COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS 3) EFFECTS OF REPEATED DOSES OF ETHINYLESTRADIOL FOR 2 AND 4 WEEKS ON THE MALE REPRODUCTIVE ORGANS

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ABSTRACT — Male Sprague-Dawley rats were subcutaneously administered 0.3 and 3.0 mg/kg/day of ethinylestradiol for 2 and 4 weeks. Two weeks treatment decreased body weight gain, food consumption, absolute weights of testis, epididymis, prostate and seminal vesicle, and relative weights of epididymis, prostate and seminal vesicle. On the other hand, it increased absolute and relative weights of pituitary and adrenal glands, and induced atrophy of Leydig cells, degeneration/necrosis of pachytene spermatocytes, vacuolar degeneration of Sertoli cells, and retention of spermatids. In addition to the changes found after 2 weeks treatment, 4 weeks treatment induced exfoliation of germinal cells, decreases in spermatid and multinucleated giant cell formation and relative weights of testis. These results suggest that examination of prostate and seminal vesicle weights and histopathological changes in the testis are important for evaluation of male reproductive toxicity of ethinylestradiol and 2 weeks treatment is sufficient to detect toxicity on male reproductive organs.

KEY WORDS: Ethinylestradiol, Male reproductive toxicity, Rats, Histopathological examination

INTRODUCTION

Before first administration of drug candidates to humans, results of repeated dose toxicity studies are necessary. However, the period of repeated dose studies for reproductive organs was not harmonized in ICH (1998). Japan and the EU/US recommend 4 weeks and 2 weeks, respectively. This difference came about because evaluation of influence on male reproduction was necessary before first clinical trials in Japan and there were not enough data indicating 2 weeks to be sufficient to detect effects on male reproductive potential (1998). Therefore, the Japan Pharmaceutical Manufacturing Association and National Institute of Health Sciences have organized a validation study to obtain information on the validity and the limitations of 2-week repeated dose toxicity studies to detect effects on male reproductive organs in rats. As a part of this

study, we administered ethinylestradiol for 2 weeks and compared the effects to those encountered with a 4-week administration.

Ethinylestradiol (19-nor-17 α -pregna-1,3,5 (10)-trien-20-yne-3,17-diol) is one of the most active steroidal estrogens known when administered orally (IARC, 1979). In a recent study, when ethinylestradiol was orally administered to male Sprague-Dawley rats for 4 weeks, atrophy of testis, epididymis, prostate and seminal vesicles and degenerative changes of spermatocytes, spermatids, Sertoli cells and Leydig cells were observed. (Iwase *et al.*, 1995).

The purpose of the present study was to assess the testicular toxicity of subcutaneously administered ethinylestradiol for 2 and 4 weeks.

MATERIALS AND METHODS

Chemicals

Ethinylestradiol and sodium carboxymethyl cellulose (CMC) of reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Ethinylestradiol was suspended in 0.5% CMC solution immediately before each dosing.

Animals

Male Sprague-Dawley rats (Crj:CD(SD)) were purchased from Charles River Japan, Inc. (Hino). After 4 days acclimatization, 30 rats were employed in the experiments. The animal room was maintained at 21–25°C, with a relative humidity of 45–65%, 20 times/hr ventilation and 12 hr lighting (7:00–19:00). The rats were housed in plastic cages (2 rats/cage) with chip bedding (Beta chip, Oriental Yeast Inc., Tokyo) and given solid food (CRF-1, Oriental Yeast Inc., Tokyo) and tap water *ad libitum*.

Experimental design

The experimental design is shown in Fig. 1. Ethinylestradiol was subcutaneously administered to 5 groups of at dose levels of 0, 0.3 and 3.0 mg/kg/day for 2 and 4 weeks.

1. Toxicological signs, body weight and food consumption

Rats were observed twice daily for toxicological signs, and weighed twice weekly.

2. Macroscopic examination and organ weights

Six rats from each group were sacrificed under anesthesia the day after 2 and 4 weeks treatment and examined macroscopically. At sacrifice, testes, epididymides, seminal vesicle, prostate, adrenal gland and

pituitary were weighed and their weights relative to the final body weight calculated. Testes were fixed in formalin-sucrose-acetic acid (FSA) solution, and the other organs were fixed in 10% neutral buffered formalin solution, embedded in paraffin, sectioned and stained with hematoxylin-eosin. Histopathological examination of the testis and epididymis was performed under a light microscope.

3. Serum hormone level

Concentrations of 17β -estradiol in serum were measured with an enzyme-immunoassay (EIA) kit from Assay Designs, Inc. (USA).

Statistical analysis

Parametric data were statistically analyzed by one way analysis of variance (ANOVA) techniques with post-hoc analysis by Dunnett's multiple (Dunnett, 1955) comparison procedure.

RESULTS

Mortality, toxicological signs, body weight and food consumption

No significant changes in mortality were evident during the experiment. Body weight gain and food consumption were suppressed in the ethinylestradiol treated groups.

Organ weights

The results are shown in Table 1. After 2 and 4 weeks treatment with ethinylestradiol, the absolute and/or relative weights of the testes, epididymides, prostate and seminal vesicle showed statistically significant decreases, while weights of pituitary and adrenal gland were statistically significant increased.

Hormone measurements

The results are shown in Table 2. Concentrations of 17β -estradiol showed statistically significant decreases in both treated groups.

Macroscopic findings

After 2 weeks treatment, atrophy of the prostate and seminal vesicle was evident. In addition, atrophy of the testis and epididymis was found after 4 weeks treatment.

Histopathological findings

Histopathological findings for the testis and epididymis are summarized in Table 3. In the testis, after 2

Fig. 1. Experimental design.

weeks administration, atrophy of Leydig cells (photo 1), degeneration/necrosis of spermatocytes (photo 2), vacuolar degeneration of Sertoli cells and retention of spermatids (photo 3) were observed in most of the treated animals. In addition to the changes found after 2 weeks, exfoliation of germinal cells and decrease of spermatid and multinucleated giant cell formation were observed after 4 weeks treatment. In the epididymis, atrophy of seminiferous tubules, decreases in spermatids and cell debris were observed after 4 weeks and with the high dose after 2 weeks treatment.

DISCUSSION

In the present study, ethinylestradiol was administered to male Sprague-Dawley rats for 2 and 4 weeks to allow male reproductive toxicity to be assessed.

With both 2 weeks and 4 weeks treatment, ethinylestradiol suppressed body weight gain and food consumption. Suppression of body weight gain and food consumption by ethinylestradiol was also reported by Iwase *et al.* (1995), since other estrogens have also been shown to suppress body weight and food consumption (Sullivan and Smith, 1957; Ikegawa *et al.*, 1995; Cook *et al.*, 1998), these effects appeared characteristic of estrogens such as ethinylestradiol.

Table 1. Organ weights of rats treated subcutaneously with ethinylestradiol.

Dose (mg/kg/day)	4 weeks			2 weeks		
	0	0.3	3.0	0.3	3.0	
No. of rats examined	6	6	6	6	6	
Final body weight (g)	355.7 ± 35.7	232.5 ± 18.5**	189.5 ± 8.88**	300.7 ± 8.28**	277.8 ± 13.8**	
Absolute						
Testes (g)	2.95 ± 0.29	1.06 ± 0.51**	0.72 ± 0.14**	2.36 ± 0.30*	2.21 ± 0.40**	
Epididymides (g)	0.87 ± 0.11	0.26 ± 0.14**	0.19 ± 0.04**	0.55 ± 0.06**	0.44 ± 0.16**	
Prostate (g)	0.52 ± 0.18	0.04 ± 0.04**	0.02 ± 0.01**	0.11 ± 0.03**	0.09 ± 0.02**	
Seminal vesicle (g)	1.21 ± 0.09	0.13 ± 0.04**	0.13 ± 0.04**	0.22 ± 0.03**	0.20 ± 0.02**	
Pituitary (mg)	7.85 ± 2.28	15.7 ± 5.06**	23.5 ± 5.58**	16.4 ± 2.73**	21.9 ± 2.16**	
Adrenal glands (mg)	38.0 ± 16.5	80.1 ± 9.88**	61.2 ± 9.43**	90.4 ± 12.2**	71.1 ± 7.08**	
Relative						
Testes (g/100 gBW)	0.832 ± 0.081	0.447 ± 0.176**	0.380 ± 0.062**	0.788 ± 0.117	0.794 ± 0.125	
Epididymides (g/100 gBW)	0.245 ± 0.025	0.108 ± 0.049**	0.102 ± 0.016**	0.182 ± 0.020*	0.157 ± 0.053**	
Prostate (g/100 gBW)	0.144 ± 0.046	0.015 ± 0.014**	0.010 ± 0.006**	0.038 ± 0.011**	0.031 ± 0.007**	
Seminal vesicle (g/100 gBW)	0.346 ± 0.054	0.056 ± 0.015**	0.069 ± 0.022**	0.073 ± 0.008**	0.073 ± 0.007**	
Pituitary (mg/100 gBW)	2.210 ± 0.598	6.759 ± 2.116**	12.39 ± 2.994**	5.456 ± 0.920*	7.871 ± 0.757**	
Adrenal glands (mg/100 gBW)	10.42 ± 4.023	34.43 ± 2.851**	32.25 ± 4.431**	30.03 ± 3.444**	25.66 ± 2.898**	

Mean ± S.D.

Relative organ weights was calculated as organ weights/final body weights.

*: p<0.05, **: p<0.01: Significant difference from the control.

Table 2. Serum concentrations of 17 β -estradiol in rats treated subcutaneously with ethinylestradiol.

Dose (mg/kg/day)	4weeks			2weeks		
	0	0.3	3.0	0.3	3.0	
No. of rats examined	6	6	6	6	6	
17 β -estradiol (ng/ml)	14.33 ± 1.66	3.83 ± 0.81**	0.85 ± 0.07**	4.36 ± 1.27**	1.46 ± 0.20**	

Mean ± S.E.

*: P<0.05, **: P<0.01 : Significantly different from the control.

Weight reduction and atrophy of the testis, epididymides, prostate and seminal vesicle were also observed after both 2 weeks and 4 weeks treatment. Atrophic changes in testis corresponded to the decrease in organ weights and weight decrease in epididymides was observed with 2 weeks administration 0.3 mg/kg/day, where obvious atrophic changes were not observed. Therefore, these organ weights may be sensitive parameters for evaluation of male reproductive toxicity of ethinylestradiol.

Our study showed atrophy of Leydig cells, along with degeneration/necrosis of pachytene spermatocytes, vacuolar degeneration of Sertoli cells and retention of spermatids. These latter histopathological changes in

the testis may be directly related to decrease in serum testosterone levels, since the major androgenic steroid testosterone is synthesized by the Leydig cells. Pachytene spermatocytes at stages VII-VIII of the spermatogenic cycle (the androgen-dependent stages) are the first germ cells to degenerate with abnormal retention of step 19 spermatids at stages IX-XI or later following testosterone withdrawal in the rat (Knobil and Neill, 1994). In addition, in this study, the concentration of 17β -estradiol was found to be decreased. Estradiol is formed from testosterone. The pathway of biosynthesis estradiol is as follows: testosterone is converted to estrogenic C18 steroids first by removing the 19-methyl group and subsequently an aromatic or phenolic

Table 3. Histopathological findings for rats treated subcutaneously with ethinylestradiol.

4weeks	Dose (mg/kg)	0					0.3					3.0				
Organs	No. of Animals	6					6					6				
Histopathological findings	Grade	—	±	+	++	+++	—	±	+	++	+++	—	±	+	++	+++
Testis																
atrophy of Leydig cells		6	0	0	0	0	0	1	2	3	0	0	0	0	6	0
degeneration/necrosis of pachytene spermatocyte		6	0	0	0	0	0	0	6	0	0	0	0	0	0	6
vacuolar degeneration of Sertoli cells		6	0	0	0	0	1	0	2	3	0	0	0	0	6	0
retention of spermatids		6	0	0	0	0	0	1	0	2	3	0	0	0	0	*6
exfoliation of germinal cells		6	0	0	0	0	1	0	2	1	2	0	0	0	0	6
decreases in spermatids		6	0	0	0	0	1	0	2	1	2	0	0	0	5	1
multinucleated giant cell formation		6	0	0	0	0	2	2	0	0	2	0	0	3	3	0
Epididymis																
atrophy of seminiferous tubules		6	0	0	0	0	0	1	2	3	0	0	0	0	6	0
decreases in spermatids		6	0	0	0	0	1	0	0	3	2	0	0	0	0	6
cell debris		6	0	0	0	0	1	0	2	3	0	0	2	4	0	0
2weeks	Dose (mg/kg)	0.3					3.0									
Organs	No. of Animals	6					6									
Histopathological findings	Grade	—	±	+	++	+++	—	±	+	++	+++					
Testis																
atrophy of Leydig cells		0	0	6	0	0	0	0	4	2	0					
degeneration/necrosis of pachytene spermatocyte		0	0	4	2	0	0	0	3	2	1					
vacuolar degeneration of Sertoli cells		1	4	1	0	0	1	1	4	0	0					
retention of spermatids		0	0	6	0	0	0	0	6	0	0					
exfoliation of germinal cells		5	0	1	0	0	4	0	2	0	0					
decreases in spermatids		5	1	0	0	0	4	0	2	0	0					
multinucleated giant cell formation		6	0	0	0	0	5	0	1	0	0					
Epididymis																
atrophy of seminiferous tubules		6	0	0	0	0	2	0	4	0	0					
decreases in spermatids		5	0	1	0	0	4	0	0	2	0					
cell debris		1	4	0	1	0	0	2	2	2	0					

—: no remarkable change, ±: slight change, +: mild change, ++: moderate change, +++: severe change.

*: impossible to assess due to severe change of seminiferous tubules.

Effects of ethinylestradiol on male reproductive organs in rats.

Photo 1. Testis from a rat treated with ethinylestradiol, showing atrophy of Leydig cells.
HE stain. $\times 475$.
A: 0 mg/kg group (control group).
B: 3.0 mg/kg group for 4 weeks.

Photo 2. Testis from a rat treated with 3.0 mg/kg/day of ethinylestradiol for two weeks, showing degeneration / necrosis of pachytene spermatocytes at stage VII. HE stain. $\times 557$.

Photo 3. Testis from a rat treated with 3.0 mg/kg/day of ethinylestradiol for two weeks, showing retention of elongate spermatids at stage X. HE stain. $\times 557$.

steroid A ring (aromatase reaction) is formed, giving estradiol and estrone.

In conclusion, our study found that accessory sex organ weights and histopathological changes in the testis are important parameters for evaluation of male reproductive toxicity of ethinylestradiol. Provided these parameters are used, 2 weeks treatment is as effective as 4 weeks treatment to detect toxic effects of ethinylestradiol on male reproductive organs.

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