COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS

27) REPEATED TOXICITY STUDY ON ETHYLENE GLYCOL MONOMETHYL ETHER FOR 2 AND 4 WEEKS TO DETECT EFFECTS ON MALE REPRODUCTIVE ORGANS IN RATS

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ABSTRACT — As part of a collaborative project to determine the minimum administration period to detect compound effects on male reproductive organs in Sprague-Dawley (Crl:CD(SD)) male rats, 6- and 8-week-old rats were administered ethylene glycol monomethyl ether (EGME) daily at 100 and 200 mg/kg/day for 2 weeks or 100 mg/kg/day for 4 weeks, and histopathological changes in the testes and epididymides were examined.

Testis and epididymis weights in the 2-week 200 mg/kg and 4-week 100 mg/kg groups were obviously decreased.

On histopathological examination, severe degenerative changes in the testis such as atrophy of the seminiferous tubules and multinucleated giant cell formation were observed in all 2-week 200 mg/kg group rats. Degeneration of pachytene spermatocytes in Stages X IV, II, III and a decrease in the number of germ cells was observed with both 2 and 4 weeks treatments at 100 mg/kg/day. In the epididymides, the number of sperm in the caput decreased with 100 mg/kg/day groups after both 2 and 4 weeks. In addition, degeneration of pachytene spermatocytes induced by EGME was found to be exclusively due to apoptotic death.

Similar testicular and epididymal changes were observed with 2 and 4 weeks treatments at 100 mg/kg/day of EGME. Therefore, we conclude that a 2 weeks administration period is sufficient for detection of EGME effects on male reproductive organs.

KEY WORDS: Ethylene glycol monomethyl ether, Testicular toxicity, Male fertility, Histopathology, Rat

INTRODUCTION

The effects of drug candidates on male reproductive organs must be evaluated first before conducting human clinical studies in Japan. While a 2 week exposure period is considered sufficient for this purpose in the EU and US, in Japan a collaborative study of the Japanese Pharmaceutical Manufacturing Association (JPMA) and National Institute of Health Sciences (NIHS), only indicated that 4 weeks repeated dosing could detect toxicity (Takayama et al., 1995). There has hitherto been no consensus in Japan on the validity of 2 weeks studies. This investigation is part of another collaborating study conducted to obtain information about limitation of 2 weeks dosing.

Ethylene glycol monomethyl ether (EGME) is a water-soluble organic solvent widely used in the chemical industry. The ability of EGME to induce testicular damage was first demonstrated in rabbits (Wiley et al., 1938). EGME has been known as a toxic compound to
pachytene spermatocytes (Matsui and Takahashi, 1999). EGME-induced testicular damage is characterized, at low doses, by the death of pachytene spermatocytes undergoing meiotic division in stage X-IV seminiferous tubules (Foster et al., 1983; Chapin et al., 1984). As the dose is increased, other germ cell types are also affected, but to a much lesser extent (Chapin et al., 1985). The damage was found to be stage-specific and related to the exposure concentration-time period (Lee and Kinney, 1989). We selected EGME for comparison of the effects of 2 and 4 weeks treatments on male reproductive organs in rats.

**MATERIAL AND METHODS**

**Animals**

Five-week-old Sprague-Dawley (Crl:CD(SD)) male rats were purchased from Charles River Japan, Inc., Hino. After an acclimatization period of 6 or 20 days, healthy rats were used. They were housed individually in stainless steel cages in a controlled air-conditioned animal room at an ambient temperature of 23 ± 1°C, with a relative humidity of 55 ± 10%, a 12-hr light/dark cycle, and ventilation 10 changes/hr. The rats were allowed free access to tap water and a laboratory animal diet (CRF-1, Oriental Yeast Co., Ltd. Tokyo).

**Chemicals**

Ethylene glycol monomethyl ether (EGME) was obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan. It was orally administered by gavage to rats at dose levels of 0 and 100 mg/kg/day for the 4 weeks administration groups and 0, 100 and 200 mg/kg/day for the 2 weeks groups. EGME was dissolved in distilled water and administered to rats at 5 ml/kg.

**Experimental design**

1. **Four weeks dosing study**

   Six-week-old male rats were divided into 2 groups of 6 rats each. One group was administered 100 mg/kg of EGME orally for 4 weeks, and the other group was administered an equal volume of distilled water.

2. **Two weeks dosing study**

   Eight-week-old male rats were divided into 3 groups of 6 rats each. Two groups were administered 100 mg/kg or 200 mg/kg of EGME, the other was given an equal volume of distilled water.

**General observation and pathological examination**

Rats were observed once a day throughout the administration period. Body weights and food consumption were measured twice a week. The day after the last administration, rats were sacrificed by exsanguination under ether anesthesia. At necropsy, bilateral testes and epididymides were weighed separately. Relative weights (absolute weight/body weight) were calculated based on the final body weights of the animals. The testes were fixed in FSA fixative (mixture of 37% formalin, 5% sucrose solution and acetic acid with a 5.0:15.0:0.8 volume ratio) and the epididymides in 10% neutral buffered formalin. They were embedded in paraffin, sectioned, and stained with hematoxylin-eosin and for the periodic acid-Schiff reaction (H-E+PAS stain).

**Statistical analysis**

Data for the treated and control groups were compared using the F-test before application of the Student’s or Aspin-Welch’s t-tests (Snedecor and Cochran, 1980). The Student’s t-test (Snedecor and Cochran, 1980) was applied with homogeneity of variance, and the Aspin-Welch’s t-test (Snedecor and Cochran, 1980) with non-homogeneity. In tables in which the individual results differed significantly from those of the control group, this is indicated by asterisks (*: p<0.05, **: p<0.01).

**Analysis of germ cell death by TdT-mediated dUTP-biotin nick end labeling (TUNEL)**

Sectioned testes were treated with 20 μg/ml of proteinase K for 15 min at room temperature. Endogenous peroxidases were inactivated by incubation in 3% hydrogen peroxide in PBS for 5 min at room temperature. The sections were then labeled with TUNEL solution (Apop Tag Peroxidase kit; Oncor Appligene, Basel, Switzerland) for 60 min at 37°C followed by anti-digoxigenin antibody conjugated with peroxidase for 30 min at room temperature. Finally, brownish coloration of apoptotic cell nuclei was achieved by exposure to 0.2% 3,3'-diaminobenzidine for 5 min. Sections were counterstained with 2% methyl green solution for 20 min.

For verification of the present TUNEL procedures, sections of normal rat testis treated with the above proteinase K and hydrogen peroxide were incubated in 10 μg/ml of DNase and then labeled with TUNEL solution with or without TdT. The former was examined as a positive control and the latter as a negative one.
RESULTS

Clinical signs, body weights and food consumption data

None of the animals died during the experiment period and no clinical signs were observed in any animals of the 2- and 4-weeks dosing study.

In the 4-weeks dosing study, there were no significant changes in body weight and food consumption with 100 mg/kg/day. (Fig. 1)

In the 2-weeks dosing study, body weights were decreased significantly on days 7, 10 and 14 in the 200 mg/kg group. Food consumption in treated groups was significantly lower than in the control group on days 7, 10 and 14 in the 100 mg/kg group and on days 3, 7, 10 and 14 in the 200 mg/kg. (Fig. 2)

Organ weights

In the 4- and 2-weeks dosing study, testis and epididymis weights were decreased in the 100 mg/kg and 200 mg/kg groups, respectively. (Table 1)

Macroscopic examination

Atrophy of the testes and epididymides was observed at necropsy in the 200 mg/kg group of the 2-weeks dosing study.

Histopathological findings

1. Four weeks dosing study

In the testes, decreased numbers of spermato-
Table 1. Organ weights for rats treated with EGME for 2 or 4 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control(4W)</th>
<th>EGME-100(4W)</th>
<th>Control(2W)</th>
<th>EGME-100(2W)</th>
<th>EGME-200(2W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute organ weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis (L) (g)</td>
<td>1.550 (6) ± 0.060</td>
<td>1.293 (6) ± 0.187**</td>
<td>1.495 (6) ± 0.111</td>
<td>1.370 (6) ± 0.131</td>
<td>0.758 (6) ± 0.085**</td>
</tr>
<tr>
<td>Testis (R) (g)</td>
<td>1.562 (6) ± 0.069</td>
<td>1.270 (6) ± 0.197**</td>
<td>1.482 (6) ± 0.128</td>
<td>1.355 (6) ± 0.149</td>
<td>0.733 (6) ± 0.072**</td>
</tr>
<tr>
<td>Epididymis (L) (mg)</td>
<td>387 (6) ± 15</td>
<td>312 (6) ± 63*</td>
<td>383 (6) ± 29</td>
<td>342 (6) ± 33*</td>
<td>259 (6) ± 25**</td>
</tr>
<tr>
<td>Epididymis (R) (mg)</td>
<td>401 (6) ± 11</td>
<td>316 (6) ± 45**</td>
<td>376 (6) ± 28</td>
<td>344 (6) ± 42</td>
<td>259 (6) ± 27**</td>
</tr>
<tr>
<td>Relative organ weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testis (L) (g%)</td>
<td>0.429 (6) ± 0.040</td>
<td>0.370 (6) ± 0.032*</td>
<td>0.415 (6) ± 0.035</td>
<td>0.388 (6) ± 0.039</td>
<td>0.243 (6) ± 0.031**</td>
</tr>
<tr>
<td>Testis (R) (g%)</td>
<td>0.432 (6) ± 0.038</td>
<td>0.363 (6) ± 0.032**</td>
<td>0.412 (6) ± 0.042</td>
<td>0.384 (6) ± 0.039</td>
<td>0.235 (6) ± 0.025**</td>
</tr>
<tr>
<td>Epididymis (L) (mg%)</td>
<td>107 (6) ± 6</td>
<td>89 (6) ± 11**</td>
<td>106 (6) ± 10</td>
<td>97 (6) ± 7</td>
<td>83 (6) ± 8**</td>
</tr>
<tr>
<td>Epididymis (R) (mg%)</td>
<td>111 (6) ± 7</td>
<td>90 (6) ± 5**</td>
<td>105 (6) ± 10</td>
<td>97 (6) ± 8</td>
<td>83 (6) ± 9**</td>
</tr>
</tbody>
</table>

Mean(N)±S.D.
Significantly different from the Control-Group (*: p<0.05,**: p<0.01).
cytes (Photo 1), round spermatids in stages I-VIII and elongated spermatids were observed, and degeneration of dividing spermatocytes in stage X IV and pachytene spermatocytes in stages I-II. III was also observed in the 100 mg/kg group. In the epididymides, degeneration of the epithelium and a decrease in the number of sperm were noted in the caput (Table 2).

2. Two weeks dosing study

In the 100 mg/kg group, similar changes in the testes and epididymides were recognized. Although degeneration of dividing spermatocytes in stage X IV (Photo 2) and pachytene spermatocytes in stages I-II, III was also observed, decreased numbers of elongate spermatids were not observed. In the 200 mg/kg group, testicular and epididymal changes were more remarkable. In addition, the testes showed atrophy of seminiferous tubules, decrease in the number of elongated spermatids, and formation of multinucleated giant cell in the seminiferous tubules (Photo 3) (Table 3).

Furthermore, in the 2- and 4-weeks 100 mg/kg groups, degenerative spermatocytes were positive by TUNEL staining (Photo 4). In the 2-weeks 200 mg/kg group, spermatocytes with TUNEL-positive nuclei were even more prevalent.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg/day)</th>
<th>Control (4W)</th>
<th>EGME-100 (4W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tests</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Atrophy of seminiferous tubules</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multinuclear giant cells</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in number of spermatogonia</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in number of spermatocytes</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in number of round spermatids</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in number of elongate spermatids</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Epididymis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degeneration of epithelium</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in number of sperm in the caput</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*: no remarkable changes, +: slight, ++: moderate, +++: severe.

*: With degeneration of dividing spermatocytes in stage X IV and pachytene spermatocytes in stages I-II. III
†: Stage diagnosis was impossible.
‡: Stages I-VIII.

Table 3. Histopathological findings in the testes and epididymides for male rats treated with EGME for 2 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg/day)</th>
<th>Control (2W)</th>
<th>EGME-100 (2W)</th>
<th>EGME-200 (2W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tests</td>
<td></td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Atrophy of seminiferous tubules</td>
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<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in number of round spermatids</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in number of elongate spermatids</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Epididymis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degeneration of epithelium</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in number of sperm in the caput</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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*: With degeneration of dividing spermatocytes in stage X IV and pachytene spermatocytes in stages I-II. III
†: Stage diagnosis was impossible.
‡: Stages I-VIII.
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**Photo 1.** Decrease in numbers of spermatocytes with degeneration in 4-weeks dosing study of EGME-100 mg/kg group. HE+PAS stain. ×435.

**Photo 2.** Seminiferous tubule with degeneration of dividing spermatocytes (Stage X IV) in 2-weeks dosing study of EGME-100 mg/kg group. HE+PAS stain. ×435.
Effects of EGME on the male reproductive system in rats.

**Photo 3.** Seminiferous tubule with a decrease in the number of germ and multinucleated giant cell formation in 2-weeks dosing study of EGME-200 mg/kg group. HE+PAS stain. ×435.

**Photo 4.** Seminiferous tubule with terminal dUTP nick end labeling (TUNEL)-positive dividing spermatocytes (Stage X IV) in 2-weeks dosing study of EGME-100 mg/kg group. TUNEL method. ×435.
DISCUSSION

To determine an appropriate period for evaluation of toxicity on male reproductive organs, the model compound, EGME was administered to male rats for 2 or 4 weeks.

It has been known that each population of germ cells has its own sensitivity to different chemical toxicants. In the early stages of damage, many chemicals have been shown to have cell specific effects that are restricted to specific stages of the spermatogenic cycle. The previous study, the initial and major site of damage following EGME treatment was restricted to the primary spermatocytes undergoing postzygotene meiotic maturation and division. Additionally, within the spermatocyte population, differential sensitivity was observed depending on the precise stage of meiotic maturation: dividing spermatocytes (stage X IV) > early pachytene spermatocytes (stage I-II) > late pachytene spermatocytes (stage VIII-XIII) > mid-pachytene spermatocytes (stage III-VII)(Creasy, 1997).

In our study, decreased numbers of spermatocytes, and degeneration of dividing spermatocytes(stage X IV) and pachytene spermatocytes in stages I-II. III were observed in the 100 mg/kg group of the 4- and 2-weeks dosing study. Moreover, decreased numbers of spermatids were also observed in the 100 mg/kg group of the 4- and 2-weeks dosing study. These changes were more sever in the 4-week than the 2-week dosing study. In addition, testicular and epididymal changes were more remarkable in the 200 mg/kg group of the 2-week dosing study. Thus, the intensity of damages was observed depending on the dosing period and dose level.

Previous report described that death of pachytene spermatocytes induced by EGME was exclusively of apoptotic death(Matsu and Takahashi, 1999). This apoptotic features were confirmed using TUNEL staining in our study and could be detectable in the 100 mg/kg group of the 2-week dosing study.

In organ weight, testis weight was decreased with 2-weeks at 200 mg/kg and 4-weeks at 100 mg/kg. On the other hand, histopathological changes in the testes were already detectable after 2 weeks at 100 mg/kg/day.

In this study, male reproductive organ damages observed in the 100 mg/kg group of the 2-weeks dosing study reflected those of the 4-weeks dosing study. Therefore, we can draw the conclusion that 2-weeks administration is for the detection of EGME effects on male reproductive organs.

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REFERENCES