COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS
19) EFFECTS OF TWO-WEEK REPEATED DOSING OF ENOXACIN ON THE MALE REPRODUCTIVE ORGANS

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ABSTRACT — The toxicity of Enoxacin (ENX), a fluoroquinolone antibacterial agent, on the testis and epididymis was studied in rats. ENX was administered to 5 male rats orally once daily for 2 weeks at the dose level of 3000 mg/kg/day. ENX-treated rats showed a marked decrease in body weight, and two of them died on Day 10. At the end of the dosing period, absolute weights of the epididymis were decreased; in contrast, relative weights of testis were increased in the ENX-treated group. On histopathological examination, testis of ENX-treated rats exhibited the following regressive changes: degeneration of spermatids and spermatocytes, retention of Step 19 spermatids, chromatin margination in nuclei of spermatids, multinucleated giant cell formation, and/or vacuolar degeneration of Sertoli cells. Additionally, desquamated cell debris was observed in the epididymis. Degenerative spermatids and spermatocytes were strongly positive by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). From these results, it is concluded that a 2-week treatment is sufficient to detect toxic effects of ENX on reproductive organs in male rats, and that testicular toxicity induced by ENX is associated with germ cell apoptosis.

KEY WORDS: Enoxacin, Testicular toxicity, Rat, Apoptosis, TUNEL

INTRODUCTION

Before administration of drug candidates to human, results of repeated dose toxicity studies must to evaluated. However, the period of repeated dose study was not harmonized in the International Conference on Harmonization. Japan and EU/US recommend 4 weeks and 2 weeks, respectively. This difference came about because evaluation of the effects on male reproduction was necessary before first clinical trials in Japan and there were not enough data indicating that 2 weeks are sufficient to detect effects on male reproductive potential. Therefore, the Japanese Pharmaceutical Manufacturing Association and the National Institute of Health Sciences (NIHS) 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 Enoxacin (ENX), a fluoroquinolone antibacterial agent, for 2 weeks to evaluate effects on the testis and epididymis. An earlier subacute (1-month) toxicity study of ENX in rats revealed toxicity on male reproductive organs (Takemoto et al., 1984).

MATERIALS AND METHODS

Animals and husbandry
Sixteen 7-week-old Sprague-Dawley male rats were purchased from Clea Japan Inc. (Fuji, Japan) and housed in metal cages in an animal room maintained at the temperature of 23±2°C with a relative humidity of 50±10% and a 12 hr: 12 hr light-dark cycle (light, 6:00-18:00). The animals were allowed free access to diet (CE-2, Clea Japan Inc.) and filtered and UV-radiation sterilized water. After one week of quarantine and

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acclimation, 10 healthy 8-week-old rats, weighing 262-276 g, were divided into two groups consisting of 5 rats each.

**Test substances and treatment**

ENX (Lot No. 37H0945, Sigma Chemical Co., St Louis, USA) was suspended at a concentration of 15 w/v% in a 0.2 w/v% aqueous solution of sodium carboxymethyl cellulose (CMC; Lot No. PAM7925, Wako Pure Chemical Ind. Ltd., Osaka, Japan). The animals were dosed once daily by gavage at the dosing volume of 20 ml/kg for 2 weeks. The actual dosing volumes were calculated based on the most recent body weight. Control animals received the same volume of 0.2 w/v% CMC. It has been reported that 1-month oral repeated dosing of ENX at the dose of 3000 mg/kg/day in the rats resulted in atrophy of seminiferous tubules and appearance of degenerative or necrotic spermatids in the epididymis (Takemoto et al., 1984). Based on this result, the same dose level was selected in the present study.

**Observation**

The animals were weighed twice weekly. They were exsanguinated under ether anesthesia at the end of the dosing period for pathological examination. The testes and epididymides were weighed and fixed in Bouin's solution and 10% neutral buffered formalin, respectively. The tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E) for histopathological examination. Additional sections of testis were stained for periodic acid-Schiff's reaction for quantitative morphometry of germ cells.

Histological findings for the testis were graded as follows: −, no remarkable change; ±, minimal (change in a few seminiferous tubules); +, slight (change in less than 20% of seminiferous tubules); ++, moderate (change in 20 to 50% of seminiferous tubules); ++++, severe (change in more than 50% of seminiferous tubules). Histological findings for the epididymis were graded as follows: −, no remarkable change; ±, minimal (change in a few epididymal ducts); +, slight (change in several epididymal ducts in the head); ++, moderate (change in almost all epididymal ducts of the head); ++++, severe (change in epididymal ducts from the head to the body).

In the sections of testis, cells undergoing apoptosis were detected by a modified terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method using an apoptosis detection kit (ApopTag® Plus In situ Apoptosis Detection kit; S7101-kit, Oncor Inc., MD, USA).

**Statistical analysis**

For body weight and organ weights of the testis and epididymis, mean values and standard deviations were calculated for the control and the ENX-treated groups. Parameters were analyzed for homogeneity of variance by the F-test (Snedecor and Cochran, 1980). For homogeneous data, analysis with the Student's t-test (Snedecor and Cochran, 1980) was performed, while for heterogeneous data, the Aspin-Welch's t-test was used (Snedecor and Cochran, 1980). Significant differences were analyzed at the 5 and 1% levels (one-tailed test). All of the above-mentioned statistical analyses were conducted using commercially available computer software (The SAS system, release 6.12 edition, SAS Institute Japan Inc.).

**RESULTS**

Data for body weight changes are shown in Table 1. In the ENX-treated group, the body weights were lower than those in the control group during the entire dosing period. Two ENX-treated animals died on Day 10; one was found dead and another was sacrificed due to deterioration in general condition.

Male reproductive organ weights are shown in Table 2. An increase in relative weight of the testis and a decrease in absolute weight of the epididymis were observed in the ENX-treated group.

Histopathological findings in the present study are summarized in Table 3. In the testis of the ENX-treated rats, decrease in numbers of and degeneration of spermatids and spermatocytes was remarkable in the seminiferous tubules. In addition, retention of Step 19 spermatids, chromatin margination in nuclei of spermatids and multinucleated giant cell formation were observed in the same animals (Photos 1, 2). Vascular degeneration of Sertoli cells was detected in the rat sacrificed on Day 10 (Photo 3). The testes of the rat which died on Day 10 were not examined because of severe autolysis. In the epididymides of ENX-treated rats, desquamated cell debris was observed in the ductal lumina (Photo 4). Evaluation of germ cells by quantitative morphometry was not conducted because obvious histological changes in the testis were detected in the H&E stained sections.

Regarding evaluation of apoptosis, occasional seminiferous tubules contained a few TUNEL-positive cells in control rats. Degenerative spermatids and spermatocytes were strongly positive by the TUNEL
Effects of Enoxacin on reproductive organs in male rats.

Table 1. Body weight changes of male rats administered ENX orally for 2 weeks.

<table>
<thead>
<tr>
<th>Dose (mg/kg/day)</th>
<th>No. of rats examined</th>
<th>Body weight (g)</th>
<th>Dosing period (Days)</th>
<th>0</th>
<th>3</th>
<th>7</th>
<th>10</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>268 ± 5</td>
<td>288 ± 8</td>
<td>313 ± 8</td>
<td>292 ± 21</td>
<td>352 ± 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3000</td>
<td>5</td>
<td>269 ± 5</td>
<td>271 ± 12*</td>
<td>241 ± 33**</td>
<td>238 ± 50*</td>
<td>305 ± 15**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean ± S.D.
* : Significantly different from the control (p<0.05).
**: Significantly different from the control (p<0.01).

Table 2. Organ weights of male rats administered ENX orally for 2 weeks.

<table>
<thead>
<tr>
<th>Dose (mg/kg/day)</th>
<th>No. of rats examined</th>
<th>Testis (g)</th>
<th>Epididymis (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>1.66 ± 0.11</td>
<td>0.88 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.47 ± 0.05)</td>
<td>(0.25 ± 0.03)</td>
</tr>
<tr>
<td>3000</td>
<td>3</td>
<td>1.66 ± 0.12</td>
<td>0.67 ± 0.04**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.54 ± 0.02)*</td>
<td>(0.22 ± 0.01)</td>
</tr>
</tbody>
</table>

Mean ± S.D.
* : Significantly different from control (p<0.05).
**: Significantly different from control (p<0.01).

Values in parentheses are relative weights calculated by (absolute weight/final body weight) × 100.

Table 3. Histopathological findings for male rats administered ENX orally for 2 weeks.

<table>
<thead>
<tr>
<th>Dose (mg/kg/day)</th>
<th>Organs</th>
<th>Histopathological findings</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>- ± + ++ +++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tests</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. of rats examined</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Degeneration, spermatids and spermatocytes</td>
<td>5 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retention, Step 19 spermatids</td>
<td>5 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chromatin margination, spermatids, nucleus</td>
<td>5 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multinucleated giant cell formation</td>
<td>5 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vacuolar degeneration, Sertoli cells</td>
<td>5 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epididymis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. of rats examined</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Desquamated cell debris, epididymal ducts</td>
<td>5 0 0 0 0</td>
</tr>
</tbody>
</table>

Histological grade: −, No remarkable change; ±, Minimal; +, Slight; ++, Moderate; +++, Severe.
a: including one rat sacrificed in a moribund condition on Day 10.
b: including one rat found dead and one rat sacrificed in a moribund condition on Day 10.
c: along with decrease in the numbers of spermatids and spermatocytes.
Photo 1. Seminiferous tubules from a rat treated with 3000 mg/kg/day of ENX. Note degeneration of spermatids and spermatocytes and decrease in their number. A multinucleated giant cell (arrow) and chromatin margination in nuclei of spermatids are evident. HE stain. ×450.

Photo 2. Seminiferous tubules from a rat treated with 3000 mg/kg/day of ENX. Note retention of Step 19 spermatids (arrows). HE stain. ×600.
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**Photo 3.** Seminiferous tubules from a rat treated with 3000 mg/kg/day of ENX. Note vacuolar degeneration of Sertoli cells. HE stain. ×600.

**Photo 4.** Epididymal ducts from a rat treated with 3000 mg/kg/day of ENX. Desquamated cell debris is observed in the lumen. HE stain. ×300.
method in ENX-treated rats (Photo 5).

DISCUSSION

The present study was conducted to examine the effects of 2-week treatment with ENX on male reproductive organs. Daily oral administration of 3000 mg/kg/day of ENX reduced the body weight gain, and two animals died during the dosing period. Histopathological examination revealed abnormalities of the testis and epididymis attributable to ENX: an increase in relative weight of the testis, a decrease in absolute weight of the epididymis, regressive changes of the testis and desquamated cell debris in the epididymis. Chromatin margination in nuclei of round spermatids and multinucleated giant cells were observed in ENX-treated rats; it is known that degenerating round spermatids show such changes (Creasy and Foster, 1991). Vacuolar degeneration of Sertoli cells, a primary morphological event produced by treatment with Sertoli cell toxicants, was observed in one ENX-treated rat.

A comparison of our results with findings of the 4-week study reported by Takemoto (1984) is given in Table 4. Reproductive organ weight changes were similar in the two cases. On histopathological examination of the testis, atrophy of seminiferous tubules was observed in the 4-week study. Histological features of the testis in the present study are in line with a transition to testicular atrophy. In the epididymis, histological changes associated with testicular atrophy were observed in each study. This 2-weeks exposure to ENX provided results similar to those obtained in the 4-week study. Therefore, a 2-week treatment is sufficient to detect toxic effects of ENX on male reproductive organs.

Changes of germ cells appeared to be related to apoptosis in the present study. In the testis, spontaneous germ cell degeneration occurs during spermatogenesis and this involves apoptosis (Blanco-Rodriguez and Martínez-García, 1996). Apoptosis in the testis is an important physiological mechanism to limit the number of germ cells, and is closely associated with the Sertoli cells, which are supportive cells in the seminiferous tubules and regulate proliferation and differentiation of germ cells. Apoptosis of germ cells is induced under several conditions such as treatment with germ cell toxicants and disturbance of the sex hormonal environment (Blanco-Rodriguez and Martínez-García, 1998;
Matsui and Takahashi, 1999; Sinha Hikim et al., 1997; Woolveridge et al., 1999). It is increased by treatment with Sertoli cell toxicants such as monoethylnhexyl phthalate, 2, 5-hexanediol and 1, 3-dinitrobenzene (Richburg and Boekelheide, 1996; Blanchard et al., 1996; Strandgaard and Miller, 1998). Injured Sertoli cells can not support germ cells adequately (Lee et al., 1999). Furthermore, the Fas system has been identified as a key regulator of apoptosis of germ cells, and treatment with Sertoli cell toxicants results in up-regulated expression of both Fas ligand (FasL) by Sertoli cells and Fas by germ cells (Lee et al., 1999). It is thus possible that the testicular injury induced by ENX may be attributable to Sertoli cell injury resulting in germ cell apoptosis under regulation by the Fas/FasL pathway, because vacuolar degeneration of Sertoli cells was here observed in one ENX-treated rat.

However, in cases where both spermatids and spermatocytes are affected by a testicular toxicant, it is difficult to discriminate between the true primary effects on germ cells and those resulting from Sertoli cell toxicity (Creasy and Foster, 1991). According to Allenby et al. (1990) and Williams and Foster (1988), Sertoli cell toxicants produce significant increases in secretion of lactate, pyruvate and/or inhibit in rat Sertoli cell cultures. Therefore, it is necessary that this be examined by in vitro assay to prove the hypothesis that ENX affects primarily Sertoli cells in the seminiferous tubules.

**ACKNOWLEDGMENT**

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**REFERENCES**


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**Table 4. Summary of the effects of ENX on reproductive organs in male rats.**

<table>
<thead>
<tr>
<th>Dosing period</th>
<th>2 weeks</th>
<th>4 weeks*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg/day)</td>
<td>3000</td>
<td>3000</td>
</tr>
<tr>
<td>Organ weights</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>(- (↑))</td>
<td>(- (↑))</td>
</tr>
<tr>
<td>Epididymis</td>
<td>(↓ (-))</td>
<td>(↓ (-))</td>
</tr>
<tr>
<td>Histopathological findings (main findings)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>Degeneration, spermatids/ spermatocytes</td>
<td>Atrophy, seminiferous tubules</td>
</tr>
<tr>
<td>Epididymis</td>
<td>Desquamated cell debris, epididymal ducts</td>
<td>Appearance of degenerative/ necrotic spermatids</td>
</tr>
</tbody>
</table>

*a: refer to the report of Takemoto et al..
Signs in parentheses represent changes in relative weights.
\(-\): No change.
\(↑\): Increase.
\(↓\): Decrease.
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