EFFECTS OF DIETHYLSTILBESTROL ON MALE AND FEMALE PUBERTAL DEVELOPMENT: AN EVALUATION OF THE PROTOCOL FOR THE ASSESSMENT OF PUBETAL DEVELOPMENT AND THYROID FUNCTION

Jae-Ho Shin, Hyung Sik Kim, Hyun Ju Moon, Il Hyun Kang, Tae Sung Kim, Ji Hyun Seok, In Young Kim, and Soon Young Han

Endocrine Toxicology Division, National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul 122-704, Korea

Introduction

For assessing the potential of pesticides and other chemicals to disrupt endocrine function in humans and wildlife, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended a screening and testing program[1]. In developing the recommended Tier 1 Screening (T1S) battery, many existing and potential assays were evaluated for their relative strengths and weaknesses. Rodent 20-day thyroid/pubertal assays are one of the Tier 1 screening assays (female) and one of the alternative assays (male), which are expected to detect some biological activities such as estrogen agonism, androgen agonism/antagonism, and thyroid related effects. The estrogen receptors are distributed widespread in the reproductive tract from fetal life through adulthood, and the exposure to the developing male and females to exogenous estrogenic compounds either in utero or neonatally can result in a range of abnormalities of reproductive development and function. Therefore, estrogens are considered to play a critical role in normal male and female reproductive organ development[2-4]. In the present report, we document the results of the rodent 20-day thyroid/pubertal assay of DES performed in our laboratory and discuss the practical application of the assays for detecting endocrine-related effects of test chemicals.

Materials and Methods

Study design

Sprague-Dawley Crl:CD male and female rats were obtained from the Laboratory Animal Resources NITR/KFDA (Seoul, Korea) under SPF-conditions. Litters of 18-day old male and female rats were housed together by litter in clear polycarbonate cages for 3 days prior to the start of dosing. At 21-day of age, all animals were allocated to the various treatment groups by random sorting in accordance with body weight. Immature male rats (33 day of age, 10 rats/dose) were treated by gavage with DES (0, 10, 20, 40 μg/kg/day) for 20 days. Weaning female rats (21 days of age) were administered by oral gavage with DES (0, 0.2, 1.0, and 5.0 μg/kg/day) for 20 days. The corn oil was

used as the vehicle, and the dose volume was 5.0 ml/kg body weight. Throughout the study period, clinical signs and body weights were observed at least once a day after treatment. Twenty-four hours after the last treatment, each rat was anesthetized with CO_2.

Preputial separation (PPS)

Male rats were inspected daily 9:00 and 10:00 for prepuce separation (PPS). PPS is considered complete when the prepuce can be completely retracted to exposure of the glans penis.

Vaginal opening (VO)

Female rats were examined daily for VO. On the day that VO was first detected, the age and body weights were recorded.

Organ weights

In males rats, testes, epididymes, ventral prostate, seminal vesicles plus coagulating glands and fluid (SVCGF), Cowper's gland, glans penis and levator ani plus bulbocarvernosus muscles (LABC) were weighed. In female rats, the uterus and ovary were carefully dissected and weighed at once. The liver, heart, kidney, thyroid, thymus, pituitary glands, and adrenal glands were carefully dissected and weighed also.

Hormonal measurements

Commercially available radioimmunoassay (RIA) kits were used to measure serum concentrations of E2 and T4 (Amersham Corp., Arlington Height, IL, USA).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) and the Dunnett's test; p values of $<0.05(*)$ were considered to be significant.

Results and Discussion

During the study period, statistically significant decreases in mean body weights were observed in male rats treated with DES (40 μg/kg/day), whereas no significant effects on mean necropsy body weight or body weight gains in female rats. No clinical signs of toxicity were observed in any treatment group (Data not present).

Control male rats represented PPS at 39.5±2.5 day and mean body weight of 237.6±3.5. DES (20 and 40 μg/kg/day) significantly delayed the PPS, but no significant changes in the mean body weight at PPS were observed (Fig. 1).

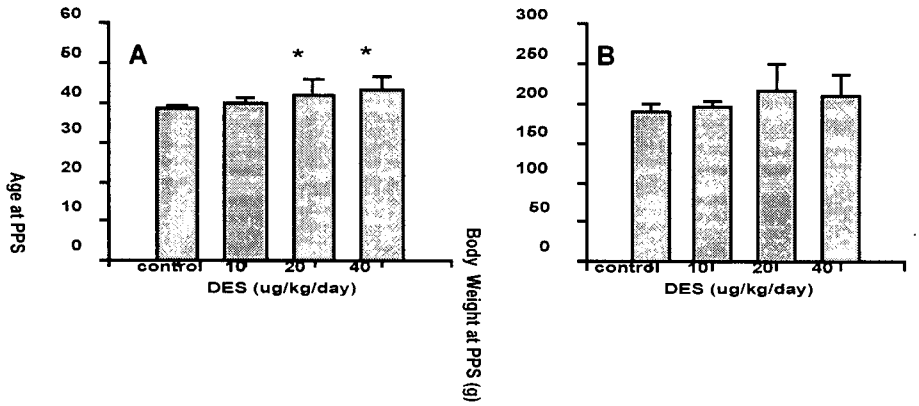

Fig. 1. Effects of DES on the onset of PPS (A) and the body weight at PPS (B) in Sprague-Dawley male rats treated with DES. *Significantly different from the control group ($p<0.05$)

The weight of ventral prostate and SVCGF were significantly reduced in the 20 and 40 μg/kg/day (Table 1). The LABC and glans penis weights were reduced at all doses and the adjacent cowper's gland was significantly smaller at 20 and 40 μg/kg/day (Table 1). The adrenal gland weight was increased at all doses examined (Table 2). The weight of liver was increased in 10 and 20, As for other point such as food intake and weights of thyroid glands, hypophysis, kidney, there were no effects of DES treatment on the serious systemic toxicity. No microscopic changes were observed in the thyroid gland after DES administration (data not shown).

For hormone measurement, serum testosterone and LH levels were significantly decreased at all DES-treated group but serum estradiol levels were increased. But, no significant difference with respect to thyroxin levels was observed (Table 3).

To investigate the role of estrogen agonist on the female pubertal development, we examined the effects of DES on VO day. The mean age at VO was 32.4±1.6 days (range; 30-35 days) and the mean body weight at the age of VO was 117.4±14.8 in control animals. DES significantly advanced the age of VO to 24 days for animals treated with 5.0 μg/kg DES (Fig. 2A). VO was first detected at 24 days of age in all animals treated with 5.0 μg/kg DES. The mean body weight at the time of VO was also significantly reduced in 5.0 μg/kg doses of DES (64.7±4.9)(Fig. 2B). Clark[5] reported that VO in rodents usually occurs around 33-42 days after birth with the body weight just above 100 g. In the present study, the mean range of VO was 30-35 days in Sprague-Dawley female rats, which is consistent with those of previously reported data.[6-8] Several studies have demonstrated that estrogenic chemicals such as methoxychlor, nonylphenol, and octylphenol have been affected VO in rodents[9]. In addition to estrogens, the age at VO and uterine growth can be affected by alteration of several other endocrine mechanisms, including alterations of the growth hormone and steroid hormone synthesis.

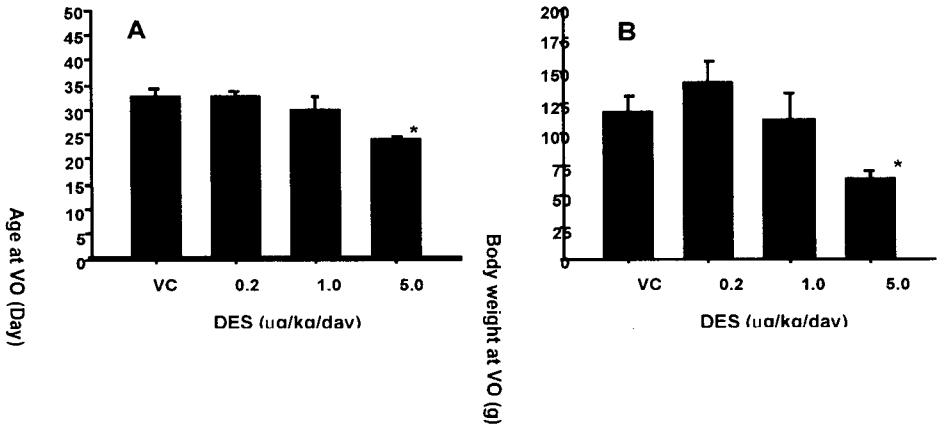

Fig. 2. Effects of DES on the onset of the VO (A) and the body weight at the age of VO (B) in Sprague-Dawley female rats treated with DES. *Significantly different from the control group. ($p<0.05$)

These results are agreement with previous data that DES (silastic implants) advanced VO days followed by 12-17 days of persistent diestrus before regular estrus cycles.[9,10] In the present study, DES (1.0 μg/kg) also significantly reduced ovarian weights, whereas no significant differences in necropsy body weight and uterine weights were observed at any treatment group. DES (5.0 μg/kg/day) significantly decreased the absolute ovary weights (64% of control) as compared with control, whereas DES (5 ug/kg/day) significantly increased the thyroid weight (Table 4). No significant changes in weights of the other organs (liver, heart, kidney, thymus, pituitary, and adrenals) were observed for any DES dose group (Table 5). Serum hormone concentrations were measured individually in all male female rats regardless of stage of estrous cycle. Serum TSH levels were decreased by DES 1.0 and 5.0 ug/kg treatment, but not significant. Serum T4 concentrations were unaffected by DES treatment.

In summary, a potent estrogen agonist (DES) was evaluated to validate the assay for the detection of EDs using male and female pubertal assay, respectively. DES significantly delayed the PPS without any significant changes in the body weight at PPS. In addition, DES significantly advanced the age at VO day. Our data suggest that the male and female pubertal assays may be useful to detect the chemicals having estrogenic activity. Although the compound used in the present study was strong endocrine-active agents, age at VO or at PPS were the most sensitive endpoint to evaluate the these assays. However, further validation study will be necessary to detect weak EDs.

Acknowledgement

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Table 1. Absolute organ weights in Sprague-Dawley immature male rats treated with DES for 20 days.

Group (ug/kg)	Combined Epididymides (g)	Ventral Prostate (g)	Seminal Vesicle (g)	LABC (g)	Cowper's Gland (g)	Glans Penis (g)
Corn oil	0.33±0.03	0.28±0.04	0.54±0.09	0.76±0.07	0.056±0.009	0.085±0.006
DES 10	0.32±0.03	0.29±0.02	0.52±0.04	0.63±0.03*	0.051±0.007	0.073±0.003*
DES 20	0.30±0.03	0.19±0.02*	0.43±0.08*	0.62±0.04*	0.036±0.007*	0.074±0.005*
DES 40	0.26±0.01*	0.14±0.03*	0.13±0.02*	0.39±0.03*	0.021±0.005*	0.061±0.004*

Note. n = 10 animals per treatment group

*Significantly different from control using Dunnett's test ($p < 0.05$)

Table 2. Absolute organ weights in Sprague-Dawley immature male rats treated with DES for 20 days.

Group	Liver (g)	Combined Adrenals (g)	Combined Testes (g)	Thyroid Glands (g)	Hypophysis (g)
Control	9.61±0.07	0.046±0.007	2.73±0.16	0.013±0.003	0.011±0.001
DES 10ug/kg	12.12±0.65*	0.069±0.006*	2.64±0.12	0.017±0.003*	0.011±0.002
DES 20ug/kg	12.94±0.58*	0.071±0.011*	2.56±0.13*	0.010±0.003	0.010±0.003
DES 40ug/kg	9.84±0.73	0.073±0.005*	2.47±0.13*	0.013±0.001	0.011±0.001

Note. n = 10 animals per treatment group

*Significantly different from control using Dunnett's test ($p < 0.05$)

Table 3. Serum hormone levels in Sprague-Dawley immature male rats treated with DES for 20 days.

Group		Testosterone (ng/ml)	Estradiol (pg/ml)	T4 (ng/ml)	LH (pg/ml)
Corn oil		2.1±0.31	23.6±5.46	56.6±5.51	23.3±8.52
DES	10ug/kg	0.5±0.19*	31.2±8.11	75.6±17.43	7.9±1.03*
	20ug/kg	0.6±0.69*	16.2±2.59	69.1±13.05	10.7±5.18*
	40ug/kg	0.3±0.21*	32.2±9.39	54.1±13.48	5.1±0.54*

Note. n = 10 animals per treatment group.

*Significantly different from control using Dunnett's test ($p < 0.05$)

Table 4. Absolute organ weights and serum hormone concentrations in Sprague-Dawley immature female rats treated with DES for 20 days

		Final B.W. (g)	Thyroid gland (mg)	Ovary (mg)	Uterus (g)	TSH (ng/ml)	T4 (ng/ml)
Control	0	156.7±5.72	8.91±1.68	54.77±9.11	0.28±0.07	0.77±0.14	32.5±8.9
DES	0.2μ/kg	164.0±17.4	9.69±3.10	60.28±15.2	0.28±0.05	0.79±0.16	38.7±7.5
	1μg/kg	158.6±14.1	10.17±3.44	47.22±7.90	0.23±0.03	0.46±0.22	37.2±9.6
	5μg/kg	160.2±7.71	10.52±3.42*	35.18±10.3*	0.29±0.06	0.57±0.24	36.4±7.1

Note. n = 10 animals per treatment group

*Significantly different from control using Dunnett's test ($p < 0.05$)

Table 5. Absolute organ weights in Sprague-Dawley immature female rats treated with DES for 20 days

		Liver (g)	Heart (g)	Kidney (g)	Thymus (g)	Pituitary (mg)	Adrenals (mg)
Control	0	5.71±0.29	0.65±0.04	1.49±0.08	0.48±0.09	10.30±1.68	42.43±6.32
DES	0.2μg/kg	5.85±0.56	0.64±0.06	1.49±0.16	0.51±0.05	8.45±1.59	40.60±4.86
	1μg/kg	5.30±0.49	0.61±0.08	1.34±0.13	0.46±0.08	8.31±2.01	39.00±4.53
	5μg/kg	6.01±0.68	0.63±0.06	1.37±0.15	0.47±0.08	9.28±1.71	42.99±4.11

Note. n = 10 animals per treatment group.

*Significantly different from control using Dunnett's test ($p < 0.05$)