COLLABORATIVE WORK TO DETERMINE THE OPTIMAL ADMINISTRATION PERIOD AND PARAMETERS TO DETECT DRUG EFFECTS ON MALE RAT FERTILITY — STUDY ON ESTRADIOL BENZOATE EFFECTS —

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ABSTRACT — In order to examine the optimal administration period and parameters for male fertility assessment, male rats were subcutaneously administered 0.2, 2 or 20 $\mu\text{g/kg}$ of estradiol benzoate (E_2B), a known testicular toxicant, for 4 weeks or 9 weeks before mating.

After 4 weeks administration, suppression of body weight gain and food consumption, decreases in prostate and seminal vesicle weights, atrophy of Leydig cells, and mature spermatid retention at stages IX, X and XI were observed in the 2 and 20 $\mu\text{g/kg}$ groups. In the 20 $\mu\text{g/kg}$ group, decreases in epididymides weight and copulation index were also found but the number of sperm and sperm motility were not affected. In the 0.2 $\mu\text{g/kg}$ group, no changes were noted in any parameters. After 9 weeks administration, decreases in testis weight and the number and motility of sperm were observed in the 20 $\mu\text{g/kg}$ group, in addition to the changes found after 4 weeks administration.

These results suggest that detailed histopathological evaluation and determination of accessory sex organ weights are sensitive for evaluating the effects of E_2B on male fertility. Results with the 4-weeks treatment were comparable to those with the 9-weeks treatment in terms of these parameters.

KEY WORDS : Male fertility assessment, Male reproductive toxicity, Estradiol benzoate, Rats.

INTRODUCTION

Estradiol benzoate (E_2B) is an estrogen which is known to be a testicular toxicant. It has been reported that E_2B -treatment of male rats

causes atrophy of accessory sex organs, epididymis and testis, reductions in the number and motility of sperm, a loss of reproductive ability, inhibition of steroidogenesis in the testes and a decrease in serum testosterone levels (Chinoy and Rao, 1982 ; Rao and Chinoy, 1983 ; Chinoy *et al.*, 1984).

In the present study, male rats were sub-

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cutaneously administered E₂B for 4 weeks or 9 weeks before mating to allow examination of the optimal administration period and parameters for assessment of effects on male fertility.

MATERIALS AND METHODS

Test compound and dose level

Estradiol benzoate (β -estradiol 3-benzoate, E₂B) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Dosage levels of 0.2, 2 and 20 μ g/kg were selected based on the results of a preliminary study (see results).

Compound preparation and administration

E₂B was dissolved in ethanol and diluted with physiological saline containing 0.2% (w/v) Triton X to a final volume for each dose of 1 ml/kg. The concentration of ethanol in the dosing solution was 1% (v/v). E₂B was administered subcutaneously to male rats once daily for 4 weeks in experiment A (Exp. A) and for 9 weeks in experiment B (Exp. B). In order to compare changes observed at the initiation of the mating period with those present after mating in each experiment, male rats allowed to mate were given an additional 4-weeks treatment (8 and 13 weeks in total in Exp. A and B, respectively) throughout the mating period (Fig. 1).

Animals and husbandry

Male and female Slc : SD rats (SPF) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Female rats were used untreated for the purpose of mating. Twenty four male rats were distributed into each group in both experiments.

At the initiation of administration, male rats were 11 and 6 weeks old in Exp. A and Exp. B, respectively (Fig. 1). Except for the mating period, all rats were individually housed. The animals were allowed free access to pellet diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and chlorinated well water. The temperature and humidity in the animal room ranged from 22 to 24°C and from 50 to 60%, respectively. The animal room was maintained on a timer-controlled, 12-hr light/12-hr dark cycle (7:00–19:00).

Experimental design

Clinical signs, body weight and food consumption : Male rats were observed twice daily for mortality and clinical signs, and weighed twice weekly. Food consumption was measured weekly during the premating administration period.

Macroscopic examination and organ weights : In Exp. A, twelve males from each group were sacrificed under anesthesia and examined macroscopically after 4-weeks treatment. The remaining twelve males from each group were sacrificed after 8-weeks treatment. In Exp. B, twelve males from each group were similarly sacrificed after 9- and 13-weeks dosing (Fig. 1).

At sacrifice, testes, epididymides, seminal vesicles, prostate, adrenals and pituitary were weighed and their weights relative to body weight calculated.

Sperm examination : The heavier epididymis was selected from each male to exclude epididymides with spontaneous atrophy and placed in a plastic petri dish containing 2 ml of warmed (37°C) medium. For this purpose, Medium-199

Fig. 1. Study design.

(Gibco Lab. Co., Grand Island, N.Y., U.S.A.) supplemented with 4 mg/ml BSA(Sigma, St. Louis, U.S.A.), 1 mg/ml glucose, 55 μ g/ml sodium pyruvate, 2.5 mg/ml sodium lactate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 50 μ g/ml streptomycin and 50 units/ml penicillin (Gibco Lab. Co., Grand Island, N.Y., U.S.A.) was used with reference to the reports of Slott *et al.*(1991). The cauda epididymis was minced with a surgical blade and the specimen incubated for 5 min at 37°C to allow the sperm to diffuse into the medium. After removing the epididymis, the sample was diluted where necessary with medium to give 4×10^5 – 2×10^6 sperm/ml of medium for analysis. Sperm were counted using a hemacytometer. Sperm motile efficiency, one parameter of sperm motility, was determined using Mitsukawa's method (1979): the number of sperm crossing a line (length : 0.5 mm) in 1 min was counted with a disposable sperm counting chamber (KITAZATO SUPPLY Co. Ltd., Shizuoka, Japan, sp*ace G, depth : 20 μ m) under the microscope and the figure was divided by the sperm density of the sample. Furthermore, sperm in the chambers were videotaped and analyzed for the percentage of motile sperm.

Histopathological evaluation: Testes from males treated for 4 weeks in Exp. A and for 9 weeks in Exp. B were fixed with Bouin's solution, oriented to obtain five cross sections, and embedded in paraffin. Sections from each were cut at approximately 3 μ m and stained with hematoxylin and eosin (H & E) for histological examination. Additional sections were stained with Periodic Acid-Schiff (PAS).

For the microscopic observation, at the same stages of seminiferous tubules were selected for comparison between E₂B treated and control groups. A stage-related analysis was performed using the criteria of Russell *et al.* (1990).

Fertility examination: After 4- (Exp. A) and 9-weeks (Exp. B) treatment, males were mated with intact females (Fig. 1). One male and one female were housed for a maximum of 7 days until copulation was confirmed. Where copulation was not confirmed during this mating period, the male was housed with the another female for a maximum of 7 days. Copulation was confirmed by the appearance of vaginal plugs or the presence of sperm in vaginal smears. The day

when copulation was confirmed was designated day 0 of gestation. From the results, a copulation index (No. of males copulated/No. of males mated $\times$ 100) was calculated.

Intact females copulated were sacrificed on day 20 of gestation and the numbers of corpora lutea, implantations, resorptions, dead and live fetuses were counted. When no implantation was found, the uterus was treated with alkaline solution to confirm implantation sites according to the method of Yamada *et al.* (1988). From those observations, the fertility index (No. of males impregnated/No. of males copulated $\times$ 100) and pre- and post-implantation loss rates were calculated. Each live fetus was sexed, weighed and examined externally.

Statistical analysis

Copulation and fertility indices were analyzed using the Fisher's exact probability test.

For body weight, food consumption, organ weight and number of sperm, variance was tested using the Bartlett method. Where variance was homogenous, one-way analysis of variance was applied; where a significant difference was found between groups, the Dunnett (for uniform numbers of cases per group) or Scheffé method (for non-uniform numbers of cases per group) were applied for comparisons between E₂B-treated and control groups. Where variance was not homogeneous, the Kruskal-Wallis method was applied; where a significant difference was found between groups, the Dunnett type or Scheffé type rank method was applied for comparisons.

For percentage of motile sperm, sperm motile efficiency, the numbers of corpora lutea, implantations, resorptions, dead and live fetuses, pre- and post-implantation loss rates, and the incidence of fetus with external anomalies, the Kruskal-Wallis method was used. In cases where a significant difference was found between groups, the Dunnett or Scheffé type rank methods were then applied.

Levels of significance were set at 5% and 1%. For the items concerning fetuses, data for one litter were treated as a unit.

RESULTS

Preliminary study (Dose finding study)

To determine dose levels for Exp. A and

Photo. 1. Cross section of a testis of a rat treated with 20 μ g estradiol benzoate/kg for 4 weeks (a, b, c) and a control rat testis (d). HE stain. Degeneration/necrosis of spermatocytes (a : arrows), mature spermatid retention at stage X (b : arrows) and atrophy of Leydig cells (c) can be observed. Normal Leydig cells are shown in (d).

Exp. B, E₂B was given subcutaneously to male rats at dose levels of 5, 50 and 250 µg/kg for 2 weeks before mating. Atrophy of Leydig cells and decreases in weights of accessory sex organs were found at dose levels of more than 5 µg/kg. In addition, atrophy of seminiferous tubules, thickening of tunica albuginea, exfoliation, degeneration/necrosis of spermatocytes cells in the testes, decreases in weights of testis and epididymides as well as the number and motility of sperm, and reduced copulation or fertility indices were observed at dose levels of more than 50 µg/kg (Table 1).

Mortality, clinical signs, body weight and food consumption

None of the male rats died during the experiment and no treatment-related abnormalities were observed.

In both Exp. A and Exp. B, body weights of the 2 and 20 µg/kg groups were lower than in the control group. No significant difference was noted in body weight between the 0.2 µg/kg group and the control group. A decrease in food consumption was found in the 2 and 20 µg/kg groups of Exp. A and in the 20 µg/kg group of Exp. B.

Organ weights

After dosing for 4 weeks (Exp. A), the absolute epididymides, prostate and seminal vesicles weights of the 20 µg/kg group were lower than those of the control group, while the absolute testes weight of the 20 µg/kg group did not change. Decreases in prostate and seminal vesicles weights were observed also in the 2 µg/kg group. No changes were observed in the 0.2 µg/kg group. As to the relative organ weights, similar changes were noted except for an increase in the relative testes weight due to suppression of body weight gain. Increases in adrenal and pituitary weights were also observed in both 2 and 20 µg/kg groups (Table 2).

After dosing for 9 weeks (Exp. B), a decrease in the absolute testis weight of the 20 µg/kg group was observed in addition to the changes found after 4-weeks dosing (Table 3).

After the additional 4-weeks dosing (8 weeks in total) in Exp. A, a decrease in the absolute testis weight was detected in the 20 µg/kg group (Table 2). In Exp. B, changes in organ weight after the additional 4-weeks treatment (13 weeks

in total) were comparable with those after 9-weeks dosing (Table 3).

Macroscopic observation

Decreases in testis, epididymis, seminal vesicle and prostate size were found in both Exp. A and Exp. B. Except for this reflection of decreased organ weights, no abnormalities were observed.

Histopathological evaluation

After 4-weeks dosing (Exp. A), atrophy of seminiferous tubules was observed in one male each of the 2 and 20 µg/kg groups. Degeneration/necrosis of spermatocytes (Photo. 1a) were also noted in the 20 µg/kg group. Mature spermatid retention (Lee *et al.*, 1993) at stages IX, X and XI (Photo. 1b) and atrophy of Leydig cells (Photo. 1c) were frequently found in the 2 and 20 µg/kg groups. In the 0.2 µg/kg group, mature spermatid retention was evident in only one male (Table 4).

After 9-weeks dosing (Exp. B), thickening of tunica albuginea, exfoliation of germ cells, decrease in the number of spermatids, and formation of giant cells, in addition to the changes found after 4-weeks dosing, were observed in the 20 µg/kg group. In the 0.2 and 2 µg/kg groups, changes were similar to those found after 4-weeks dosing (Table 4).

Sperm examination

After dosing for 4 weeks (Exp. A), no treatment effects were detected in terms of the number and motility of caudal sperm (Table 5). The percentage of motile sperm in the control group was lower than those of controls in other experiments. However, the lower percentage seemed to be accidental since the sperm motile efficiency was normal.

After dosing for 9 weeks (Exp. B), decreases in the number and percentage of motile caudal sperm and a tendency for decrease of sperm motile efficiency 3 hours after sperm collection were observed in the 20 µg/kg group. In the 2 µg/kg group, the number of sperm was decreased but sperm motility was not changed. No effects were observed in the 0.2 µg/kg group (Table 5).

After the additional 4-weeks dosing (8 weeks in total) in Exp. A, decreases in the number and motility of sperm were observed for the 20 µg/kg dose. In Exp. B, the number and motility of sperm after additional treatment were compara-

Table 1. Summary of results of the preliminary study.

Dose ($\mu\text{g/kg}$)	Control	5	50	250
No. of males examined	5	5	5	5 ⁴⁾
Body weight at sacrifice (g) ¹⁾	492.82 $\pm$ 23.28	470.16 $\pm$ 33.4	455.19 $\pm$ 15.02	398.19 $\pm$ 32.56**
<i>Organ weight</i> ¹⁾				
Testis (right, g)	1.75 $\pm$ 0.11	1.68 $\pm$ 0.10	1.33 $\pm$ 0.12**	1.17 $\pm$ 0.16**
Testis (left, g)	1.78 $\pm$ 0.12	1.75 $\pm$ 0.11	1.36 $\pm$ 0.11**	1.04 $\pm$ 0.30**
Epididymides (g)	1.15 $\pm$ 0.10	1.06 $\pm$ 0.10	0.63 $\pm$ 0.08**	0.46 $\pm$ 0.08**
Prostate (g)	1.20 $\pm$ 0.18	0.77 $\pm$ 0.19**	0.46 $\pm$ 0.08**	0.46 $\pm$ 0.06**
Seminal vesicles (g)	1.17 $\pm$ 0.24	0.63 $\pm$ 0.31	0.25 $\pm$ 0.15**	0.31 $\pm$ 0.03*
<i>Histopathological evaluation</i>				
No. of animals with an abnormality				
Atrophy of seminiferous tubules	0/5	0/5	3/5	4/4
Thickening of tunica albuginea	0/5	0/5	2/5	1/4
Exfoliation of germ cells	0/5	0/5	3/5	3/4
Degeneration of spermatocytes	0/5	0/5	5/5	4/4
Necrosis of spermatocytes	0/5	0/5	2/5	4/4
Atrophy of Leydig cells	0/5	5/5	5/5	4/4
<i>Sperm examination</i> ¹⁾				
No. of sperm (10^7 cells)	2.58 $\pm$ 0.48	2.54 $\pm$ 0.57	1.16 $\pm$ 0.61**	0.24 $\pm$ 0.25**
Percentage motile	57.4 $\pm$ 20.5	39.4 $\pm$ 11.7	17.1 $\pm$ 14.6**	9.4 $\pm$ 7.6**
<i>Fertility data</i>				
Copulation index	100.0 (5/5) ²⁾	100.0 (5/5)	80.0 (4/5)	0.0 (0/5)**
Fertility index	100.0 (5/5) ³⁾	60.0 (3/5)	0.0 (0/4)**	—
<i>At cesarean section</i>				
No. of corpora lutea (mean $\pm$ S.D.)	73 (14.6 $\pm$ 2.4)	44 (14.7 $\pm$ 2.5)	0	0
No. of implantations (mean $\pm$ S.D.)	69 (13.8 $\pm$ 2.8)	40 (13.3 $\pm$ 1.5)	0	0
Pre-implantation loss rate (%)	5.9	8.4	—	—
No. of resorptions (mean $\pm$ S.D.)	3 (0.6 $\pm$ 0.5)	6 (2.0 $\pm$ 1.0)*	—	—
Post-implantation loss rate (%)	4.0	14.6*	—	—
No. of live fetuses (mean $\pm$ S.D.)	66 (13.2 $\pm$ 2.4)	34 (11.3 $\pm$ 0.6)	—	—
Sex ratio (male %)	62.4	47.5	—	—
Fetal weight (g, mean $\pm$ S.D.) : male	3.57 $\pm$ 0.13	3.93 $\pm$ 0.01*	—	—
female	3.37 $\pm$ 0.07	3.54 $\pm$ 0.23	—	—
No. of fetuses with external anomalies (%)	0	1 (3.0)	—	—
Kinky tail	0	1 (3.0)	—	—

— : Not examined.

*,** : Significantly different from the control (* : $P < 0.05$, ** : $P < 0.01$).¹⁾ : Values represent the mean $\pm$ S.D.²⁾ : No. of males with successful copulation/No. of males examined.³⁾ : No. of impregnating males/No. of males with successful copulation.⁴⁾ : One male could not be examined in the histopathology evaluation due to severe spontaneous changes.

Table 2. Mean organ weights of male rats in experiment A.

Dose ($\mu\text{g/kg}$)	Control	0.2	2	20
<i>Experiment A : 4 weeks</i>				
No. of males examined	12	12	12	12
Body weight at sacrifice (g)	425.8 $\pm$ 31.5	427.6 $\pm$ 20.2	397.6 $\pm$ 23.1*	383.6 $\pm$ 14.3**
Absolute organ weight				
Testis (right, g)	1.55 $\pm$ 0.06	1.58 $\pm$ 0.07	1.58 $\pm$ 0.14	1.54 $\pm$ 0.12
Testis (left, g)	1.59 $\pm$ 0.07	1.61 $\pm$ 0.09	1.61 $\pm$ 0.16	1.59 $\pm$ 0.11
Epididymides (g)	1.06 $\pm$ 0.06	1.06 $\pm$ 0.07	1.04 $\pm$ 0.07	0.98 $\pm$ 0.10*
Prostate (g)	1.02 $\pm$ 0.15	0.99 $\pm$ 0.11	0.84 $\pm$ 0.14*	0.67 $\pm$ 0.23**
Seminal vesicles (g)	1.01 $\pm$ 0.23	0.98 $\pm$ 0.16	0.76 $\pm$ 0.17**	0.57 $\pm$ 0.21**
Adrenals (mg)	62 $\pm$ 8	68 $\pm$ 9	75 $\pm$ 9**	78 $\pm$ 9**
Pituitary (mg)	10 $\pm$ 2	11 $\pm$ 1	13 $\pm$ 3**	13 $\pm$ 2*
Relative organ weight ¹⁾				
Testis (right, %)	0.367 $\pm$ 0.029	0.371 $\pm$ 0.026	0.399 $\pm$ 0.040	0.402 $\pm$ 0.031*
Testis (left, %)	0.375 $\pm$ 0.032	0.376 $\pm$ 0.028	0.407 $\pm$ 0.049	0.416 $\pm$ 0.027*
Epididymides (%)	0.249 $\pm$ 0.023	0.249 $\pm$ 0.021	0.263 $\pm$ 0.022	0.255 $\pm$ 0.026
Prostate (%)	0.239 $\pm$ 0.037	0.232 $\pm$ 0.027	0.211 $\pm$ 0.036	0.175 $\pm$ 0.059**
Seminal vesicles (%)	0.237 $\pm$ 0.052	0.228 $\pm$ 0.033	0.191 $\pm$ 0.043*	0.148 $\pm$ 0.052**
Adrenals (%)	0.0147 $\pm$ 0.0024	0.0158 $\pm$ 0.0020	0.0187 $\pm$ 0.0021**	0.0203 $\pm$ 0.0028**
Pituitary (%)	0.0023 $\pm$ 0.0007	0.0027 $\pm$ 0.0003	0.0033 $\pm$ 0.0006**	0.0033 $\pm$ 0.0006**
<i>Experiment A : 8 weeks</i>				
No. of males examined	12	12	12	12
Body weight at sacrifice (g)	469.3 $\pm$ 25.0	454.6 $\pm$ 33.9	434.1 $\pm$ 23.1**	397.5 $\pm$ 20.9**
Absolute organ weight				
Testis (right, g)	1.67 $\pm$ 0.13	1.63 $\pm$ 0.11	1.55 $\pm$ 0.17	1.26 $\pm$ 0.38**
Testis (left, g)	1.70 $\pm$ 0.14	1.67 $\pm$ 0.11	1.58 $\pm$ 0.17	1.32 $\pm$ 0.40**
Epididymides (g)	1.23 $\pm$ 0.10	1.18 $\pm$ 0.07	1.02 $\pm$ 0.17**	0.79 $\pm$ 0.24**
Prostate (g)	1.10 $\pm$ 0.13	1.10 $\pm$ 0.14	0.84 $\pm$ 0.28	0.50 $\pm$ 0.18**
Seminal vesicles (g)	1.18 $\pm$ 0.08	1.09 $\pm$ 0.19	0.83 $\pm$ 0.32*	0.36 $\pm$ 0.25**
Adrenals (mg)	57 $\pm$ 10	63 $\pm$ 7	79 $\pm$ 14**	87 $\pm$ 9**
Pituitary (mg)	12 $\pm$ 2	13 $\pm$ 2	14 $\pm$ 3	18 $\pm$ 4**
Relative organ weight ¹⁾				
Testis (right, %)	0.356 $\pm$ 0.027	0.360 $\pm$ 0.039	0.356 $\pm$ 0.031	0.315 $\pm$ 0.092
Testis (left, %)	0.363 $\pm$ 0.026	0.370 $\pm$ 0.039	0.364 $\pm$ 0.030	0.329 $\pm$ 0.097
Epididymides (%)	0.263 $\pm$ 0.024	0.260 $\pm$ 0.025	0.235 $\pm$ 0.034	0.198 $\pm$ 0.058**
Prostate (%)	0.235 $\pm$ 0.024	0.243 $\pm$ 0.035	0.192 $\pm$ 0.063	0.126 $\pm$ 0.041**
Seminal vesicles (%)	0.253 $\pm$ 0.028	0.241 $\pm$ 0.044	0.190 $\pm$ 0.071	0.088 $\pm$ 0.061**
Adrenals (%)	0.0121 $\pm$ 0.0019	0.0139 $\pm$ 0.0014	0.0183 $\pm$ 0.0034**	0.0220 $\pm$ 0.0029**
Pituitary (%)	0.0025 $\pm$ 0.0004	0.0029 $\pm$ 0.0006	0.0032 $\pm$ 0.0008*	0.0045 $\pm$ 0.0012**

Values represent the mean $\pm$ S.D.*,** : Significantly different from the control (* : $P < 0.05$, ** : $P < 0.01$).¹⁾ : Organ weight/Body weight at sacrifice $\times 100$.

Table 3. Mean organ weights of male rats in experiment B.

Dose ($\mu\text{g/kg}$)	Control	0.2	2	20
<i>Experiment B : 9 weeks</i>				
No. of males examined	12	12	12	12
Body weight at sacrifice (g)	452.9 $\pm$ 54.8	437.3 $\pm$ 30.4	400.9 $\pm$ 30.1**	350.7 $\pm$ 35.2**
Absolute organ weight				
Testis (right, g)	1.58 $\pm$ 0.10	1.58 $\pm$ 0.09	1.58 $\pm$ 0.11	1.14 $\pm$ 0.28**
Testis (left, g)	1.59 $\pm$ 0.10	1.59 $\pm$ 0.10	1.63 $\pm$ 0.12	1.18 $\pm$ 0.27**
Epididymides (g)	1.05 $\pm$ 0.09	1.01 $\pm$ 0.19	0.96 $\pm$ 0.07	0.67 $\pm$ 0.24**
Prostate (g)	0.96 $\pm$ 0.14	0.98 $\pm$ 0.15	0.75 $\pm$ 0.17**	0.36 $\pm$ 0.16**
Seminal vesicles (g)	1.01 $\pm$ 0.18	0.98 $\pm$ 0.23	0.78 $\pm$ 0.17*	0.29 $\pm$ 0.16**
Adrenals (mg)	61 $\pm$ 8	65 $\pm$ 11	81 $\pm$ 11**	89 $\pm$ 10**
Pituitary (mg)	10 $\pm$ 2	13 $\pm$ 2*	13 $\pm$ 2	14 $\pm$ 3**
Relative organ weight ¹⁾				
Testis (right, %)	0.354 $\pm$ 0.046	0.360 $\pm$ 0.023	0.398 $\pm$ 0.045*	0.330 $\pm$ 0.086
Testis (left, %)	0.356 $\pm$ 0.047	0.363 $\pm$ 0.024	0.408 $\pm$ 0.048*	0.341 $\pm$ 0.086
Epididymides (%)	0.235 $\pm$ 0.033	0.231 $\pm$ 0.044	0.242 $\pm$ 0.024	0.193 $\pm$ 0.072
Prostate (%)	0.215 $\pm$ 0.035	0.224 $\pm$ 0.033	0.188 $\pm$ 0.044	0.104 $\pm$ 0.048**
Seminal vesicles (%)	0.224 $\pm$ 0.042	0.222 $\pm$ 0.053	0.195 $\pm$ 0.048	0.081 $\pm$ 0.045**
Adrenals (%)	0.0135 $\pm$ 0.0012	0.0148 $\pm$ 0.0022	0.0203 $\pm$ 0.0030**	0.0256 $\pm$ 0.0030**
Pituitary (%)	0.0023 $\pm$ 0.0004	0.0030 $\pm$ 0.0004	0.0032 $\pm$ 0.0004**	0.0039 $\pm$ 0.0009**
<i>Experiment B : 13 weeks</i>				
No. of males examined	12	12	12	12
Body weight at sacrifice (g)	460.7 $\pm$ 42.6	462.8 $\pm$ 39.2	439.0 $\pm$ 25.0	382.8 $\pm$ 26.4**
Absolute organ weight				
Testis (right, g)	1.57 $\pm$ 0.10	1.59 $\pm$ 0.12	1.63 $\pm$ 0.09	1.30 $\pm$ 0.16**
Testis (left, g)	1.58 $\pm$ 0.08	1.64 $\pm$ 0.13	1.64 $\pm$ 0.07	1.35 $\pm$ 0.16*
Epididymides (g)	1.13 $\pm$ 0.11	1.15 $\pm$ 0.10	1.07 $\pm$ 0.09*	0.68 $\pm$ 0.14**
Prostate (g)	1.02 $\pm$ 0.14	1.00 $\pm$ 0.14	0.77 $\pm$ 0.16**	0.42 $\pm$ 0.18**
Seminal vesicles (g)	1.05 $\pm$ 0.25	1.17 $\pm$ 0.23	0.79 $\pm$ 0.14*	0.30 $\pm$ 0.19**
Adrenals (mg)	62 $\pm$ 10	67 $\pm$ 9	91 $\pm$ 9**	97 $\pm$ 9**
Pituitary (mg)	10 $\pm$ 3	12 $\pm$ 1	14 $\pm$ 2**	17 $\pm$ 6**
Relative organ weight ¹⁾				
Testis (right, %)	0.344 $\pm$ 0.028	0.346 $\pm$ 0.032	0.371 $\pm$ 0.022	0.340 $\pm$ 0.045
Testis (left, %)	0.344 $\pm$ 0.027	0.356 $\pm$ 0.029	0.375 $\pm$ 0.021	0.354 $\pm$ 0.046
Epididymides (%)	0.247 $\pm$ 0.032	0.249 $\pm$ 0.027	0.243 $\pm$ 0.020	0.177 $\pm$ 0.038**
Prostate (%)	0.222 $\pm$ 0.032	0.216 $\pm$ 0.032	0.176 $\pm$ 0.034**	0.107 $\pm$ 0.041**
Seminal vesicles (%)	0.228 $\pm$ 0.057	0.255 $\pm$ 0.056	0.181 $\pm$ 0.033	0.078 $\pm$ 0.049**
Adrenals (%)	0.0136 $\pm$ 0.0022	0.0146 $\pm$ 0.0017	0.0207 $\pm$ 0.0022**	0.0225 $\pm$ 0.0025**
Pituitary (%)	0.0021 $\pm$ 0.0006	0.0026 $\pm$ 0.0003	0.0032 $\pm$ 0.0005**	0.0044 $\pm$ 0.0016**

Values represent the mean $\pm$ S.D.*** : Significantly different from the control (* : $P < 0.05$, ** : $P < 0.01$).¹⁾ : Organ weight/Body weight at sacrifice $\times 100$.

Table 4. Summary of results of histopathological evaluation.

Dose ($\mu\text{g/kg}$)	Control	0.2	2	20
<i>Experiment A : 4 weeks</i>				
No. of males examined	12	12	12	12
No. of animals with an abnormality				
Atrophy of seminiferous tubules (focal)	0	0	1	1
Degeneration/necrosis of spermatocytes	0	0	0	5
Mature spermatid retention at stages IX, X and XI	0	1	3	5
Atrophy of Leydig cells	0	0	4	11
<i>Experiment B : 9 weeks</i>				
No. of males examined	12	12	12	12 ¹⁾
No. of animals with an abnormality				
Atrophy of seminiferous tubules (diffuse)	0	0	0	4
Atrophy of seminiferous tubules (focal)	0	0	0	1
Thickening of tunica albuginea	0	0	0	3
Exfoliation of germ cells	0	0	0	1
Degeneration/necrosis of spermatocytes	0	0	0	10
Decrease in number of spermatids	0	0	0	1 ¹⁾
Formation of giant cells	0	0	0	1
Mature spermatid retention at stages IX, X and XI	1	2	5	6
Atrophy of Leydig cells	0	0	9	11

¹⁾ : In one male, the spermatogenic stage could not be identified due to severe atrophy of seminiferous tubules. Therefore, a stage-related analysis could not be performed.

ble to data after 9 weeks (Table 5).

Fertility data and observation at cesarean section

In Exp. A, a significant reduction of copulation index and a tendency for decreased fertility index were evident in the 20 $\mu\text{g/kg}$ group. In Exp. B, tendencies for reduction of the copulation and fertility indices were also observed with the 20 $\mu\text{g/kg}$ dose (Table 6).

At cesarean section, pre-implantation loss rates for the 20 $\mu\text{g/kg}$ group were found to be significantly increased in both Exp. A and Exp. B. This was accompanied by tendencies for decreased numbers of implantations and live fetuses. In both experiments, post-implantation loss rate, fetal weights and sex ratio for each treated group were comparable to control values. In Exp. A, exencephaly, anophthalmia with cleft

plate and lip, and omphalocele were found in one fetus of the 0.2, 2 and 20 $\mu\text{g/kg}$ groups, respectively. In Exp. B, hypoplasia of the maxilla and mandible was found in only one fetus of the 20 $\mu\text{g/kg}$ group (Table 6).

DISCUSSION

As shown in Table 7, histopathological changes in the testes were detected without alterations in testis weight. Therefore, we conclude that a detailed histopathological evaluation is most sensitive for determining whether or not a test compound exerts testicular toxicity. With respect to organ weights, decreases in accessory sex organ weights were detected at a lower dose level than decreases in testis and epididymides

Table 5. Summary of results of sperm examination.

Dose ($\mu\text{g/kg}$)		Control	0.2	2	20
<i>Experiment A : 4 weeks</i>					
No. of males examined		12	12	12	12
No. of sperm (10^7 cells)		13.66 $\pm$ 3.75	13.42 $\pm$ 4.68	13.39 $\pm$ 3.74	10.94 $\pm$ 2.10
Percentage motile	0 time	54.7 $\pm$ 13.3	68.1 $\pm$ 14.2	71.0 $\pm$ 11.2*	62.1 $\pm$ 14.3
	3 hr	55.6 $\pm$ 15.2	55.8 $\pm$ 21.2	55.3 $\pm$ 24.5	58.3 $\pm$ 21.7
	6 hr	61.1 $\pm$ 13.6	54.2 $\pm$ 9.7	53.6 $\pm$ 19.4	46.9 $\pm$ 17.4
Sperm motile efficiency ¹⁾	0 time	0.911 $\pm$ 0.739	0.990 $\pm$ 0.579	1.027 $\pm$ 0.328	0.928 $\pm$ 0.522
	3 hr	0.444 $\pm$ 0.274	0.522 $\pm$ 0.341	0.759 $\pm$ 0.462	0.488 $\pm$ 0.338
	6 hr	0.695 $\pm$ 0.586	0.546 $\pm$ 0.423	0.579 $\pm$ 0.458	0.490 $\pm$ 0.390
<i>Experiment A : 8 weeks</i>					
No. of males examined		12	12	12	12
No. of sperm (10^7 cells)		15.95 $\pm$ 3.90	16.13 $\pm$ 2.86	12.72 $\pm$ 4.60	7.59 $\pm$ 5.28**
Percentage motile	0 time	84.7 $\pm$ 11.9	83.5 $\pm$ 8.7	80.1 $\pm$ 9.2	64.5 $\pm$ 31.9
	3 hr	80.3 $\pm$ 9.6	72.5 $\pm$ 11.2	71.5 $\pm$ 15.0	51.8 $\pm$ 29.1**
	6 hr	70.1 $\pm$ 12.9	64.5 $\pm$ 10.7	70.4 $\pm$ 16.8	42.7 $\pm$ 28.4*
Sperm motile efficiency	0 time	0.817 $\pm$ 0.537	0.733 $\pm$ 0.508	0.843 $\pm$ 0.401	0.450 $\pm$ 0.439
	3 hr	0.583 $\pm$ 0.358	0.394 $\pm$ 0.243	0.623 $\pm$ 0.227	0.251 $\pm$ 0.226*
	6 hr	0.403 $\pm$ 0.276	0.482 $\pm$ 0.321	0.527 $\pm$ 0.367	0.376 $\pm$ 0.401
<i>Experiment B : 9 weeks</i>					
No. of males examined		12	12	12	12
No. of sperm (10^7 cells)		12.52 $\pm$ 3.21	12.97 $\pm$ 3.35	9.04 $\pm$ 2.54*	3.37 $\pm$ 2.24**
Percentage motile	0 time	86.8 $\pm$ 7.3	88.6 $\pm$ 4.0	85.0 $\pm$ 8.6	67.5 $\pm$ 27.2
	3 hr	78.5 $\pm$ 6.5	79.0 $\pm$ 6.2	72.1 $\pm$ 10.0	51.7 $\pm$ 24.4**
	6 hr	74.8 $\pm$ 11.8	69.1 $\pm$ 20.0	66.9 $\pm$ 11.6	59.5 $\pm$ 20.7
Sperm motile efficiency	0 time	0.720 $\pm$ 0.422	0.945 $\pm$ 0.467	0.827 $\pm$ 0.320	0.414 $\pm$ 0.449
	3 hr	0.557 $\pm$ 0.271	0.738 $\pm$ 0.496	0.582 $\pm$ 0.363	0.261 $\pm$ 0.315
	6 hr	0.350 $\pm$ 0.338	0.444 $\pm$ 0.356	0.407 $\pm$ 0.284	0.215 $\pm$ 0.241
<i>Experiment B : 13 weeks</i>					
No. of males examined		12	12	12	12
No. of sperm (10^7 cells)		12.79 $\pm$ 3.00	12.03 $\pm$ 2.33	11.74 $\pm$ 1.81	3.37 $\pm$ 2.48**
Percentage motile	0 time	85.4 $\pm$ 10.1	76.1 $\pm$ 13.7	81.5 $\pm$ 9.0	51.0 $\pm$ 20.5**
	3 hr	76.9 $\pm$ 8.8	77.7 $\pm$ 7.6	70.3 $\pm$ 11.6	43.0 $\pm$ 23.2**
	6 hr	63.3 $\pm$ 16.9	67.9 $\pm$ 8.0	60.1 $\pm$ 15.3	42.1 $\pm$ 12.0**
Sperm motile efficiency	0 time	0.661 $\pm$ 0.290	0.778 $\pm$ 0.519	0.851 $\pm$ 0.815	0.289 $\pm$ 0.234*
	3 hr	0.847 $\pm$ 0.601	0.685 $\pm$ 0.241	0.468 $\pm$ 0.226	0.175 $\pm$ 0.220**
	6 hr	0.654 $\pm$ 0.433	0.699 $\pm$ 0.220	0.450 $\pm$ 0.210	0.137 $\pm$ 0.199**

Values represent the mean $\pm$ S.D.*** : Significantly different from the control (* : $P < 0.05$, ** : $P < 0.01$).¹⁾ : No. of sperm crossing the line in 1 min/Sperm density of the sample.

Table 6. Summary of fertility data.

Dose ($\mu\text{g/kg}$)	Control	0.2	2	20
<i>Experiment A</i>				
No. of males examined ¹⁾	12	12	12	12
Fertility data				
Copulation index	91.7 (11/12) ³⁾	91.7 (11/12)	83.3 (10/12)	50.0 (6/12)*
Fertility index	100.0 (11/11) ⁴⁾	90.9 (10/11)	90.0 (9/10)	83.3 (5/6)
At cesarean section				
No. of corpora lutea (mean $\pm$ S.D.)	160 (14.5 $\pm$ 1.4)	152 (15.2 $\pm$ 1.8)	132 (14.7 $\pm$ 1.3)	62 (12.4 $\pm$ 4.4)
No. of implantations (mean $\pm$ S.D.)	152 (13.8 $\pm$ 1.7)	142 (14.2 $\pm$ 1.8)	123 (13.7 $\pm$ 1.9)	40 (8.0 $\pm$ 5.6)
Pre-implantation loss rate (%)	5.1	6.4	6.8	42.1*
No. of resorptions (mean $\pm$ S.D.)	25 (2.3 $\pm$ 2.1)	27 (2.7 $\pm$ 2.8)	17 (1.9 $\pm$ 1.4)	6 (1.2 $\pm$ 1.3)
Post-implantation loss rate (%)	16.7	19.5	14.5	12.4
No. of live fetuses (mean $\pm$ S.D.)	127 (11.5 $\pm$ 2.8)	115 (11.5 $\pm$ 3.5)	106 (11.8 $\pm$ 2.6)	34 (6.8 $\pm$ 4.6)
Sex ratio (male %)	51.7	57.5	58.1	58.5
Fetal weight (g, mean $\pm$ S.D.) : male	3.66 $\pm$ 0.16	3.34 $\pm$ 0.30	3.45 $\pm$ 0.23	3.74 $\pm$ 0.32
female	3.43 $\pm$ 0.26	3.09 $\pm$ 0.40	3.26 $\pm$ 0.26	3.45 $\pm$ 0.06
No. of fetuses with external anomalies (%)	0	1 (0.7)	1 (1.1)	1 (1.8)
Exencephaly	0	1 (0.7)	0	0
Anophthalmia, Cleft palate and lip	0	0	1 (1.1)	0
Omphalocele	0	0	0	1 (1.8)
<i>Experiment B</i>				
No. of males examined ²⁾	12	12	12	12
Fertility data				
Copulation index	100.0 (12/12) ³⁾	100.0 (12/12)	100.0 (12/12)	75.0 (9/12)
Fertility index	91.7 (11/12) ⁴⁾	100.0 (12/12)	100.0 (12/12)	66.7 (6/9)
At cesarean section				
No. of corpora lutea (mean $\pm$ S.D.)	162 (14.7 $\pm$ 1.0)	184 (15.3 $\pm$ 1.5)	177 (14.8 $\pm$ 1.3)	82 (13.7 $\pm$ 2.5)
No. of implantations (mean $\pm$ S.D.)	160 (14.5 $\pm$ 1.1)	182 (15.2 $\pm$ 1.3)	160 (13.3 $\pm$ 1.1)	47 (7.8 $\pm$ 7.0)
Pre-implantation loss rate (%)	1.3	1.0	9.0	48.5*
No. of resorptions (mean $\pm$ S.D.)	23 (2.1 $\pm$ 1.6)	15 (1.3 $\pm$ 1.0)	21 (1.8 $\pm$ 1.8)	4 (0.7 $\pm$ 1.0)
Post-implantation loss rate (%)	14.1	9.5	12.1	4.5
No. of live fetuses (mean $\pm$ S.D.)	137 (12.5 $\pm$ 1.6)	165 (13.8 $\pm$ 1.7)	141 (11.8 $\pm$ 1.8)	43 (7.2 $\pm$ 6.2)
Sex ratio (male %)	48.5	53.3	50.3	48.3
Fetal weight (g, mean $\pm$ S.D.) : male	3.79 $\pm$ 0.22	3.63 $\pm$ 0.23	3.73 $\pm$ 0.28	3.75 $\pm$ 0.30
female	3.52 $\pm$ 0.15	3.47 $\pm$ 0.14	3.32 $\pm$ 0.39	3.63 $\pm$ 0.49
No. of fetuses with external anomalies (%)	0	0	0	1 (16.7)
Hypoplasia of maxilla and mandible	0	0	0	1 (16.7)

* : Significantly different from the control ($P < 0.05$).¹⁾ : These males were mated from day 28 after the initiation of dosing and sacrificed after dosing for 8 weeks.²⁾ : These males were mated from day 63 after the initiation of dosing and sacrificed after dosing for 13 weeks.³⁾ : No. of males with successful copulation/No. of males examined.⁴⁾ : No. of impregnating males/No. of males with successful copulation.

Table 7. Summary of maximum no effect dose levels for each parameter.

Experiment	Maximum no effect dose ($\mu\text{g/kg}$)				Results of comparison between short term and long term administration
	Experiment A		Experiment B		
Length of administration period	4 weeks	8 weeks	9 weeks	13 weeks	
Paternal mortality	20	20	20	20	S=L ¹⁾
Clinical sings	20	20	20	20	S=L
Body weight	0.2	0.2	0.2	0.2	S=L
Food consumption	0.2	—	2	—	S≠L
Organ weight					
Testes	20	2	2	2	S≠L
Epididymides	2	0.2	2	0.2	S≠L
Prostate	0.2	0.2	0.2	0.2	S=L
Seminal vesicle	0.2	0.2	0.2	0.2	S=L
Macroscopic observation	2	0.2	2	0.2	S≠L?
Histopathological evaluation ²⁾	0.2	—	0.2	—	S=L
Sperm examination					
Sperm number	20	2	0.2	2	S≠L
Sperm motility	20	2	2	2	S≠L
Fertility test					
Copulation	2	—	2	—	S=L?
Fertility	20	—	2?	—	S≠L?
Cesarean section	2	—	2	—	S=L

— : Not examined.

¹⁾ : S=L represents cases where the maximum no effect dose of short term administration was comparable with that of long term administration. S≠L represents cases where short term administration was not comparable with long term administration.²⁾ : Testis only.

? : Unclear.

weights. Thus accessory sex organ weight may be sensitive parameter for evaluating the reproductive toxicity of a compound which has a hormonal influence, such as E₂B. No effects on copulation and fertility were observed at a dose level causing reduced accessory sex organ weights. This indicates that mating results alone are not sufficient to detect compound effects on male fertility. As for sperm examination, E₂B influence on motility was remarkable 3 hours after sperm collection. It is known that hamster epididymal sperm become fully capacitated when they are incubated *in vitro* for 3 hours or more (Yanagimachi, 1969). Therefore, the sperm

motility at about 3 hours after collection is assumed to be related to the fertility index and may be an important parameter for evaluation of male reproductive function.

With respect to the length of the administration period before mating, the results of histopathological evaluations after 4-weeks treatment were comparable to those after 9-weeks treatment. Decreases in weights of accessory sex organs were noted after both 4 and 9 weeks treatment. Thus, the effects of E₂B could be detected by these sensitive parameters, even if a premating administration period of only 4 weeks is applied. Decreases in testis weights, number

and motility of sperm, and a decreasing tendency of fertility index were in contrast found only after 9 weeks treatment. Optional sperm examination methods for experimental animals have not been fully established as yet and it is to be hoped that sensitive parameters will be found with improvements in this area.

Rao and Chinoy (1983) have proposed that the changes caused by E₂B treatment are due to direct effects on testosterone production in the testis rather than effects on negative feedback on the pituitary gonadotropins (FSH and LH), since the compound induces a decrease in serum testosterone level but does not affect serum FSH and LH levels in male rats. Several reports have suggested the presence of estrogen receptors in the cytoplasm of Leydig cells (Brinkmann *et al.*, 1972; Abney, 1976) and that estrogen has a direct effect on their testosterone biosynthesis (Sairam and Berman, 1979; Kalla *et al.*, 1980). The atrophy of Leydig cells found in this study is in line with the published data for serum testosterone.

We conclude that a detailed histopathological evaluation and determination of accessory sex organ weights allow sensitive evaluation of the effects of E₂B on male fertility. Provided these parameters are used, 4-weeks treatment is as effective as 9-weeks treatment.

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REFERENCES

- Abney, T. O. (1976) : A cytoplasmic estradiol receptor in rat testicular tissue. *Endocrinology*, **99**, 555–566.
- Brinkmann, A. O., Mulder, E., Lamers-Stahlhofen, G. J. M. and Van der Molen, H. J. (1972) : An oestradiol receptor in rat testis interstitial tissue. *FEBS Letters*, **26**, 301–305.
- Chinoy, N. J. and Rao, M. V. (1982) : Effect of estradiol benzoate on hormonal profile and steroid genetic organs of immature and adult male rats. *Endocrinology*, **80**, 287–293.
- Chinoy, M. R., Sharama, J. D. and Chinoy, N. J. (1984) : Altered structural and functional integrity of the reproductive tissues in estradiol benzoate-treated intact male albino rats. *Int. J. Fertil.*, **29**, 98–103.
- Kalla, N. R., Nisula, B. C., Menard, R. and Loriaux, D. L. (1980) : The effect of estradiol on testicular testosterone biosynthesis. *Endocrinology*, **106**, 35–39.
- Lee, K-P., Frame, S. R. and Valentine, R. (1993) : Testicular degeneration and spermatid retention in young male rats. *Toxicol. Pathol.*, **21**, 292–302.
- Mitsukawa, S. (1979) : A new method for determining sperm motility : the clinical application of sperm motile efficiency. *Nippon Hinyokika Gakkai Zasshi*, **70**, 1221–1231.
- Rao, M. V. and Chinoy, N. J. (1983) : Effect of estradiol benzoate on reproductive organs and fertility in the male rat. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **15**, 189–198.
- Russell, L. D., Ettlin, R. A., Hikim, A. P. S. and Clegg, E. D. (1990) : Histological and histopathological evaluation of the testis. pp. 62–194, Cache River Press, Clearwater, FL.
- Sairam, M. R. and Berman, M. I. (1979) : Direct inhibitory effects of estrogens on rat Leydig cells *in vitro*. *Steroids*, **33**, 233–241.
- Slott, V. L., Suarez, J. D. and Perreault, S. D. (1991) : Rat sperm motility analysis : methodologic considerations. *Reprod. Toxicol.*, **5**, 449–458.
- Yamada, T., Ohsawa, K. and Ohno, H. (1988) : The usefulness of alkaline solution for clearing the uterus and staining implantation sites in rats. *Exp. Anim.*, **37**, 325–331.
- Yanagimachi, R. (1969) : *In vitro* capacitation of hamster spermatozoa by follicular fluid. *J. Reprod. Fert.*, **18**, 275–286.