COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS 2) TESTICULAR TOXICITY IN RATS TREATED ORALLY WITH ETHINYLESTRADIOL FOR 2 WEEKS

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ABSTRACT — Parameters of ethinylestradiol-induced testicular toxicity were evaluated with organ weight determination, histopathological examination and quantitative morphometry. Male Sprague-Dawley rats were administered ethinylestradiol orally at 3 or 10 mg/kg/day for 2 weeks and 3 mg/kg/day for 4 weeks. Final body weights in all treated groups were lower than in the respective control group. Decreased absolute and/or relative organ weights of epididymides, prostate, seminal vesicles and testes were observed in all treated groups. In the testes, apoptosis of round spermatids, atrophy of seminiferous tubules, exfoliation of spermatids or spermatocytes, and vacuolar degeneration of Sertoli cells were only observed with 4 weeks treatment. Apoptosis of pachytenes spermatocytes and atrophy of Leydig cells were also more marked in the 4 week treated group than after 2 weeks. Therefore, degenerative histopathological changes in testes were more remarkable after 4 weeks treatment than in the 2 weeks treatment groups. However, retention of spermatids was less after 4 weeks treatment and the TUNEL index, calculated as the number of TUNEL-positive spermatocytes or spermatids, was increased in all treated groups.

These results suggest that ethinylestradiol-induced testicular toxicity can be detected in male rats administered the compound for 2 weeks and that the TUNEL method for in situ detection of apoptosis is effective for evaluation of testicular toxicity.

KEY WORDS: Ethinylestradiol, Testicular toxicity, PCNA, Apoptosis, Quantitative morphometry

INTRODUCTION

Ethinylestradiol (19-nor-17alpha-pregna-1,3,5(10)-trien-20-yen-3,17-diol) is a synthetic steroid 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 has no detectable mutagenicity (Stenchever et al., 1969; Badr and Badr, 1974; Kraemer et al., 1974; Ishidate, 1987) or initiating activity in liver carcinogenesis (Yager, 1983), but has been associated with increased incidences of hepatic and endometrial neoplasias (Baum et al., 1973; IARC, 1979). It induces acute hepatic hypertrophy in the normal liver (Ochs et al., 1986) and when administered chronically, increases levels of hepatocellular proliferation (Vickers and Lucier, 1991; Mayol et al., 1991 and 1992). It is considered to be a transplacental carcinogen inducing endometrial carcinomas in mice (Yasuda et al., 1976). The chemical is also reported to cause testicular toxicity (Nakayama et al., 1979; Iwase et al., 1995; Takayama et al., 1995).

In the present paper, we evaluated ethinylestradiol-induced testicular toxicity by histopathological observation and quantitative morphometry using proliferating cell nuclear antigen (PCNA) immunohistochemistry and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. PCNA immunohistochemistry is well estab-
lished as a tool to study cell proliferation in the testes (Chandra et al., 1997; Nambu and Kumamoto, 1995; Santamaría et al., 1995; Schlatt and Weinbauer, 1994) and the TUNEL method has recently found wide application for assessment of germ cell apoptosis (Blanco-Rodríguez and Martínez-García, 1997; Cai et al., 1997; Matsui and Takahashi, 1999; Shinoda et al., 1998; Strandgaard and Miller, 1998). The purpose of this study was to investigate, in a comparative fashion, ethinylestradiol effects on male reproductive organs following treatment for 2 or 4 weeks.

The present study was conducted as part of a collaborative project of JPMA and NIH to obtain information on the validity and limitations of 2-week repeated dose toxicity studies to detect effects on male reproductive organs in rats.

MATERIALS AND METHODS

Experimental animals
Six or eight week-old male Sprague-Dawley rats [Crl:CD(SD)] were supplied by Charles River Japan, Inc. They were maintained in an air-conditioned barrier-system animal room with an ambient temperature of 22±3°C, relative humidity of 55±5%, and a 12-h light/dark cycle. Food and water were available ad libitum. The rats were allocated randomly to 4 groups, each comprising 6 animals.

Chemicals and treatment
Ethinylestradiol was obtained from Wako Pure Chemical Ind. Ltd., Japan, suspended in 0.5% sodium carboxymethyl cellulose (CMC, Wako Pure Chemical Ind. Ltd., Osaka, Japan) solution and administered orally by gavage for 2 weeks at doses of 3 and 10 mg/kg/day or for 4 weeks at a dose of 3 mg/kg/day. Control animals received the same dose volume of CMC solution (0.5% in distilled water) for 4 weeks. The dosage volume was 5 mL/kg. Dosing was started when the animals were 8 weeks old for 2 weeks treatment and at 6 weeks of age for 4 weeks treatment.

Tissue preparation
All rats were sacrificed by exsanguination from the abdominal aorta under sodium pentobarbital anesthesia and autopsied one day after the end of the administration period. Each animal was weighed before sacrifice, and the pituitary, prostate, seminal vesicles, epididymides and testes were removed and their absolute and relative weights were determined. The right testes were fixed in formalin sucrose surfactant acetic acid (FSA) solution for 5 days, and the left ones in Boulin's solution overnight. After fixation, all testes were dehydrated through a graded alcohol series and embedded in paraffin. Cross sections were cut at 4 μm in thickness and mounted onto poly-L-lysine coated slides. For each animal, several slides from the right testes were stained with hematoxylin-eosin (HE) and PAS for light microscopy, while those from the left testes were used for PCNA immunohistochemistry and the TUNEL method.

PCNA immunohistochemistry
Deparaffinized and rehydrated tissue sections were treated for 30 min with hydrogen peroxide (0.3% in methanol) to block endogenous peroxidase. After rinsing with phosphate buffered saline (PBS), sections were treated for 20 min with normal horse serum to block non-specific binding. To detect PCNA immunoreactivity, sections were incubated for 2 hr at room temperature with a mouse monoclonal antibody (PC10 clone) against PCNA, obtained from Medac Diagnostika, GmbH (Hamburg, Germany) diluted in PBS containing 1% bovine serum albumin (1:200). After rinsing with PBS, sections were treated for 30 min at room temperature with a horse anti-mouse biotinylated immunoglobulin (Vectastain ABC Peroxidase kit, Vector Laboratories, California, USA), then rinsed in PBS and incubated for 30 min with an avidin-biotin-peroxidase complex (Vectastain ABC Peroxidase kit). Subsequently, the antibody complex was developed with diaminobenzidine tetrachloride (DAB) plus 3% hydrogen peroxide. Negative control sections were processed in parallel with omission of the primary antibody. Duodenum sections were used for positive controls. Sections were counterstained with Mayer's hematoxylin.

TUNEL method
The TUNEL method was used for in situ detection of DNA strand breaks in apoptotic cells in testes. Staining was performed using ApopDETEx Cell Death Assay System (ENZO, New York, USA). Deparaffinized, rehydrated and dried tissue sections were treated for 15 min at room temperature with proteinase K (ENZO) at 25 μg/ml. After rinsing with PBS, they were treated for 30 min with hydrogen peroxide (0.3% in methanol) to block endogenous peroxidase and then incubated at 37°C in a humidified chamber for 30 min with terminal deoxynucleotidyl transferase (TdT) and biotin-16-dUTP. For the next step they were treated at 37°C in a humidified chamber for 30 min with a strep-
tavidin-biotinylated horseradish peroxidase complex. After rinsing with PBS, sections were developed with DAB plus 3% hydrogen peroxide. Negative control sections were processed with omission of TdT and biotin-16-dUTP. Thymus sections were used for positive controls. For microscopic observation sections were counterstained with Mayer's hematoxylin.

Morphometry
Twenty cross-sectioned seminiferous tubules randomly selected at all stages (Leblond and Clermont, 1952) from 4-6 sections per right or left testis were scanned with a 40X objective. Target cells or positive staining cells and Sertoli cells were counted per right or left testis in each experimental and control group. The following indexes were calculated according to Nambu and Kumamoto (1995).

Spermatogenesis was evaluated with PAS-staining sections as a spermatid index, calculated as: Spermatid index = Mean of (No. of spermatids / No. of Sertoli cells per seminiferous tubule).

The DNA synthetic activity in spermatogenesis was evaluated as a PCNA index, calculated as: PCNA index = Mean of (No. of germ cells with positive staining / No. of Sertoli cells per seminiferous tubule).

The incidence of apoptotic cells was evaluated as a TUNEL index, calculated as: TUNEL index = Mean of (No. of germ cells with positive staining / No. of Sertoli cells per seminiferous tubule).

Statistical analysis
Final body weights, absolute and relative weights of the pituitary, prostate, seminal vesicles, epididymides and testes, spermatogenesis indexes, PCNA indexes and TUNEL indexes were statistically analyzed by one-way analysis of variance (ANOVA) and LSD multiple comparisons (Snedecor and Cochran, 1980) conducted using STATISTICA (Statsoft Inc., Oklahoma, USA). Significance was set as p<0.05 or p<0.01. All results are presented as mean±SD values.

RESULTS

Body and organ weights
Final body weights and organ weights, including these for the pituitary, epididymides, prostate, seminal vesicles and testes, are given in Table 1. Final body weights were significantly decreased in all treated groups, but particularly after 4 weeks treatment. Absolute and/or relative weights of the epididymides, prostate and seminal vesicles also showed consistent significant decrease. Absolute organ weights of testes were significantly decreased in all treated groups, while the relative organ weights were significantly decreased only in the 4 week treated group. Absolute organ weights of pituitaries in all treated groups showed values similar to the controls, whereas relative weights were significantly increased in the 3 mg/kg/day×4 week and 10 mg/kg/day×2 week treated groups.

Histopathological examination
Histopathological findings for testes are summarized in Table 2. After 4 weeks treatment they included apoptosis of round spermatids, atrophy of seminiferous tubules, exfoliation of spermatids or spermatocytes and vacuolar degeneration of Sertoli cells (Photos 1 and 2), findings that were not observed in the 2 weeks treatment groups. Apoptosis of pachytene spermatocytes in stages VII - IX and atrophy of Leydig cells were also more marked in the 4 week (Photo 2) than in the 2 week treated groups (Photo 3a). Retention of spermatids in stages IX - XIII was less after 4 weeks treatment than in any of the 2 weeks treatment groups (Photo 3b). Slight decrease in spermatocytes was observed in one rat of the 4 weeks treatment group. Slight multinucleated giant cell formation was found in two rats after 4 weeks treatment and in one rat receiving 3 mg/kg/day for 2 weeks. Slight swelling of spermatocytes was found in two rats in the 4 weeks treatment group. Slight decrease in elongated spermatids was found in one rat each of the 4 and 2 week treated groups at 3 mg/kg/day.

Quantitative morphometry
Results of quantitative morphometry are summarized in Fig. 1.
Spermatids, identified according to the method described by Leblond and Clermont (1952), were present in the apical part of the seminiferous epithelium. The spermatid index for spermatogenesis was significantly decreased only in the 4 week treated group.
PCNA-positive cells were detected in the testes of all animals analyzed. The label was confined to the nucleus and the labeled testicular germ cells were spermatogonia and spermatocytes. Negative control sections showed a complete lack of staining in all germ cell types. PCNA-positive cells were present in the basal part of the seminiferous epithelium (Photos 4a and 4b). The PCNA index for DNA synthetic activity in the 4 week treated group (Photo 4b) was significantly decreased as compared to the control group (Photo 4a).
TUNEL-positive cells were detected in the testes of all animals analyzed. The label was confined to the nucleus and the labeled testicular germ cells were again spermatocytes and spermatids. Negative control sections showed a complete lack of staining in all germ cell types. Most TUNEL-positive cells were pachytene spermatocytes of the seminiferous tubules (Photos 5a and 5b). TUNEL indices for in situ apoptosis showed significant increase after the 4 and 2 weeks treatments at 3 mg/kg/day, and less prominent increase in the 2 week treated group given 10 mg/kg/day.

Table 1. Body weights and organ weights for male rats treated orally with ethinyloestradiol for 2 or 4 weeks.

<table>
<thead>
<tr>
<th>Dose</th>
<th>No. of animals</th>
<th>0 mg/kg/day × 4w</th>
<th>3 mg/kg/day × 4w</th>
<th>3 mg/kg/day × 2w</th>
<th>10 mg/kg/day × 2w</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td></td>
<td>350 ± 28</td>
<td>202 ± 20**</td>
<td>270 ± 14**</td>
<td>272 ± 16**</td>
</tr>
<tr>
<td>Absolute organ weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pituitary (mg)</td>
<td></td>
<td>9.6 ± 1.6</td>
<td>8.0 ± 1.0</td>
<td>8.4 ± 1.8</td>
<td>10.2 ± 2.0</td>
</tr>
<tr>
<td>Epididymides (mg)</td>
<td></td>
<td>836 ± 59</td>
<td>236 ± 114**</td>
<td>453 ± 81**</td>
<td>460 ± 49**</td>
</tr>
<tr>
<td>Prostate (mg)</td>
<td></td>
<td>872 ± 190</td>
<td>98 ± 38**</td>
<td>256 ± 18**</td>
<td>257 ± 43**</td>
</tr>
<tr>
<td>Seminal vesicle (g)</td>
<td></td>
<td>0.75 ± 0.12</td>
<td>0.06 ± 0.01**</td>
<td>0.14 ± 0.02**</td>
<td>0.14 ± 0.03**</td>
</tr>
<tr>
<td>Testes (g)</td>
<td></td>
<td>3.13 ± 0.32</td>
<td>1.13 ± 0.66**</td>
<td>2.26 ± 0.30**</td>
<td>2.34 ± 0.15**</td>
</tr>
<tr>
<td>Relative organ weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pituitary (mg/100 g BW)</td>
<td></td>
<td>2.73 ± 0.40</td>
<td>3.98 ± 0.36**</td>
<td>3.08 ± 0.58</td>
<td>3.75 ± 0.58**</td>
</tr>
<tr>
<td>Epididymides (mg/100 g BW)</td>
<td></td>
<td>240 ± 22</td>
<td>116 ± 53**</td>
<td>167 ± 28**</td>
<td>169 ± 17**</td>
</tr>
<tr>
<td>Prostate (mg/100 g BW)</td>
<td></td>
<td>248 ± 45</td>
<td>48 ± 17**</td>
<td>95 ± 10**</td>
<td>95 ± 18**</td>
</tr>
<tr>
<td>Seminal vesicle (g/100 g BW)</td>
<td></td>
<td>0.216 ± 0.048</td>
<td>0.029 ± 0.008**</td>
<td>0.052 ± 0.011**</td>
<td>0.050 ± 0.007**</td>
</tr>
<tr>
<td>Testes (g/100 g BW)</td>
<td></td>
<td>0.894 ± 0.069</td>
<td>0.546 ± 0.302**</td>
<td>0.836 ± 0.097</td>
<td>0.862 ± 0.053</td>
</tr>
</tbody>
</table>

Values are means ± S.D.
Significantly different from the controls (0 mg/kg/day × 4w), *: p<0.05, **: p<0.01.

Table 2. Histopathological findings for the testes of male rats treated orally with ethinyloestradiol for 2 or 4 weeks.

<table>
<thead>
<tr>
<th>Findings</th>
<th>0 mg/kg/day × 4w (6)</th>
<th>3 mg/kg/day × 4w (6)</th>
<th>3 mg/kg/day × 2w (6)</th>
<th>10 mg/kg/day × 2w (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis of pachytene spermatocytes</td>
<td>6*</td>
<td>0 0 0</td>
<td>0 2 4</td>
<td>0 1 5 0</td>
</tr>
<tr>
<td>Apoptosis of round spermatids</td>
<td>6 0 0 0</td>
<td>2 1 3 0</td>
<td>6 0 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Atrophy of Leydig cell</td>
<td>6 0 0 0</td>
<td>0 1 1 4</td>
<td>0 0 6 0</td>
<td>0 2 4 0</td>
</tr>
<tr>
<td>Atrophy of seminiferous tubule</td>
<td>6 0 0 0</td>
<td>2 0 4 0</td>
<td>6 0 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Cellular swelling of spermatocyte</td>
<td>6 0 0 0</td>
<td>4 2 0 0</td>
<td>6 0 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Decrease in spermatocytes</td>
<td>6 0 0 0</td>
<td>5 1 0 0</td>
<td>6 0 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Decrease in elongate spermatids</td>
<td>6 0 0 0</td>
<td>5 1 0 0</td>
<td>5 1 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Exfoliation of elongate spermatids</td>
<td>6 0 0 0</td>
<td>2 0 0 4</td>
<td>6 0 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Exfoliation of round spermatids</td>
<td>6 0 0 0</td>
<td>3 1 2 0</td>
<td>6 0 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Exfoliation of spermatocytes</td>
<td>6 0 0 0</td>
<td>3 1 2 0</td>
<td>6 0 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Multinucleated giant cell formation</td>
<td>6 0 0 0</td>
<td>4 2 0 0</td>
<td>5 1 0 0</td>
<td>6 0 0 0</td>
</tr>
<tr>
<td>Retention of spermatids</td>
<td>6 0 0 0</td>
<td>4 2 0 0</td>
<td>0 5 1 0</td>
<td>0 6 0 0</td>
</tr>
<tr>
<td>Vacular degeneration of Sertoli cells</td>
<td>6 0 0 0</td>
<td>1 1 4 0</td>
<td>6 0 0 0</td>
<td>6 0 0 0</td>
</tr>
</tbody>
</table>

* Grade: −, negative; 1, slight; 2, moderate; 3, marked.
* Grade: −, negative; 1, slight; 2, moderate; 3, marked.
Testicular toxicity of ethinylestradiol with 2 weeks treatment.

Photo 1. Normal seminiferous tubules and Leydig cells in a control rat. HE stain. ×150.

Photo 2. Atrophic seminiferous tubules showing degeneration of spermatocytes, spermatids and Sertoli cells, and atrophy of Leydig cells in a rat treated with ethinylestradiol at 3 mg/kg/day for 4 weeks. HE stain. ×150.

Photo 3. Seminiferous tubules showing apoptosis of pachytene spermatocytes and retention of spermatids, and atrophy of Leydig cells in a rat treated with ethinylestradiol at 3 mg/kg/day for 2 weeks. HE stain. (a) ×150. (b) ×600.
DISCUSSION

In a previous study (Iwase et al., 1995), ethinylestradiol, a synthetic estrogen, was found to reduce body weight gain and food consumption without clinical symptoms in male rats treated for 4 weeks. Similar change was here noted after a dosing period of 2 weeks, which was concluded to therefore be sufficient to detect effects of ethinylestradiol on body weight in male rats.

Two weeks treatment with ethinylestradiol caused organ weight reduction of the epididymides, prostate, seminal vesicles and testes as with 4 weeks treatment in this study and in line with the literature (Gibson, et al., 1967; Das, 1977, Iwase et al., 1995).

Histopathological findings, like apoptosis of spermatocytes/spermatids, atrophy of Leydig cells, exfoliation of spermatocytes/spermatids and vacuolar degeneration of Sertoli cells were more marked or solely found in rats treated with ethinylestradiol for 4 weeks. The changes suggest that ethinylestradiol acts indirectly on spermatogenesis by decreasing testosterone secretion and interfering with release of LH and FSH (Takahashi and Matsui, 1993). Apoptosis of pachytene spermatocytes in stages VII - IX and retention of spermatids in stages IX - XIII observed frequently in rats treated for 2 weeks can be considered as early symptoms of hypospermatogenesis.

Using an antibody against PCNA, specific immunostaining of spermatogonia (although it was not possible to identify the specific differentiation stage) and pachytene spermatocytes was observed. Comparable results in Sprague-Dawley rats have been reported by Schlatt and Weinbauer (1994). A gonadotropin-releasing hormone antagonist (Schlatt and Weinbauer, 1994) and 1,3,5-trinitrobenezene (Chandra et al., 1997) were found to induce maximal testicular regression with decreased testicular weight and seminiferous tubular diameter, but the seminiferous tubules still contained a number of PCNA-positive cells. In this study, the PCNA index in rats treated with ethinylestradiol for 4 weeks was significantly decreased, but DNA synthetic activity was retained, as in the former investigations. These results indicate that spermatogonia as stem cells are probably not a target for ethinylestradiol toxicity. Therefore, other factors need to be investigated.

In the present study, apoptotic germ cells apparent in HE-stained sections were reflected by TUNEL staining, the latter being more sensitive for in situ detection of DNA strand breaks. Numbers of TUNEL-positive pachytene spermatocytes or round spermatids in rats treated with 3 mg/kg ethinylestradiol for 2 or 4 weeks were significantly increased as compared to the control group value.

In most cases, induction of apoptosis in these cell types occurs at stages I, XII and XIV (Blanco-Rodríguez

Fig. 1. Quantitative morphometric data for the testes of male rats treated orally with ethinylestradiol for 2 or 4 weeks. Values are means±S.D. Significantly different from the controls, *: p<0.05, **: p<0.01.
Testicular toxicity of ethinylestradiol with 2 weeks treatment.

Photo 4. Seminiferous tubules immuno-stained for PCNA in a rat treated with ethinylestradiol at 3 mg/kg/day for 4 weeks (b) and a control rat (a). PCNA-positive germ cells are more frequent in the 4 week treated rat (b). ×300.

Photo 5. Seminiferous tubules labeled with terminal dUTP nick end labeling (TUNEL) in a rat treated with ethinylestradiol at 3 mg/kg/day for 2 weeks (b) and a control rat (a). TUNEL-positive germ cells (arrows) are more frequent in the 2 week treated rat (b). ×300.
and Martínez-García, 1997), when dying cells are also found in control animals (Billig et al., 1995; Blanco-Rodríguez and Martínez-García, 1996a and 1996b; Kerr, 1992). Stage- and cell-specific occurrence of apoptosis in male germ cells has also been reported after withdrawal of testosterone (Bartke, 1995; Billig et al., 1995; Henrikson et al., 1995; Sinha et al., 1995; Troiano et al., 1994). Adriamycin, ethylene glycol monomethyl ether and 1,3-dinitrobenzene exclusively induce apoptotic death of spermatogonia, pachytene spermatocytes or round spermatids (Matsui and Takahashi, 1999). In developing and adult rats treated with a gonadotropin antagonist such as Azalin B that reduces serum LH and FSH (Billig et al., 1995; Sinha et al., 1995), or in adult rats administered ethane dimethane sulfonate that causes serum and intratesticular testosterone reduction by selective destruction of Leydig cells (Henrikson et al., 1995; Troiano et al., 1994), preleptotene or meiotic spermatocytes and post-meiotic spermatids were found to be predominantly apoptotic in stages VII and VIII; testosterone supplementation prevented the induction of apoptosis of these cells (Henrikson et al., 1995). A role for decrease in male hormone production can also be presumed for the ethynylestradiol-induced apoptosis observed here. Proteins probably involved in Sertoli-germ cell communication (Griswold, 1995; Jégou et al., 1993; Skinner, 1991) may be responsible for stage-specific control of cell survival or death. Death signals have been characterized, as well as the important Fas-Fas ligand system (Lee et al., 1997). Future, investigators should focus on apoptotic-death signals induced by ethynylestradiol.

In conclusion, while histopathological findings and reduced organ weights evidencing testicular toxicity were not as severe in rats treated with ethynylestradiol for 2 weeks in the present study as previously reported (Iwase et al., 1995), they were still unequivocal. Thus 2 weeks is a sufficient period for sensitive detection of adverse effects. Furthermore, our data suggest that the TUNEL index for quantitative morphometry is effective for evaluation of testicular toxicity.

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