MALE REPRODUCTIVE TOXICITY OF ETHINYLESTRADIOL ASSOCIATED WITH 4 WEEKS DAILY DOSING PRIOR TO MATING IN RATS

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ABSTRACT — Parameters of male reproductive toxicity of ethinylestradiol were assessed by conducting a mating test, sperm assay, organ weight determination and histopathological examination. Male Sprague Dawley rats were orally administered 0.1, 0.3, 3 or 10 mg/kg/day ethinylestradiol for 4 weeks prior to mating. Body weight gain and food consumption were suppressed in all treated groups. Reproductive ability of the 3 and 10 mg/kg/day males disappeared. Slightly low copulation indices were observed in the 0.1 and 0.3 mg/kg/day groups, although fertility indices were not affected. Sperm could hardly be found in the epididymis of 3 and 10 mg/kg/day males. Sperm counts were also decreased in the other treated groups, but sperm motility was not affected. Decreased absolute and/or relative weights of testes, epididymides, prostate and seminal vesicles were observed in all treated groups along with testis, epididymis, seminal vesicle and prostate atrophy, and degenerative changes of spermatocytes, spermatids, Sertoli cells and Leydig cells. These results suggest that sperm quantification and histopathological assessment are more appropriate for assessing male reproductive toxicity of ethinylestradiol than performance of copulation and fertility tests.

KEY WORDS : Ethinylestradiol, Reproductive toxicity test, Sperm count, Sperm motility, Histopathological examination.

INTRODUCTION

Ethinylestradiol (19-Nor-17 α -pregna-1,3,5(10)-trien-20-yen-3,17-diol), a synthetic estrogen, has been used as a major component of oral contraceptives and a carcinostatic agent for prostate and breast carcinomas. Therapeutic doses are reported to be 0.3 to 100 mg/day in man (Nakayama *et al.*, 1979). Ethinylestradiol is not

mutagenic in the Ames test, *in vitro* chromosomal aberration test or mouse dominant lethal test (Stenchever *et al.*, 1969 ; Badr *et al.*, 1974 ; Kraemer *et al.*, 1974 ; Ishidate, 1987), but has shown carcinogenicity for the rodent liver and endometrium (IARC, 1979). Tumor promotion activity of ethinylestradiol was demonstrated in a two stage model of hepatocarcinogenesis (Mayol *et al.*, 1991) and it is considered to be a transplacental carcinogen inducing endometric carcinomas in mice (Yasuda *et al.*, 1976). This chemical is also reported to be a teratogen causing

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hypertrophic mammary nipples in mouse fetuses (Yasuda *et al.*, 1981). In a 27-day rat subacute study (Nakayama *et al.*, 1979), ethinylestradiol caused atrophy of Leydig cells but no inhibition of spermatogenesis in the testes of male rats at 0.1 mg/kg/day, and inhibition of spermatogenesis and atrophy of seminiferous tubules, Leydig cells, prostate and seminal vesicles at 1 mg/kg/day or more. These findings indicate that ethinylestradiol should be classified as a reproductive toxicant causing testicular toxicity. However, little is known of the effects of ethinylestradiol on sperm and male reproductive ability. From this reason, ethinylestradiol was chosen as a model compound to assess parameters of reproductive toxicity in male rats. The dosing period comprised 28 days prior to mating, 7 days mating period, and the following 3 days or more during recovery of reproductive organs and glands from the effects of ejaculation.

The major aim of this reproductive toxicity study was to comparatively evaluate sperm examination, reproductive performance testing, determination of reproductive organ weights and histopathological assessment, for detecting chemical effects on male fertility in experimental animals.

This study was conducted as part of a collaborative work to determine the optimal treatment period and optimal parameters for detecting drug effects on male fertility.

MATERIALS AND METHODS

Chemicals and treatment

Ethinylestradiol and sodium carboxymethyl cellulose (CMC) were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Ethinylestradiol (Fig. 1) is a white to slightly

Fig. 1. Chemical structure of ethinylestradiol.

yellowish white crystalline compound, insoluble in water and soluble in pyridine, ethanol and ether (M. W. ; 296.39, CASRN ; 57-63-6). It was suspended in 0.5% CMC solution immediately before each dosing. Male rats were dosed once daily, by oral gavage at a dosing volume rate of 5 ml/kg. Actual dosing volumes were adjusted weekly, according to body weights recorded on the nearest day. Control animals received the same dose volume of CMC solution.

Experimental animals and husbandry conditions

Male Sprague Dawley (SD) rats, 9 weeks old, were purchased from Charles River Japan Co. (Atsugi-shi, Japan), and acclimated for 1 week prior to the initiation of dosing. Female SD rats, 12 weeks old, were also obtained from the same breeder and acclimated for 2 weeks prior to pairing. The animal room was maintained at a temperature of $22 \pm 3^\circ\text{C}$ and $55 \pm 20\%$ room humidity with a 12-hr light/dark cycle (light, 7:00–19:00). Rats were housed in plastic cages (2 rats/cage) with chip bedding (Beta Chip, Charles River Japan Co.) for the test period. Mated female rats were housed individually under the same conditions. Autoclaved standard diet CRF-1 (Charles River Japan Co.) and tap water were given *ad libitum*.

Experimental design and performance

The experimental design in terms of the life phase is illustrated in Fig. 2. Experiment A (Exp. A) was carried out first. However, severe damage exceeding our expectations occurred in the reproductive system of male rats treated with 3 and 10 mg/kg/day ethinylestradiol. Accordingly, experiment B (Exp. B) was planned to achieve assessment of the parameters examined using the lower doses of 0.1 and 0.3 mg/kg/day. Male rats (10 weeks old) were treated orally with ethinylestradiol for 28 days prior to mating. With the exception of animals in the recovery test, male rats were continuously treated with ethinylestradiol until at least 3 days after the day of copulation or termination of the 7-day mating period. Total lengths of the dosing period in each experiment were almost the same (Exp. A, 38 to 41 days ; Exp. B, 34 or 40 days) and necropsies were performed at the day after termination of ethinylestradiol-treatment. This small difference in the dosing length between Exp. A and B was mainly due to variation in the days taken to copulate

Fig. 2. Experimental design to detect reproductive toxicity of ethinylestradiol after 4 weeks daily dosing prior to mating.

with female rats.

All animals were observed daily for clinical signs, and body weights and food consumption were measured weekly.

Experiment A : Ethinylestradiol was administered by oral gavage to male rats at 0, 3, 10 mg/kg/day for 4 weeks prior to mating. After this pre-mating period, 10 males in each group were continuously dosed and mated with untreated female rats (14 weeks old) on the basis of one male to one female for 7 days. After copulation or the termination of 7-day mating period, males were maintained on ethinylestradiol for at least 3 days, during the period of recovery of their reproductive organs and glands from the effects of ejaculation. Copulation was confirmed by the presence of sperm in vaginal smears and the day when sperm became positive was considered as day 0 of gestation. All males were necropsied and examined for epididymal sperm motility and sperm counts. Male reproductive organs and the pituitary were weighed and examined histopathologically. In order to assess the effects of treatment on male fertility, copulated females were necropsied on days 13 to 16 of gestation and the others 13 to 16 days after the end of the mating period.

In the recovery test, 6 males in each group

were maintained for 28 days after the end of the 4 weeks ethinylestradiol-treatment and then mated with untreated females for 7 days to allow examination of the recovery of reproductive ability. Male rats were necropsied 67 or 68 days after initiation of treatment and examined as described above.

Experiment B : Ethinylestradiol was administered by oral gavage to male rats at 0, 0.1, 0.3 mg/kg/day for 28 days. After this pre-mating period, treatment was continued while 10 males in each group were mated with untreated female rats on the basis of one male to one female for 7 days. After copulation or the termination of 7-day mating period, males were maintained on ethinylestradiol for at least 3 days, during the period of recovery of their reproductive organs and glands from the effects of ejaculation. All male and female rats were necropsied and examined as in Exp. A. In this experiment, no recovery test was included.

Necropsy : All male rats were autopsied as scheduled in Fig. 2 by exsanguination from the abdominal aorta under ether anesthesia. Paired epididymides, pituitary, prostate, seminal vesicles and paired testes were weighed. The left epididymis of each rat was stored at -40°C for determination of sperm counts. The right epi-

didymis of each rat was used for sperm motility testing and then applied for histopathological examination. Testes were fixed in Bouin's solution, and the right epididymis and the remaining organs were fixed in 10% buffered formalin solution for routine histopathological processing. Paraffin sections were stained with hematoxylin and eosin for microscopic examination. Females were also sacrificed as detailed above and the numbers of corpora lutea, implantation sites, live embryos and dead embryos were counted to assess the effects of ethinylestradiol on male reproductive ability.

Sperm examination

Sperm motility and sperm counts in caudal epididymis were examined for each male rat. Sperm motility was assessed by the method of Morrissey and co-workers (1988) with the following modifications. One drop of caudal fluid was taken from a small epididymal tubule in the right epididymis. This fluid was immediately placed into a tube containing 5 ml of 199 medium containing 0.5% bovine serum albumin and incubated for at least 5 min at 37°C. The suspension was dispersed in a Thoma hemocytometer kept at 37°C. Four independent fields of each sample were recorded on video tape using inverted phase-contrast microscopy. Thereafter, the observer determined the actual number of motile and non-motile sperm for each field of playback vision. Sperm were considered motile if they showed any movement. The total number of motile sperm divided by the sum of motile and non-motile sperm was expressed as a motility index (percentage).

Sperm counts were determined using the left epididymis, stored at -40°C. After thawing at room temperature, each caudal epididymis was separated and weighed, placed in a petri dish and minced in 1 ml of Hanks' balanced salt solution (HBSS). The homogenized solution was replaced into a tube, HBSS was added to obtain 5 ml and after gentle repeated pipetting with a Pasteur pipette, this suspension was diluted approximately 40 fold with HBSS, if a suitable density of sperm in the first homogenized solution was obtained. The actual volume of added HBSS varied depending on the sperm density. This sperm suspension was placed in a 0.1 mm³ chamber of a Thoma hemocytometer. The

observer counted sperm in all fields. Sperm count was calculated using the following formula; the number of sperm $\times 10^4 \times$ dilution/caudal epididymis weight (g).

Statistical analysis

Parametric data were statistically analyzed by one-way analysis of variance (ANOVA) techniques with post-hoc analysis by Dunnett's multiple comparison procedure. Non-parametric data expressed as indexes or percentages except for copulation and fertility indexes were analyzed by the Kruskal-Wallis test with the Dunnett type test. Copulation and fertility indexes were analyzed with the Fisher exact test. Statistical analysis was not performed on macroscopic findings and histopathological data.

RESULTS

General symptoms

No clinical signs of toxicity or mortalities were encountered in Exp. A and B. Body weight gains and food consumption of all ethinylestradiol-treated male rats were reduced dose-dependently during the dosing period (Fig. 3). During the recovery period of Exp. A, however, in the groups given 3 and 10 mg/kg/day, food consumption returned to the control level with rapid increase in body weight.

Reproductive ability and sperm examinations

Results of reproductive ability and sperm examinations are shown in Table 1.

Reproductive examination: In Exp. B, successful copulation of all male rats used in the control group (Cont B) with observed and of 7 male rats each of the groups treated with 0.1 and 0.3 mg/kg/day ethinylestradiol. The difference was not statistically significant between the control and ethinylestradiol-treated groups. Subsequently, to examine ability to impregnate, fertility indices was estimated from the numbers of copulating males and pregnant female rats. Fertility indexes were comparable between the 0.1 and 0.3 mg/kg/day groups and the concurrent control group. In Exp. A, successful copulation was observed for all male rats of the control group (Cont A). At least 2 male rats each in the groups of 10 male rats treated with 3 and 10 mg/kg/day ethinylestradiol copulated successfully, but no impregnation occurred. In the groups

Fig. 3. Body weight, body weight gain and food consumption of ethinylestradiol-treated males.
*, **:Significantly difference from the control value ($P < 0.05$ and $P < 0.01$, respectively).

maintained for 28 days after 4 weeks exposure to ethinylestradiol at 3 and 10 mg/kg/day, moderate recovery of copulation and fertility abilities was noted. Our data thus showed that ethinylestradiol induces reversible reproductive impairment at high dose levels.

Sperm examination: Sperm examination was performed by means of counts of sperm taken from the caudal epididymis and observation of their motility. Decrease in sperm count was clearly observed in all ethinylestradiol-treated groups. In Exp. B, the number of sperm was moderately decreased in the groups given 0.1 and 0.3 mg/kg/day and, the results of sperm motility

demonstrated virtually identical levels for both treated and control rats. Small numbers of abnormal sperm were observed in all specimens examined. In Exp. A, sperm could hardly be found in the groups receiving 3 and 10 mg/kg/day. After the 4-week recovery period, however, the sperm in both ethinylestradiol-treated groups had become numerous, although still fewer than in controls, and normal sperm motility values were obtained. Infrequent abnormal sperm were observed in all specimens examined. Our data showed that ethinylestradiol decreases the number of sperm in all treated groups whereas sperm motility is not affected by treatment with 0.1 and

Table 1. Reproductive performance and sperm assessment for male rats treated orally with ethinylestradiol.

Dose (mg/kg/day)	Experiment B			Experiment A			Recovery (Experiment A)		
	Cont B	0.1	0.3	Cont A	3	10	Control	3	10
No. of males mated	10	10	10	10	10	10	6	6	6
Copulation index (%)	100 (10/10)	70 (7/10)	70 (7/10)	100 (10/10)	20** (2/10)	20** (2/10)	83 (5/6)	67 (4/6)	50 (3/6)
Fertility index (%)	90 (9/10)	100 (7/7)	86 (6/7)	90 (9/10)	0 (0/2)	0 (0/2)	100 (5/5)	50 (2/4)	33 (1/3)
No. of males tested	10	10	10	10	10	10	6	6	6
Males with no sperm ^{a)}	0	0	0	0	8**	7**	0	0	0
Sperm count ^{b)}	662±145 ^{c)} (10)	461±184 (10)	467±237* (10)	695±140 (10)	0.01±0.01 (2)	0±0 (3)	701±95 (6)	128±53** (6)	109±81** (6)
Motility index (%)	96.5±1.1 ^{c)} (10)	92.6±7.2 (9)	93.6±5.2 (9)	95±3.4 (10)	— (0)	— (0)	97±1.5 (6)	82.8±5.6** (5)	82.7 (2)

Copulation index (%) : (No. of males with successful copulation / No. of males mated) × 100.

Fertility index (%) : (No. of females impregnated / No. of males with successful copulation) × 100.

^{a)} : No. of males having no sperm in the caudal epididymis.

^{b)} : Sperm count, (× 1000000 / g caudal epididymis)

^{c)} : Values represent group mean ± S.D. and in parentheses indicate No. of males tested.

Motility index (%) : (the total number of motile sperm / the sum of motile and nonmotile sperm) × 100.

*, ** : Significant difference from the control value ($P < 0.05$ and $P < 0.01$, respectively).

Table 2. Reproductive parameters for dams mated with ethinylestradiol-treated males.

Dose (mg/kg/day)	Experiment B			Experiment A			Recovery (Experiment A)		
	Cont B	0.1	0.3	Cont A	3	10	Control	3	10
No. of dams examined	9	7	6	9	0	0	5	2	1
No. of corpora lutea	17.0±1.7	15.9±1.6	17.0±3.8	16.2±1.72	—	—	15.8±5.93	11	14
No. of implantations	15.4±1.2	14.9±1.5	14.3±4.1	14.6±3.00	—	—	14.2±7.69	6.5	14
No. of dead embryos	0.6±0.5	0.9±0.9	0.3±0.5	1.4±1.33	—	—	1.6±1.52	0.5	0
No. of live fetuses	14.9±1.4	14.0±1.8	14.0±4.1	13.1±4.20	—	—	12.6±6.66	6	14
Pre-implantation loss (%)	8.6±9.4	6.3±3.3	15.6±15.3	10.5±14.8	—	—	20.4±35.3	48.3	0
Post-implantation loss (%)	3.5±3.5	5.9±5.9	2.3±3.6	12.1±12.6	—	—	7.9±8.1	4.2	0

Values represent group mean ± S.D.

Pre-implantation loss (%) : ((No. of corpora lutea-No. of implantations) / No. of corpora lutea) × 100.

Post-implantation loss (%) : (No. of dead embryos / No. of implantations) × 100.

Table 3. Organ weights of male rats treated orally with ethinylestradiol.

Dose (mg/kg/day)	Experiment B				Experiment A			
	Cont B	0.1	0.3		Cont A	3	10	
No. of males examined	10	10	10		10	10	10	
Final Body weight (g)	497 ± 27	410 ± 22**	380 ± 39**		475 ± 27	290 ± 22**	249 ± 13**	
Absolute								
Pituitary (mg)	14.5 ± 2.4	14.2 ± 2.1	13.7 ± 1.7		13.1 ± 1.8	12.3 ± 1.7	13.5 ± 2.1	
Testes (g)	3.39 ± 0.24	2.82 ± 0.55*	2.76 ± 0.59**		3.30 ± 0.12	0.69 ± 0.20**	0.63 ± 0.11**	
Epididymides (g)	1.07 ± 0.10	0.93 ± 0.22	0.81 ± 0.20**		1.17 ± 0.08	0.34 ± 0.24**	0.25 ± 0.02**	
Prostate (g)	0.83 ± 0.11	0.50 ± 0.18**	0.31 ± 0.17**		0.73 ± 0.19	0.07 ± 0.04**	0.07 ± 0.02**	
Seminal vesicles (g)	2.53 ± 0.26	1.63 ± 0.53**	1.18 ± 0.50**		2.69 ± 0.49	0.28 ± 0.04**	0.30 ± 0.04**	
Relative								
Pituitary ($\times 10^{-3}$)	2.9 ± 0.5	3.5 ± 0.4*	3.6 ± 0.4**		2.8 ± 0.4	4.2 ± 0.8**	5.4 ± 1.0**	
Testes	0.68 ± 0.05	0.69 ± 0.13	0.73 ± 0.14		0.70 ± 0.04	0.23 ± 0.06**	0.25 ± 0.04**	
Epididymides	0.22 ± 0.02	0.22 ± 0.05	0.21 ± 0.05		0.25 ± 0.02	0.12 ± 0.08**	0.10 ± 0.01**	
Prostate	0.17 ± 0.02	0.12 ± 0.05*	0.02 ± 0.01**		0.15 ± 0.04	0.03 ± 0.01**	0.08 ± 0.04**	
Seminal vesicles	0.51 ± 0.04	0.39 ± 0.12*	0.10 ± 0.20**		0.57 ± 0.10	0.12 ± 0.02**	0.31 ± 0.11**	

Organ weights of testes and epididymides are expressed as paired weights.

Values represent group mean ± S.D.

Relative organ weight is calculated as organ weight (g)/final body weight (g).

*, ** : Significant difference from the control value ($P < 0.05$ and $P < 0.01$, respectively).

0.3 mg/kg/day.

Other reproductive parameters: Other reproductive parameters assayed were numbers of corpora lutea, implantations, dead and live embryos, and percentages of pre-implantation and post-implantation losses (Table 2). In Exp. B, comparable values for all parameters were found for control and treated groups. In Exp. A, low values for corpora lutea, implantations and live fetuses and a high frequency of pre-implantation loss was found in the 3 mg/kg/day recovery group, when compared to the controls. This result was primarily due to one abnormal pregnant rat which had only eight corpora lutea, one implantation site and one live embryo. No abnormal values were evident in the 10 mg/kg/day recovery group. Our data thus indicated that ethinylestradiol treatment does not interfere with early embryo development.

Organ weights

Reproductive organ and pituitary weights are given in Tables 3 and 4. In all treated groups of Exp. A and B, absolute and/or relative weights of the prostate, seminal vesicles and paired testes

showed statistically significant decreases, while pituitary weights were dose dependently increased (Table 3). Organ weights of paired epididymides were also decreased in the 0.3, 3 and 10 mg/kg/day groups. After the recovery period in Exp. A, absolute and/or relative weights of prostate and seminal vesicles still showed lower values, as compared to the controls (Table 4). In the 10 mg/kg/day group, absolute weights of testes gave low values, whereas relative weight of pituitary resulted in high values. Our data showed that ethinylestradiol causes decrease of reproductive organ weights which is largely reversible.

Macroscopic examination

Macroscopic findings after treatment with ethinylestradiol of the testes, epididymides, seminal vesicles and prostate are shown in Table 5. In all treated groups, atrophy was observed dose-dependently. In the recovery test of Exp. A, atrophy persisted in the epididymides with both doses and in the testes with the 10 mg/kg/day dose. Atrophic changes corresponded to decrease in organ weights (Tables 3 and 4).

Table 4. Organ weights of ethinylestradiol-treated male rats after a 4-week recovery period.

Dose (mg/kg/day)	Recovery (Experiment A)		
	Control	3	10
No. of males examined	6	6	6
Final Body weight (g)	536 ± 27	484 ± 7*	481 ± 20**
Absolute			
Pituitary (mg)	14.1 ± 2.4	14.8 ± 2.63	15.6 ± 0.59
Testes (g)	3.39 ± 0.24	3.10 ± 0.21	2.80 ± 0.25**
Epididymides (g)	1.41 ± 0.20	0.77 ± 0.09**	0.73 ± 0.10**
Prostate (g)	0.87 ± 0.19	0.84 ± 0.09	0.79 ± 0.09
Seminal vesicles (g)	3.07 ± 0.36	2.54 ± 0.33*	2.35 ± 0.15**
Relative			
Pituitary (×10 ⁻³)	2.6 ± 0.4	3.0 ± 0.5	3.2 ± 0.1*
Testes	0.63 ± 0.05	0.64 ± 0.05	0.58 ± 0.06
Epididymides	0.26 ± 0.04	0.16 ± 0.02**	0.15 ± 0.02**
Prostate	0.16 ± 0.04	0.17 ± 0.02	0.17 ± 0.02
Seminal vesicles	0.58 ± 0.09	0.52 ± 0.06	0.49 ± 0.03

Organ weights of testes and epididymides are expressed as paired weights.

Values represent group mean ± S.D.

Relative organ weights is calculated as organ weight (g)/final body weight (g).

*, **: Significant difference from the control value (P < 0.05 and P < 0.01, respectively).

Histopathological examination

Histopathological findings after treatment with ethinylestradiol are summarized in Tables 6 and 7. Representative findings are shown in Photos. 1 to 6. In all treated groups, several pathological changes were observed with dose dependence: degeneration and exfoliation of spermatocytes/spermatids, vacuolation of Sertoli cells and atrophy of Leydig cells in the testes (Photos. 1 to 3); atrophy, decrease of sperm and degenerate spermatogenic cells in the ducts in epididymis (Photos. 5 and 6); atrophy of prostate and seminal vesicles characterized by narrowed lumina and proliferation of connective tissue; and diffuse hyperplasia of anterior cells, negative for PAS-orange G double staining, in the pituitary. Sloughing of multinucleated giant cells into the lumina of the seminiferous tubules was found in one male rat treated with 0.3 mg/kg/day ethinylestradiol in Exp. B. The same change but more marked was caused by treatment with 3 and 10 mg/kg/day in Exp. A (Photo. 3). All pathological changes described above were diminished after the recovery period (testis, Photo. 4).

DISCUSSION

Many publications have been concerned with selection of the most sensitive parameter for detecting reproductive toxicity in animals. However, there has been only limited critical information relevant to male reproductive toxicity. Our present data thus should facilitate resolution of this difficult problem. In addition, our results suggest that a dosing period of 4 weeks prior to mating is sufficient to detect effects of ethinylestradiol on male reproductive ability.

In this present study, ethinylestradiol, which is pharmacologically and physiologically classified as an estrogen, reduced body weight gain and food consumption without clinical symptoms in male rats. Suppression of body weight gain and food consumption has also been reported in previous toxicity studies of ethinylestradiol (Nakayama *et al.*, 1979; Schardein, 1980). This is expected since it is known that estrogen-like chemicals contribute to a diminished efficiency of ingested protein and depression of growth hormone secretion in the animal body. Such sup-

pression of body weight gain and food consumption in rats can be antagonized by administration of progesterone (Ross and Zucker, 1976).

Ethinylestradiol caused organ weight reduction and atrophy of the testes, epididymides, prostate and seminal vesicles in the present case, in line with reported effects of estrogens on several reproductive organs and glands (Gibson, *et al.*, 1967; Das, 1977). The ability of estrogens to interfere with the release of hormones from the hypothalamus and pituitary, closely relates to the occurrence of atrophy.

Our study showed atrophy of Leydig cells, vacuolation of Sertoli cells, degeneration and exfoliation of spermatocytes/spermatids without degeneration of spermatogonia in testes and relatively rapid recovery after cessation of insult. Nakayama *et al.* (1979) reported similar findings in the testes as well as low levels of serum testosterone when male rats were treated with ethinylestradiol. As documented in current review articles (Amann, 1986; Takahashi and Matsui, 1993), lutenizing hormone (LH) stimulates Leydig cells to produce testosterone for spermatogenesis. In addition, the development of sperm is maintained through the function of Sertoli cells, under the action of follicle stimulating hormone (FSH) and testosterone. Estrogen is a well-known indirect testicular toxicant interfering with the release of LH and FSH and thus causing decrease of testosterone secretion (Takahashi and Matsui, 1993). In our study, decrease of epididymal sperm counts was observed in all ethinylestradiol-treated groups, but sperm motility was not affected. It is to be expected that decrease of sperm count would follow inhibition of spermatogenesis in the testis. Neither mutagenicity nor toxicity of ethinylestradiol were detected in germ cells using the micronucleus assay *in vivo* and *in vitro*, sperm-head abnormality test and morphometry (Pylkkänen *et al.*, 1991). Our data also support the conclusion that ethinylestradiol might not act directly on spermatogenesis and that its effects on the testis are due to its estrogenic actions with degeneration/exfoliation of spermatocytes and spermatids being secondary to degenerative changes of Sertoli cells and Leydig cells.

Reproductive ability, as reflected in copulation and fertility indices, was severely compro-

Table 5. Macroscopic findings in the male reproductive organs of rats treated orally with ethinylestradiol.

Organ/Findings/Grade	Experiment B			Experiment A			Recovery (Experiment A)		
	Dose (mg/kg/day)			Dose (mg/kg/day)			Dose (mg/kg/day)		
	Cont B	0.1	0.3	Cont A	3	10	Control	3	10
Testes	No. examined	10	10	10	10	10	6	6	6
Atrophy	+	0	2	0	0	0	0	0	2
	++	0	1	0	10	10	0	0	0
Epididymides	No. examined	10	10	10	10	10	6	6	6
Atrophy	+	0	4	0	0	0	0	5	6
	++	0	1	0	10	10	0	0	0
Seminal vesicles	No. examined	10	10	10	10	9	6	6	6
Atrophy	+	0	5	0	0	0	0	0	0
	++	0	1	0	10	9	0	0	0
Prostate	No. examined	10	10	10	10	9	6	6	6
Atrophy	+	0	3	0	0	0	0	0	0
	++	0	1	0	10	9	0	0	0

+, Slight ; ++, Moderate to Severe.

Table 6. Histopathological findings in the testes and epididymides of male rats treated orally with ethinylestradiol.

Organ/Findings/Grade	Experiment B				Experiment A			Recovery (Experiment A)		
	Dose (mg/kg/day)				Dose (mg/kg/day)			Dose (mg/kg/day)		
	Cont B	0.1	0.3		Cont A	3	10	Control	3	10
Testes	No. examined	10	10	10	10	10	10	6	6	6
Degeneration/exfoliation	+	0	0	1	0	1	0	0	0	2
of spermatocytes/spermatids	++	0	1	0	0	9	10	0	0	0
Multinucleated giant cells	+	0	0	1	0	4	5	0	0	0
in seminiferous tubules										
Vacuolation of Sertoli cells	+	0	0	1	0	2	0	0	0	0
	++	0	1	0	0	8	10	0	0	0
Atrophy of Leydig cells	+	0	0	4	0	0	0	0	0	0
	++	0	1	1	0	10	10	0	0	0
Edema	+	0	1	0	0	2	0	0	0	0
Epididymides	No. examined	10	10	10	10	10	10	6	6	6
Atrophy	+	0	0	2	0	0	0	0	1	2
	++	0	1	1	0	10	10	0	0	0
Decrease of sperm in ducts	+	0	0	2	0	0	0	0	3	2
	++	0	1	1	0	10	10	0	0	4
Degenerative spermatogenic	+	0	1	1	0	7	7	0	3	4
cells in ducts	++	0	0	1	0	1	0	0	0	0
Granuloma	+	0	0	1	0	0	0	0	0	0
Infiltration of leukocytes	+	0	1	1	0	1	2	0	0	0

+, Slight ; ++, Moderate to Severe.

Table 7. Histopathological findings in the reproductive glands and pituitary of male rats treated orally with ethinylestradiol.

Organ/Findings/Grade	Experiment B				Experiment A				Recovery (Experiment A)			
	Dose (mg/kg/day)				Dose (mg/kg/day)				Dose (mg/kg/day)			
	Cont B	0.1	0.3		Cont A	3	10		Control	3	6	10
Seminal vesicles	No. examined	10	10	10	10	10	10	9	6	6	5	
Atrophy	+	0	0	3	0	0	0	0	0	0	0	
	++	0	1	3	0	10	9	9	0	0	0	
Infiltration of eosinophils	+	0	0	3	0	0	0	0	0	0	1	
Prostate	No. examined	10	10	10	10	10	10	9	6	6	6	
Atrophy	+	0	1	1	0	0	0	0	0	0	0	
	++	0	1	3	0	10	9	9	0	0	0	
Infiltration of leukocytes	+	5	4	3	5	0	0	0	3	2	2	
	++	0	0	1	1	0	0	0	1	1	0	
Inflammation	+	1	0	0	0	1	0	0	0	0	0	
	++	0	0	0	0	1	0	0	0	0	0	
Pituitary	No. examined	9	10	9	10	10	10	10	5	6	6	
Diffuse hyperplasia of anterior cells	+	0	2	2	0	1	3	3	0	1	0	
	++	0	0	0	0	2	7	7	0	0	0	

+, Slight ; ++, Moderate to Severe.

Photo. 1. Testis from a control rat, showing the normal appearance. HE stain, $\times 180$.

Photo. 2. Testis from a rat treated with 0.3 mg/kg/day of ethinylestradiol for four weeks, showing degeneration/exfoliation of spermatocytes/spermatids, vacuolation of Sertoli cells and atrophy of Leydig cells. HE stain, $\times 180$.

Photo. 3. Testis from a rat treated with 10 mg/kg/day of ethinylestradiol for four weeks, showing degeneration/exfoliation of spermatocytes/spermatids, multinucleated giant cell formation, vacuolation of Sertoli cells and atrophy of Leydig cells. HE stain, $\times 180$.

Photo. 4. Testis from a rat allowed a 4-week withdrawal period following treatment with 10 mg/kg/day of ethinylestradiol for four weeks, showing no remarkable changes. HE stain, $\times 180$.

Photo. 5. Epididymis from a control rat, demonstrating the normal appearance. HE stain, $\times 113$.

Photo. 6. Epididymis from a rat treated with 10 mg/kg/day of ethinylestradiol for four weeks, showing atrophy, decrease of sperm and degenerative spermatogenic cells in ducts. HE stain, $\times 113$.

mised in males receiving 3 and 10 mg/kg/day of ethinylestradiol in this study. We consider that this effect is mainly related to severe damage of reproductive organs and glands with reduction of androgen release. The observed diffuse hyperplasia of anterior cells in the pituitary involved chromophobe cells.

Consideration of the present results for various parameters leads to the conclusion that counting of sperm in the epididymis and histopathological examination of the testis are effective for detecting reproductive toxicity of ethinylestradiol. Our data suggest that the order of sensitivity for this purpose is: sperm counts, organ weights of prostate and seminal vesicles, histopathological examination > organ weights of testis and epididymis > reproductive performance and sperm motility.

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