COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS
21) COMPARISON OF THE EFFECTS OF 2- AND 4-WEEKS PYRIMETHAMINE TREATMENT IN RATS

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ABSTRACT — Pyrithamine (PYR), clinically used as an antimalarial or antiparasitic agent, is known to exert testicular toxicity in rats on oral administration for 8 weeks. This study was performed to examine whether 2-weeks daily repeated doses of PYR might induce detectable toxicity on the male reproductive organs in rats. PYR was orally administered to Slc:SD male rats at doses of 12.5, 25 or 50 mg/kg for 2 weeks, or 6.25, 12.5 or 25 mg/kg for 4 weeks. No effects were seen in any group on physical examination and body weight measurement. Gross findings in autopsy exhibited no abnormalities in any of the animals. There were no meaningful changes in the weights of reproductive organs, or in the number and activity of sperm in the epididymides. Loss and degenerative changes of pachytenes spermatocytes in stages I to III seminiferous tubules were observed in the rats given 50 mg/kg PYR for 2 weeks.

From the results obtained, it is concluded that 2-weeks repeated dosing with PYR is sufficient to detect toxicity on male reproductive organs in rats using histopathological examination.

KEY WORDS: Pyrithamine, Infertility, Spermatogenesis, Reproductive toxicity, Rat

INTRODUCTION

Pyrithamine (PYR) is an inhibitor of dihydrofolate reductase (DHFR) (Miller et al., 1989) clinically used as an antimalarial or antiparasitic agent.

In terms of the effects of pyrithamine on male reproductive organs, Awoniyi et al. (1993) reported that 8-weeks PYR daily treatment (400 mg/kg) reduced the weights of the testes and epididymides, the testicular and epididymal sperm counts, and fertility without effects on pituitary LH, FSH and intratesticular testosterone concentrations. The results pointed to direct effects on the seminiferous epithelium. However, no data have been obtained regarding whether effects of PYR on male reproductive organs can be detected with a shorter period of treatment such as 2 or 4 weeks.

The present study was conducted as part of a collaborative work of JPMA and NIHS to obtain the information on the validity and limitations of 2-weeks repeated dose toxicity studies to detect effects on male reproductive organs in rats. Therefore PYR was administered to male rats for 2 and 4 weeks to determine whether 2-weeks treatment would allow toxicity on male reproductive organs to be detected.

MATERIALS AND METHODS

Test and control materials

The test material was pyrithamine (PYR, 5-(4-Chlorophenyl)-6-ethyl-2, 4-pyrimidinediamine), purchased from Wako Pure Chemical Industries, Ltd. The label claim purity was 99.0%.
The vehicle, 0.5% carboxymethylcellulose sodium salt (Wako Pure Chemical Industries, Ltd.) in water for injection (Otsuka Seiyaku Kojo Co., Ltd.), was also used as the control material.

**Animals and housing conditions**

Sprague-Dawley 5-week-old male rats were purchased from Japan SLC (Shizuoka) and used following one (4-weeks treatment group) or 3-weeks (2-weeks treatment group) quarantine and acclimation. Animals were assigned to each treatment group based on the body weight on the previous day to the dosing start. The range of body weights at the start of dosing was 233.7 to 263.5 g (6 weeks of age) and 319.4 to 363.2 g (8 weeks of age) in the 4-weeks and 2-weeks treatment groups, respectively.

Animals were housed individually in suspended stainless steel wire mesh cages (W200×D300×H180 mm) in a barrier system animal room with a temperature of 20-26°C, relative humidity of 40-70%, ventilation of 12 cycles/hr and a 12-hr light-dark cycle (light, 7:00-19:00). Animals received a commercial food standard diet CE-2 (CLEA Japan, Inc., Tokyo, Japan) and tap water *ad libitum*.

**Dose and administration**

In a preliminary oral repeated dose study, animals used were Crl:CD(SD) rats and 50 and 100 mg/kg were administered daily to 5 animals each. However, 9 of 10 rats died or were euthanized in a moribund condition within 2 weeks. Therefore, 50 mg/kg was considered as a lethal daily dose with 2-weeks treatment. The doses for the present study were selected on 12.5, 25 and 50 mg/kg for the 2-weeks treatment groups, and 6.25, 12.5 and 25 mg/kg for the 4-weeks treatment groups.

PYR was suspended in a 0.5% carboxymethylcellulose sodium salt solution using a mortar and pestle at a concentration of 6.25 mg/mL. Dosing suspensions or the vehicle were administered orally to rats by gavage daily for 2 or 4 weeks (14 or 28 days). Animals given the vehicle served as controls. The dosing volume was adjusted weekly based on individual body weight.

**Observations and measurements**

All animals were observed daily for survival and for changes in gross motor and behavioral activity and in appearance. General conditions of each animal were observed daily. Individual body weights were recorded on days 0 (first day of dosing), 3, 7, 10, 14, 17, 21, 24 and 28 of the study.

All surviving animals were euthanized for necropsy on days 14 or 28 of the study (at 10 weeks of age). The testes, epididymides, prostate and seminal vesicles were excised for measurement of weights following gross examination of thoracic and abdominal viscera. Relative weights of these excised organs were calculated on the basis of the terminal body weights.

Sperm were collected by mincing the right epididymis in modified Krebs-Ringer bicarbonate solution with 4 mg/mL of bovine serum albumin at 37°C and suspensions were incubated at 37°C for 1 hr in an incubator. Sperm activity was then determined with a sperm quality analyzer (SQA). SQA detects variation in optical density caused by the motility of sperm and provides a sperm motility index (SMI), considered suitable to assess rat sperm viability and motility (Hara et al., 1995). An aliquot of the suspension (0.1 mL) was diluted 100 times with cold physiological saline and the number of sperm was counted under a microscope using a hemacytometer.

Following organ weight measurements, the testes were fixed in FSA solution (20 mL of formalin, 60 mL of 5% sucrose and 3.2 mL of acetic acid mixture) and the left epididymis, prostate and seminal vesicles were fixed in 10% neutral buffered formalin. All the fixed organs were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Additional sections were stained with Periodic Acid-Schiff (PAS) and utilized to identify spermatogenic stages with dosage according to the criteria proposed by Russell et al. (1990). For quantitative evaluation of germ cells in the testes, six seminiferous tubules in stages I, II and III were randomly selected from six cross sections, and the numbers of normal and degenerated spermatocytes and Sertoli cells were counted in the controls and the rats given 25 and 50 mg/kg.

**Statistical analysis**

One-way analysis of variance was used to compare the means of data obtained from all treatment groups. Significant differences between the control and PYR treated group were assessed by the Dunnett test (God and Weil, 1994). The level of significance was taken as p<0.05 or 0.01.

**RESULTS**

There were no clinical findings of note during the treatment period in any rats. No effects on body weight gain were seen in any group (data not shown).

Gross examination at autopsy revealed no abnormalities in any of the animals. Table 1 summarizes data
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose mg/kg/day</th>
<th>Duration of treatment</th>
<th>Body weight (g)</th>
<th>Absolute</th>
<th>Relative</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tests (g)</td>
<td>Epididymides (mg)</td>
<td>Prostate (mg)</td>
<td>Seminal vesicles (mg)</td>
<td>Tests (10-3%)</td>
<td>Epididymides (10-3%)</td>
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<td>2 weeks</td>
<td>Mean 391.7</td>
<td>3.4</td>
<td>918.8</td>
<td>680.5</td>
<td>830.9</td>
<td>865.5</td>
<td>235.9</td>
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<tr>
<td></td>
<td>±SD</td>
<td></td>
<td>27.4</td>
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<td>133.2</td>
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<td>Mean 384.5</td>
<td>3.4</td>
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<td>714.7</td>
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<td>893.2</td>
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<td>14.0</td>
<td>2.1</td>
<td>46.8</td>
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<td></td>
<td>25</td>
<td></td>
<td>Mean 387.2</td>
<td>3.7</td>
<td>1024.0*</td>
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<td>2.0</td>
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<td>95.2</td>
<td>153.2</td>
<td>99.5</td>
<td>27.7</td>
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<td></td>
<td>50</td>
<td></td>
<td>Mean 370.1</td>
<td>3.6</td>
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<td>26.6</td>
<td>0.3</td>
<td>49.0</td>
<td>116.7</td>
<td>79.2</td>
<td>87.2</td>
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<tr>
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<td>4 weeks</td>
<td>Mean 393.6</td>
<td>3.4</td>
<td>885.6</td>
<td>758.7</td>
<td>881.1</td>
<td>872.3</td>
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<tr>
<td></td>
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<td></td>
<td>24.3</td>
<td>0.3</td>
<td>61.8</td>
<td>208.7</td>
<td>131.8</td>
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<tr>
<td>Pyrimethamine</td>
<td>6.25</td>
<td></td>
<td>Mean 383.7</td>
<td>3.5</td>
<td>917.1</td>
<td>609.0</td>
<td>760.9</td>
<td>901.8</td>
<td>239.0</td>
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<tr>
<td></td>
<td>±SD</td>
<td></td>
<td>18.1</td>
<td>0.2</td>
<td>98.4</td>
<td>146.9</td>
<td>129.7</td>
<td>33.8</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td></td>
<td>Mean 408.1</td>
<td>3.4</td>
<td>901.1</td>
<td>725.9</td>
<td>806.2</td>
<td>842.9</td>
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<tr>
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<td>0.1</td>
<td>43.4</td>
<td>103.0</td>
<td>68.5</td>
<td>53.9</td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td></td>
<td>Mean 381.3</td>
<td>3.5</td>
<td>896.6</td>
<td>610.5</td>
<td>692.4*</td>
<td>915.9</td>
<td>235.3</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td></td>
<td>16.5</td>
<td>0.1</td>
<td>73.7</td>
<td>112.3</td>
<td>120.3</td>
<td>38.3</td>
<td>18.0</td>
</tr>
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</table>

Significantly different from controls *p < 0.05, by the Dunnett test.
for absolute and relative weights of reproductive organs. Absolute weights of the epididymides were increased significantly in the rats given 25 mg of PYR for 2 weeks. Those of the seminal vesicles were decreased significantly in the rats given 25 mg of PYR for 4 weeks. These changes are not considered biologically meaningful because there were no significantly differences in the relative weights. On histological assessment, no treatment-related changes were observed in the epididymides, prostate or seminal vesicles.

Table 2 shows the results of the sperm analysis. There were no significant differences in any treatment group in their number in the epididymides and SMI.

However, slight to moderate loss and degeneration in spermatocytes of the seminiferous tubules were observed in rats given 50 mg/kg of PYR for 2 weeks (Photo 2a). PAS stained specimens demonstrated that loss and degenerative change of pachytene spermatocytes had occurred in stages I to III seminiferous epithelium (Photo 2b). The numbers of normal and degenerated spermatocytes, and the ratios of degenerated or normal spermatocytes to Sertoli cells are summarized in Table 2. The ratio of degenerated spermatocytes to Sertoli cells was increased, while the ratio of normal spermatocytes to Sertoli cells was decreased significantly in rats given 50 mg/kg for 2 weeks. No treatment-related changes were observed on the testes after 2-weeks treatment with the other doses and with any doses given for 4-weeks.

DISCUSSION

The present study demonstrated that 2-weeks daily treatment with 50 mg/kg PYR to rats induced loss and degenerative changes of pachytene spermatocytes in stages I to III seminiferous tubules. This change in the testis occurred without decrease in organ weight or alteration in the number or motility of sperm. Consequently, histopathological examination proved the most sensitive indicator of male reproductive toxicity of PYR in rats.

Four-weeks treatment at 25 mg/kg had no toxic effects on male reproductive organs in this study. Thus a higher dose such as 50 mg/kg might be considered necessary to induce testicular lesions, rather than a longer treatment period. Awoyie et al. (1993) administered much higher doses (100 and 400 mg/kg) of PYR.

Photo 1. Stage III seminiferous tubules in a male rat treated with the vehicle for 2 weeks. HE stain. ×73.
Table 2. Effects of pyrimethamine on the testes and sperms.

<table>
<thead>
<tr>
<th></th>
<th>2 weeks treatment</th>
<th>4 weeks treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>12.5 mg/kg</td>
</tr>
<tr>
<td>No. of animals examined</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>No. of sperm ($\times 10^6$/g)</td>
<td>5.75 ± 0.6</td>
<td>5.33 ± 0.3</td>
</tr>
<tr>
<td>Sperm motility index (SQA)</td>
<td>494.1 ± 91.9</td>
<td>478.2 ± 88.1</td>
</tr>
<tr>
<td>per gram epididymal weight</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>No. of animals with degenerated pachytene spermatocytes (%)</td>
<td>0</td>
<td>NE</td>
</tr>
<tr>
<td>Ratio of degenerated spermatocytes to Sertoli cells</td>
<td>2.31 ± 0.14</td>
<td>NE</td>
</tr>
</tbody>
</table>

Data represent mean ± S.D.
Significantly different from controls **p < 0.01, by the Dunnett test.
NE, not examined.
Photo 2a. Stage III seminiferous tubules in a male rat treated with 50 mg PYR for 2 weeks. Slight to moderate loss and degenerative changes in spermatocytes are apparent. HE stain. ×73.

Photo 2b. Stage III seminiferous tubules in a rat treated with 50 mg PYR for 2 weeks. Moderate loss of pachytene spermatocytes is evident. PAS stain. ×73.
The effects of 2- or 4-weeks pyrimethamine treatment in rats.

for 8 weeks to rats, and atrophic change of spermatogenic tubules occurred in rats given 400 mg/kg, while no changes were seen in the rats given 100 mg/kg. According to the results obtained in this study, 100 mg/kg would be expected to induce degeneration of seminiferous tubules. A reason for the discrepancy between Awoniyi's and the present study might be differences in toxicological susceptibility to PYR existing among breeders of rats. Fifty mg/kg PYR was a lethal dose in the C6J:CD(SD) strain rats in an preliminary study, while effects on body weight and mortality were not seen in the same 50 mg/kg of the present study using the SLC:CD(SD) strain. Furthermore, according to the Awoniyi's study using the SD strain rats supplied by SASCO (Omaha, NE), only a body weight decrease was seen with a very high dose (400 mg/kg) given daily for a long period (8 weeks). Consequently, toxic effects of PYR including those on the male reproductive organs might differ among breeding strains of rats. In order to examine this possibility, toxicokinetic examinations might be useful.

Awoiyi et al. (1993) revealed that antifertility effects of PYR on rats were caused by direct toxicity against the seminiferous epithelium, spermatocoea and possibly epididymis. However, the initial target of the toxicity is still uncertain. Our present short-term treatment revealed that a possible target of PYR might be pachytyne spermatocytes in stages I to III. Other compounds inhibiting DHFR such as methotrexate (MTX) and sulfadiazine (SASP) are known to cause infertility in rodents and humans (Schilli and Przybilla, 1985; Custer et al., 1977; O'Morain et al., 1984). Since testicular toxicity of MTX in rodents was observed concomitant with other toxicity in the hematopoietic, hepatic and gastrointestinal systems, the primary target is unclear. With SASP, sixty days of treatment of rats reduced epididymal sperm motility and increased sperm abnormalities (Sharm and Kalla, 1994), while others reported that no histopathological changes were observed in the testis (Polpramool and Srihao, 1983). However, in the recent 14-days SASP treatment study (Hoyt et al., 1995), late spermatids were generally absent from stage IX tubules in contrast to the control case. After 2 weeks recovery from 14-days SASP treatment, rats had a high percentage of both stage IX and X tubules containing luminal late spermatids, with a high incidence of fragments of condensed spermatid heads in the basal layer of stage XI tubules. Consequently, 14-days SASP treatment alters sperm release into seminiferous tubules.

The 2-weeks treatment studies of SASP and the present PYR revealed testicular toxicity even at sub- or slightly toxic dose levels in terms of general condition. However, toxic effects on the testis were evident in absolutely different stages between SASP and PYR even though the two compounds are considered to have common mechanisms of action.

In the present series of collaborative studies, validity of 2-weeks treatment study for detecting toxicity toward male reproductive organs will be concluded if compounds showing toxicity with 4-weeks treatment exhibit toxicity with 2-weeks treatment. The present study did not demonstrate male reproductive toxicity with 4-weeks treatment because the 50 mg/kg whereby it was observed after 2-weeks was not applied for 4-weeks. However, as an 8-weeks treatment study has demonstrated toxicity on male reproductive organs already, it can be concluded that 2-weeks repeated dose toxicity study is sufficient to detect toxicity of PYR on male reproductive organs in rats using histopathological parameters.

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