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Involvement of Thyroxine in Ovarian Toxicity of Di-(2-ethylhexyl) Phthalate

Soichiro SEKIGUCHI, Shin ITO, Megumi SUDA and Takeshi HONMA*

Department of Health Effects Research, National Institute of Industrial Health, 6-21-1 Nagao, Tama-ku, Kawasaki 214-8585, Japan

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Abstract: Forced ovulation induced by the administration of exogenous gonadotropin is a useful marker for studying the ovarian toxicity of chemicals in experimental animals. We examined the toxicity of di-(2-ethylhexyl) phthalate (DEHP) in the ovaries of immature F344 female rats. Superovulation was induced by injections of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) in rats dosed with 125, 250, 500, 1,000 or 2,000 mg/kg body weight of DEHP for 4 consecutive days. The number of ova shed during superovulation significantly decreased in rats treated with DEHP at 500 mg/kg as compared with control, but no changes were observed in the number of ova in groups given other doses of DEHP. In control rats treated with olive oil, hypophysectomy reduced significantly the number of ovulated ova. When 2,000 mg DEHP was given to hypophysectomized (hypox) rats, the number of ova in the hypox group was significantly smaller than that in the intact group administered with the same doses of DEHP. In contrast, the numbers of ova of the intact and hypox groups did not significantly differ in rats given 500 mg DEHP. The levels of circulating thyroxine (T_4) were significantly decreased by 2,000 mg DEHP in intact rats, and a tendency for T_4 to decrease in T_4 was also observed in hypox rats given 2,000 mg DEHP. These results suggest that daily administration of 500 mg DEHP suppressed superovulation in immature F344 rats by disrupting the hypothalamic-pituitary-ovarian axis in a manner similar to that of hypophysectomy. Decreased circulating T_4 levels seemed to negate this disruption as observed in recovered superovulation after treatment with 2,000 mg DEHP.

Key words: DEHP, F344 rats, Hypophysectomized rats, Ovulation, Thyroxine

Introduction

The compound 2-bromopropane possesses reproductive toxicity in male and female humans[1, 2]. We showed that an injection of 2-bromopropane or exposure to 1,2-dichloropropane significantly decreased the number of spontaneously ovulated ova and delayed estrous cycle in female F344 rats[3, 4]. The number of ovulated ova during superovulation induced by equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) in mice is significantly reduced by 2-bromopropane[5]. Thus, ovulation is a significant marker that can be used to detect reproductive toxicity in female animals. Some studies have examined the influence of chemicals on the murine ovary *in vivo* and *in vitro* by using ovulation induced by eCG and hCG in immature female rats[6–9].

Di-(2-ethylhexyl) phthalate (DEHP) is generally used as plasticizer of vinyl products, and it exhibits ovarian toxicity after short-term administration in rats. A study *in vivo* by Davis *et al.*[10] showed that consecutive daily doses of 2,000 mg/kg of DEHP suppressed or delayed ovulation with a concomitant significant decrease in serum estradiol-17 β (E_2). A study *in vitro* found that mono- (2-ethylhexyl) phthalate (MEHP), an active metabolite of DEHP, decreases the levels of E_2 converted from testosterone (T) in incubation medium[11], and also those of mRNA for aromatase that synthesizes E_2 from T[12]. These finding suggest that the inhibiting effect on ovulation is caused by suppression of E_2 synthesis. In

*To whom correspondence should be addressed.

view of the suppressive effect of DEHP on E_2 synthesis, induced ovulation in immature female rats is useful for studying the ovarian toxicity of DEHP.

DEHP is also known to suppress enzyme activity in the liver of rats[13, 14]. When fed with diets containing DEHP, the serum thyroxine (T_4) levels declined to about half of those of control rats 3 d after the initiation of intake of DEHP in diet[13]. Administration of T_4 to intact and thyroidectomized rats significantly increased the circulating levels of thyroid hormones, and the increase of thyroid hormone levels was suppressed by DEHP administered simultaneously with T_4[14]. These results suggest that the consumption of circulating levels of thyroid hormones is accelerated by DEHP through stimulating the metabolism and/or excretion of thyroid hormones in the liver. Moreover, Tamura *et al.* reported that in thyroidectomized rats treated with eCG and hCG, the number of ova in induced ovulation and ovarian hormone levels were significantly increased compared with those in intact rats treated with eCG and hCG[15]. They suggested that thyroid hormone plays an inhibitory role in ovarian hormone synthesis and follicular development induced by eCG injection in immature female rats. The above mentioned findings seem to suggest the possibility that DEHP affects induced ovulation in immature rats through thyroid homeostasis.

In our previous study, subcutaneous injections of 500 mg/kg of DEHP administered to immature F344 rats tended to suppress superovulation induced by a single injection of eCG[16]. In the present study, in order to determine the exact effects of DEHP on reproductive function, the ability of eCG and hCG to induce superovulation in DEHP-treated rats was estimated, and we also investigated whether thyroid hormone is involved in the ovarian toxicity of DEHP.

Materials and methods

Animals and experimental designs

Intact immature females (intact females) of F344/DuCrj strain (F344) rat purchased from Charles River Japan, Inc. at 21 d of age were housed under conditions of 12 h light (08:00 to 20:00)/12 h darkness, with room temperature maintained at 23 ± 1 °C and 55 ± 5 % humidity. Sterilized pellet food, CE-2, purchased from Clea Japan, Inc. and water were freely available. Immature F344 rat females hypophysectomized at 24 d of age at the Technical Center of Charles River Japan, Inc. (hypox females) were purchased at 27 d of age. The hypophysectomy was performed by the parapharyngeal method because this method was expected to be more successful than the intra-aural method. Rats were anesthetized by an intraperitoneal injection of ketamine/xylazine. The complete sucking out of the pituitary was confirmed by the degree of body weight increase after the operation. Rats were handled according to the Animal Welfare. At the end of the experiment, the removal of the gland was verified anatomically.

The animals were housed under the same breeding conditions as intact females. Doses of 125, 250, 500, 1,000 or 2,000 mg/kg body weight (B.W.) of DEHP (Wako Pure Chemical Industries, Ltd., Japan) were diluted in olive oil (Wako). The final volume of olive oil containing each of the above doses was adjusted to 3 ml. In the first experiment, intact and hypox females were subcutaneously (sc) injected with DEHP at the above doses at intervals of 24 h from 24 to 27 d of age and from 27 to 30 d of age, respectively. In the second experiment, DEHP at 500 or 2,000 mg/kg was administered at 24-h intervals for one to three days during the four days between 24 and 27 d of age. Superovulation was induced with eCG (Teikoku Hormone MSG. Co., Ltd., Japan) at a dose of 15 iu in 0.15 ml saline injected intramuscularly (im) at 25 d of age to intact females and at 28 d of age to hypox females followed by 15 iu hCG (Teikoku Hormone) in 0.15 ml saline injected intraperitoneally (ip) at 27 or 30 d of age, 56 or 72 h after the eCG injection. All rats were sacrificed by decapitation at 28 or 31 d of age, at 72 h after the eCG injection[16], and the ovaries and uteri were weighed after removing the fat and connective tissue. Ova shed into oviducts were collected and counted[16]. Briefly, ovulated ova were flushed with physiological saline delivered from a 2.5 ml syringe attached to a 27G × 3/4 needle. Ova enveloped by cumulus cells were put into 0.1% (359 units/ml) hyaluronidase (Sigma Chemical Co., USA). The cumulus cells were dispersed at 37 °C on a warm plate and removed from the ova, which were then counted under a light microscope. We defined superovulation as ovulation in which the number of ovulated ova was more than 11[16].

Hormone measurements

Trunk blood released by decapitation was collected in conical tubes and immediately placed on ice. The tubes were centrifuged at 1,630 × g at 4 °C for 15 min, and the serum was separated and stored at −80 °C. Concentrations of T_4 in serum were measured using DELFIA kits (Perkin-Elmer Life Sciences Japan Co., Ltd.) in a time-resolved fluorometric assay. The intra- and inter-assay coefficients of variation for the T_4 assay were 7.03 and 4.74 %, respectively.

Statistical analysis

Data were analyzed by use of one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test and Fisher's exact probability tests.

Results

Ovulation

Table 1 shows the effects of 4 consecutive injections of DEHP on reproductive development in intact immature rats with induced superovulation. The number of control and DEHP-treated rats in which ovulation and superovulation were induced did not differ. A significant change was noted only in the number of ovulated ova in the 500 mg DEHP-treated groups (Table 1). Among rats in which superovulation was induced, the mean numbers of ovulated ova in control, 125 and 2,000 mg DEHP groups were 48.9, 51.6 and 51.3, respectively. The mean numbers in 250 mg DEHP group (43.9 ova) and in 1000 mg DEHP group (39.8 ova) were less than that of the control group. The mean number (30.5) of ovulated ova in rats treated with 500 mg DEHP was significantly decreased ($P<0.05$) compared with control and 125 and 2,000 mg DEHP-treated rats (Table 1).

Table 2 compares the effects of 500 or 2,000 mg DEHP injections on ovulation induced by 15 iu each of eCG and hCG in hypox and intact rats. When treated with olive oil alone, ovulation was induced in 4 of 8 hypox rats, and in all intact rats injected with eCG and hCG at a 56 h interval. The difference between the two groups was significant (50% vs. 100%; $P<0.05$). When injected with eCG and hCG at a 72 h interval, ovulation was induced in 7 of 8 hypox rats, and this ratio (87.5%) was not significantly different from that of intact rats at the 56 h interval. The numbers of rats treated with 500 or 2,000 mg DEHP and exhibiting ovulation by eCG and hCG did not significantly differ between intact and hypox rats (Table 2). We counted the numbers of rats that underwent superovulation and then calculated the ratios of superovulation to ovulation. The superovulation ratios did not significantly differ among the control, 500 and 2,000 mg/kg intact and hypox groups. The mean number of ovulated ova was counted only in the superovulated rats. The mean numbers of ovulated ova in the intact and hypox groups treated with olive oil and given eCG and hCG at a 56 h interval were 48.9 and 28.3, respectively, and 35.0 in the hypox group given eCG and hCG at a 72 h interval. These differences in numbers of ova obtained were significant ($P<0.05$). The mean numbers of ovulated ova after the administration of 2,000 mg DEHP were 51.3 in the intact group, and 31.2 and 31.0 in each of the hypox groups. The differences were statistically significant between the intact and the two hypox groups ($P<0.05$). On the other hand, the mean numbers of ovulated ova after the administration of 500 mg DEHP were 30.5 in the intact group, and 26.1 and 26.2 in each of the hypox groups (no significant differences among the three groups; Table 2).

Table 3 shows how the timing of the 500 and 2,000 mg DEHP injections affected the ovulation induced by eCG and hCG. The numbers of control and DEHP-treated rats that ovulated or superovulated were almost constant regardless of injection time or dose of DEHP. The mean number of superovulated ova was not influenced by the time of DEHP injection, and the number of ovulated ova between control and 2,000 mg DEHP-treated groups did not significantly differ. Although the mean number of ovulated ova in 500 mg DEHP-treated rats tended to decrease depending on the total amount of injected DEHP, differences between control and the 500 mg DEHP-treated groups were not statistically significant (Table 3).

Hormonal changes

Figure 1 shows the concentrations of T_4 in superovulated intact (A) and hypox rats (B). The T_4 concentration (61.5 ± 6.5 ng/ml) of intact rats treated with 500 mg DEHP was almost equal to that (67.4 ± 6.2 ng/ml) of the control group, and that (41.3 ± 4.5 ng/ml) of the 2,000 mg DEHP group was significantly lower ($P<0.05$) than the control (Fig. 1A). The mean T_4 concentrations of the two hypox groups were determined by adding the results from these groups regardless of eCG-hCG intervals because the T_4 values did not differ between them. The T_4 concentration (24.1 ± 2.9 ng/ml) of the hypox group treated with 500 mg DEHP did not significantly vary from that (26.6 ± 2.9 ng/ml) of the control group without DEHP. The T_4 concentration (21.4 ± 1.1 ng/ml) of the group treated with 2,000 mg DEHP was 20 % lower than that of the control (Fig. 1B).

Discussion

The results of the present study indicated that daily sc injections of 500 mg DEHP for 4 successive days suppressed the ovulation induced by eCG-hCG injection in immature F344 rats. The mean numbers of ovulated ova in groups treated with DEHP other than at 500 mg did not show obvious decrease from that of the control group (Table 1). The numbers of superovulated ova in all groups given 1 to 3 injections of 500 mg DEHP were not significantly lower than that of the control (Table 3), suggesting that a total amount of 2,000 mg/kg of DEHP received over 4 d is required to decrease the number of ova during superovulation. However, 1 to 4 injections of 2,000 mg DEHP failed to inhibit superovulation (Tables 1 and 3), suggesting that the effect which suppressed the number of ovulated ova in 500 mg DEHP-treated rats was revoked by the administration of 2,000 mg DEHP. It is generally thought that DEHP inhibits E_2 synthesis *in vivo*[10] and *in vitro*[11, 12]. Although 500 mg DEHP did not affect the induction ratio of ovulation (Table 1), this

Table 1. Effects of 4 consecutive injections of DEHP on body and reproductive organ weights in intact immature F344 rats

Groups[1)]	Control	125 mg	250 mg	500 mg	1,000 mg	2,000 mg
No. of rats	10	9	9	11	9	9
Induced ovulation	10 (100%)	9 (100%)	7 (77.8%)	10 (90.9%)	9 (100%)	7 (77.8%)
Superovulation	9 (90.0%)	9 (100%)	7 (100%)	10 (100%)	8 (88.9%)	7 (100%)
Ovulated ova	48.9 ± 5.0^{a}	51.6 ± 4.7^{a}	$43.9 \pm 5.3^{a,b}$	30.5 ± 4.7^{b}	$39.8 \pm 6.4^{a,b}$	51.3 ± 4.5^{a}
Autopsy B.W. (g)	62.6 ± 1.7	57.8 ± 0.7	61.5 ± 0.7	61.9 ± 1.5	61.3 ± 0.9	59.5 ± 1.1
Ovary (mg)	114.9 ± 4.6	96.8 ± 2.2	111.1 ± 5.7	114.5 ± 4.6	119.9 ± 11.8	104.7 ± 6.8
Relative ovary[2)]	1.85 ± 0.10	1.68 ± 0.04	1.82 ± 0.11	1.86 ± 0.08	1.95 ± 0.18	1.77 ± 0.13
Uterus (mg)[3)]	116.4 ± 6.9	108.1 ± 4.1	120.7 ± 6.1	108.6 ± 7.5	106.9 ± 2.4	115.9 ± 3.0
Relative uterus[3)]	1.87 ± 0.11	1.87 ± 0.06	1.96 ± 0.09	1.77 ± 0.13	1.74 ± 0.02	1.95 ± 0.07

The values are means ± SEM; different superscripts indicate significant difference.
(P<0.05 by Duncan's multiple comparison test) within the same line.
[1)] Values indicate daily doses of DEHP/3 ml vehicle/kg body weight.
[2)] Relative weights of ovary were calculated as absolute ovary weight (mg)/body weight (g) on the day of autopsy.
[3)] Uterus weights were measured after uterus fluid was blotted. Relative uterus weights were calculated as blotted weight (mg) of uterus/body weight (g) on the day of autopsy.

Table 2. Effects of 4 consecutive injections of 500 or 2,000 mg of DEHP on the superovulation in intact and hypophysectomized immature F344 rats

DEHP	Groups	Interval[1)]	Rats	Ovulation[2)]	Superovulation[3)]	Numbers of ova[4)]
Control	Intact	56 h	10	10 (100%)	9 (90.0%)	48.9 ± 5.04^{a}
	Hypox	56 h	8	4 (50.0%)[#]	3 (75.0%)	28.3 ± 4.48^{b}
	Hypox	72 h	8	7 (87.5%)	7 (100%)	35.0 ± 4.11^{b}
500 mg	Intact	56 h	11	10 (90.9%)	10 (100%)	30.5 ± 4.71
	Hypox	56 h	8	8 (100%)	8 (100%)	26.1 ± 2.95
	Hypox	72 h	7	6 (85.7%)	5 (83.3%)	26.2 ± 4.12
2,000 mg	Intact	56 h	9	7 (77.8%)	7 (100%)	51.3 ± 4.48^{a}
	Hypox	56 h	7	6 (85.7%)	5 (83.3%)	31.2 ± 5.27^{b}
	Hypox	72 h	7	5 (71.4%)	4 (80.0%)	31.0 ± 6.32^{b}

[1)] Values indicate the time intervals between eCG and hCG injections.
[2)] The number of rats that showed ovulation, and values in parentheses indicate the ovulation ratio (rats showing ovulation/rats used). [#]indicates significant difference from intact group (P<0.05) by Fisher's exact probability test.
[3)] The number of rats that showed superovulation, and values in parentheses indicate the superovulation ratio by rat numbers (superovulation/ovulation).
[4)] The values are means ± SEM; different letter of superscripts indicate significant difference (P<0.05 by Duncan's multiple comparison test) within the column on the same treatment of DEHP.

dose of DEHP for 4 successive days decreased the number of ovulated ova. A possible explanation for this phenomenon is that a dose of 500 mg DEHP for 4 successive days inhibits the growth of ovarian follicles from the primordial to the ovulable follicle. If the decreased number of ovulated ova in 500 mg DEHP-treated rats was induced by the toxicity of MEHP through suppressed estradiol synthesis, then the absence of such an effect at 2,000 mg DEHP on the number of ovulated ova would be unexplained, since this dose of DEHP should produce high peripheral levels of MEHP through metabolism.

In hypox rats, the numbers of ovulated ova in both the control and 2000 mg DEHP groups were decreased compared with those of intact rats. This result means that endogenous gonadotropic hormones (GTHs) secreted from the pituitary were involved in the superovulation in intact rats (Table 2). However, the mean numbers of ovulated ova did not significantly differ between intact and hypox rats in the 500 mg DEHP groups (Table 2). The injection of 500 mg DEHP for 4 successive days seemed to mimic the effect of hypophysectomy, which interrupts the hormonal effects on follicular growth in the hypothalamic-pituitary-ovarian axis. To investigate the cause of the absence of dose-dependency in the inhibitory effect of 500 mg DEHP on the ovulation, we examined the involvement of the thyroid hormone. Tamura *et al.*[15)] showed the role of thyroid hormone in the onset of puberty by using hormone-induced ovulation in immature female rats. In female rats thyroidectomized before puberty and treated with eCG, the serum estradiol and

Table 3. Effects of single and consecutive injections of DEHP on superovulation in intact immature F344 rats

	Single Injection	Double Injection	Triple Injection
Ovulation[1)]			
Control	7/9 (77.8%)	9/9 (100%)	9/9 (100%)
500 mg DEHP	9/9 (100%)	9/9 (100%)	8/9 (88.9%)
2,000 mg DEHP	8/9 (88.9%)	9/9 (100%)	8/9 (88.9%)
Superovulation[2)]			
Control	6/7 (85.7%)	8/9 (88.9%)	9/9 (100%)
500 mg DEHP	7/9 (77.8%)	9/9 (100%)	7/8 (87.5%)
2,000 mg DEHP	7/8 (87.5%)	8/9 (88.9%)	7/8 (87.5%)
Numbers of ova[3)]			
Control	53.7 ± 7.1	46.4 ± 4.1	54.8 ± 6.0
500 mg DEHP	42.4 ± 5.4	37.3 ± 4.6	35.7 ± 7.4
2,000 mg DEHP	48.7 ± 8.4	51.3 ± 6.6	51.9 ± 10.1

All rats in control and DEHP-treated groups were injected sc with daily doses of olive oil and DEHP solution, respectively. All injections were initiated 24 h before eCG treatment, and thereafter were conducted at intervals of 24 h.

1) Numerator and denominator indicate the number of rats that showed ovulation and the number of rats used, respectively. The values in parentheses indicate the ovulation ratio (rats showing ovulation/rats used).

2) The number of rats that showed superovulation, and the values in parentheses indicate the superovulation ratio by rat numbers (superovulation/ovulation).

3) Means ± SEM of ovulated ova in rats with superovulation.

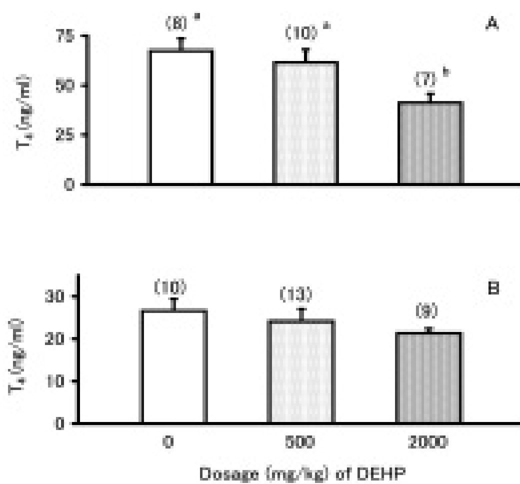

Fig. 1. Effects of DEHP on serum T_4 concentration in intact (upper panel; A) and hypophysectomized (under panel; B) rats that superovulated.

Columns, vertical bars and parentheses indicate means of T_4, SEM and number of rats in each group, respectively. Different letter of superscript indicates significant differences (P<0.05) by Duncan's multiple comparison test.

inhibin levels were elevated, and the number of large healthy follicles (>400 μm in diameter) was increased as compared with intact rats. When T_4 was administered to thyroidectomized rats, the increases in the hormone levels and in the number of developed follicles were suppressed at levels equating those of intact rats. Circulating levels of thyroid hormone were obviously low in the rats receiving DEHP[13, 14], and in our present study, the serum T_4 level was significantly lowered by treatment with 2,000 mg DEHP (P<0.05) in intact rats, whereas the T_4 level tended to be decreased by 2,000 mg DEHP in hypox rats, although the decrease was without statistical significance (Fig. 1). These results are consistent with the hypothesis that the inhibitory effects of thyroid hormone on the follicular growth were revoked by 2,000 mg DEHP through the consumption of circulating thyroid hormone. Factors other than serum T_4 level may be involved in the revocation of DEHP effects at 2,000 mg/kg on the ovulation, because basal levels of serum T_4 were lowered in hypox rats. However, the differences in T_4 levels between control and 2,000 mg/kg groups strongly suggest the role of T_4 in the regulation of ovulation.

It is generally known that E_2 is required for follicular growth in the ovary. Also DEHP is well known to suppress E_2 synthesis *in vivo* and *in vitro*. Thus, the inhibitory effect of 500 mg DEHP on superovulation found in the present study may have been caused by the disruption of ovarian hormonal relationships. However, 2,000 mg of DEHP seemed to revoke this inhibitory effect of DEHP by decreasing the serum T_4 level. We suggest that the thyroid hormone plays an important role in follicular development and ovulation.

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