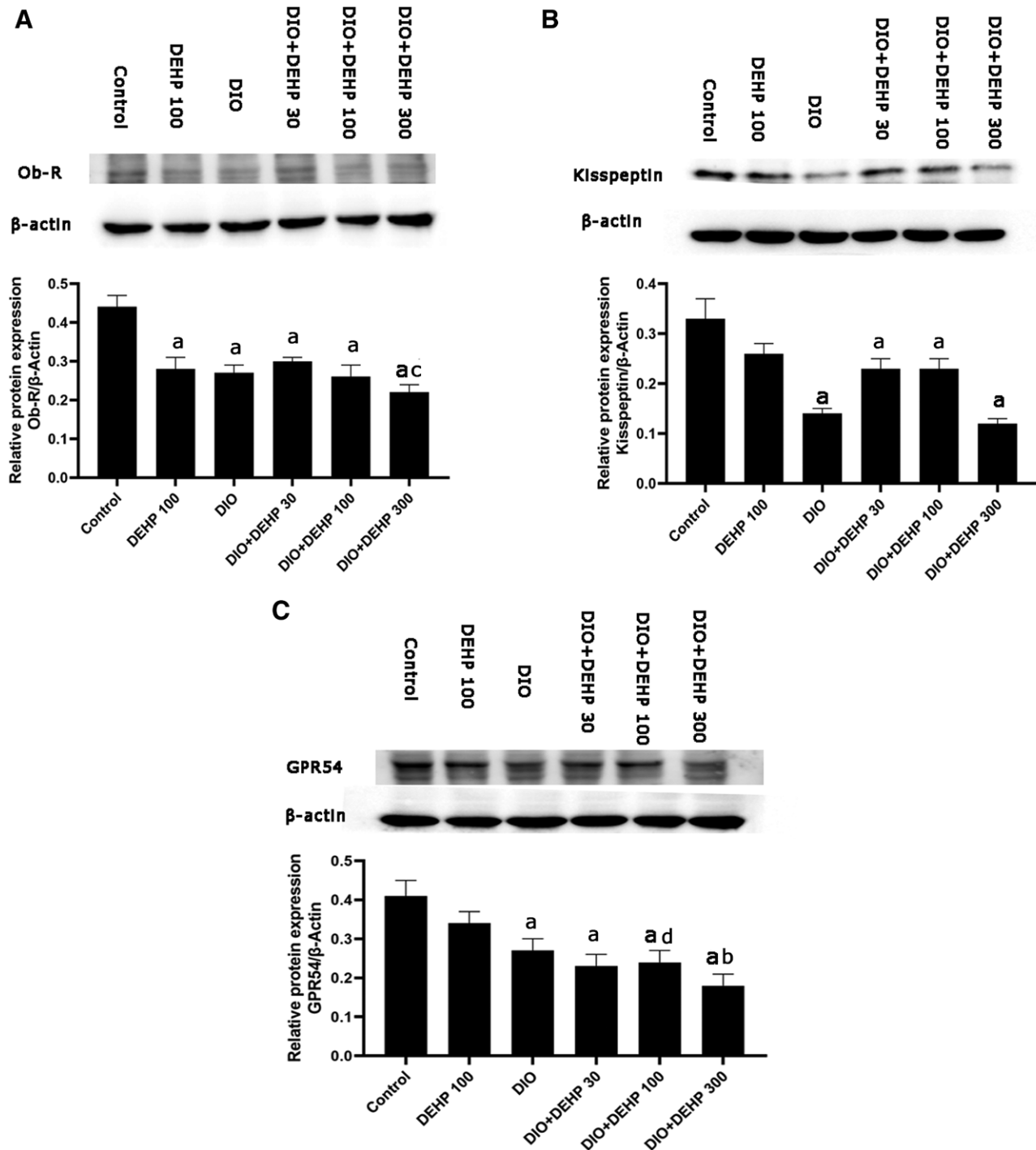
GnRH is regulated by several neuroendocrine afferents, such as leptin.<sup>23-26</sup> High leptin levels occur in obesity.<sup>40,41</sup> In our previous study, we found that GnRH levels could be inhibited by high leptin levels.<sup>16</sup> From this study, higher leptin levels were also found in DIO mice with DEHP exposure. Compared with control mice, leptin levels in DEHP100, DIO, and DIO combined with DEHP exposure mice were significantly increased. Furthermore, leptin levels in the DIO + DEHP mice were significantly higher than those in DIO- and DEHP-only mice. Finally, leptin levels in DIO mice with high-level DEHP exposure were higher than in DIO mice combined with low-level DEHP exposure. Similar to our prior study, in this study, there was also an additive effect of leptin in obese mice exposed to DEHP.<sup>17</sup> The low GnRH expression (LH levels as a surrogate for serum GnRH levels) may be inhibited by high leptin levels.

Some studies have found that leptin cannot directly regulate GnRH secretion in the hypothalamus and testes.<sup>23-26</sup> Other studies and our previous research found that kisspeptin/GPR54 plays a mitigating role in connecting leptin and GnRH.<sup>16,23-26</sup> In this study, we investigated the mechanism underlying how high leptin levels are associated with low GnRH secretion. In the hypothalamus and testis, we found that Ob-R expression was significantly decreased in DIO mice, mice exposed to DEHP, and DIO mice exposed to DEHP. High leptin levels cannot exert its normal role due to the low Ob-R expression.<sup>42</sup> We also detected kisspeptin/GPR54 expression in the hypothalamus and testes. We found that kisspeptin and GPR54 expression were inhibited in the DIO- and DEHP-only groups in the hypothalamus and testes. However, a combined role was only found for GPR54 in the testes. GPR54 expression in DIO mice with high-level DEHP exposure was significantly decreased compared with the DIO groups.

There were some limitations to this study. For example, we indirectly determined GnRH secretion by detecting LH levels. GnRH levels could be detected by the GnRH excitation test. Considering the effect of detecting other hormones, such as testosterone, we did not directly detect GnRH secretion. We only detected LH levels, which can reflect GnRH levels.<sup>39</sup> In this study, we only detected changes in hormone and protein expression. The discussed mechanism should be confirmed in further studies. However, considering that there are so few studies on



**Fig. 5** Ob-R, kisspeptin, and GPR54 protein expression in the hypothalamus (A) showed the results of Ob-R in the hypothalamus: the depict representative findings and the results of the semi-quantitative measurement in the control, DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. B showed the results of kisspeptin in the hypothalamus: the depict representative findings and the results of the semi-quantitative measurement in the control, DEHP, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. C showed the results of GPR54 in the hypothalamus: the depict representative findings and the results of the semi-quantitative measurement in the control, DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. Each bar represents mean  $\pm$  SE. n = 4. (a)  $p < 0.05$  denote statistically significant compared with control group; (b)  $p < 0.05$  denote statistically significant compared with DIO group; (d)  $p < 0.05$  denote statistically significant compared with DIO + DEHP30 group. DEHP = di-(2-ethylhexyl) phthalate; DIO = diet-induced obesity.



**Fig. 6** Ob-R, kisspeptin, and GPR54 protein expression in the testis (A) showed the results of Ob-R in the testis: the depict representative findings and the results of the semi-quantitative measurement in the control, DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. B showed the results of kisspeptin in the testis: the depict representative findings and the results of the semi-quantitative measurement in the control, DEHP100, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. C showed the results of GPR54 in the testis: the depict representative findings and the results of the semi-quantitative measurement in the control, DEHP, DIO, DIO + DEHP30, DIO + DEHP100, and DIO + DEHP300 groups. Each bar represents mean  $\pm$  SE.  $n = 4$ . (a)  $p < 0.05$  denote statistically significant compared with control group; (b)  $p < 0.05$  denote statistically significant compared with DIO group; (c)  $p < 0.05$  denote statistically significant compared with DIO + DEHP30 group; (d)  $p < 0.05$  denote statistically significant compared with DEHP100 group. DEHP = di-(2-ethylhexyl) phthalate; DIO = diet-induced obesity.

the combined effects of obesity and DEHP, these results are meaningful to report it.

In conclusion, obesity- or DEHP-only exposure had adverse effects on testosterone levels in mice, which may due to high

leptin levels and the downregulation of Ob-R, kisspeptin, and GPR54 protein expression. Obesity combined with DEHP exposure had an additive adverse effect on testosterone levels in mice, one of the potential mechanisms of which was associated with

higher leptin levels and further decreased expression of GPR54 protein in the testes.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://links.lww.com/JCMA/A56>.

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