COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE
REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS
13) EFFECTS OF A SINGLE ORAL DOSE OF CYCLOPHOSPHAMIDE

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ABSTRACT — As part of a collaborative effort to evaluate whether effects on male reproductive organs
of chemicals can be detected within two-week in toxicity studies, eight-week-old male Sprague-Dawley
rats were given a single oral administration of 100 mg/kg of Cyclophosphamide (Cp), and sacrificed at 1, 3,
7 and 14 days thereafter. The numbers of seminiferous epithelial cells were counted in the seminiferous
tubules of stages II-III, V, VII and XII of the spermatogenic cycle. Animals showed decreased spermatogonia
at Day 3, decreased spermatogonia and preleptotene spermatocytes at Day 7, and decreased spermatogonia
and zygote spermatocytes at Day 14. We also detected decrease of zygote spermatocytes on
careful routine/traditional histopathological examination at Day 14.

These results suggest that the testicular toxicity of Cp can be detected within two weeks after treatment
with a sufficient dose in rats.

KEY WORDS: Cyclophosphamide, Testicular toxicity, Quantitative morphometry, Rats

INTRODUCTION

The present study was conducted as part of a collabora-
tive project of the JPMA and NIHA to obtain
information on the validity and limitations of 2-week
repeated dose toxicity studies for detection of effects on
male reproductive organs.

Cyclophosphamide (Cp) is an antineoplastic agent
whose active metabolites are alkylating agents that
cross-link DNA. The fundamental pharmacological
activities of alkylating agents are disruption of cell
growth, mitotic activity and differentiation. Cp has also
been used effectively for the control of proteinuria
(Penso et al., 1974) and Bahcet's disease, a systemic
disorder with ocular effects (Hijikata and Matsuda,
1978). Previous studies revealed the possibility that Cp
produces serious side effects, particularly on the male
reproductive system (Fairly et al., 1972). In experi-
mental animals, investigations have shown that spermatogonia
can be killed only by very high doses of Cp (Lu and
Meistrich, 1979; da Cunha et al., 1987; Matsui et al.,
1995; Higuchi et al., 1995).

We chose Cp in this collaborative work as a repre-
sentative agent exerting testicular toxicity in the rat.
The present study was designed to assess the effects of
a single treatment on the male reproductive system. For
this purpose, a simplified quantitative morphometric
approach was adapted, counting the numbers of semi-
iferous epithelia at four selected stages of the sper-
matogenic cycle.

MATERIALS AND METHODS

Eight-week-old male Sprague-Dawley rats supplied
by Charles River Japan Inc. (Shiga, Japan) were
used. They were maintained in an air-conditioned barri-
er-system animal room controlled at an ambient tem-
perature of 23±2°C, and relative humidity of 60±
10% with a 12-hr on/off light cycle, and food and water
provided ad libitum.

Thirty rats were given a single administration of
100 mg/kg of Cp which was equal to the oral LD50, and

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5 animals each were sacrificed at 1, 3, 7 and 14 days thereafter. Control animals received distilled water, and were sacrificed similarly after 1, 3, 7 and 14 days. After weighting the animals, rats were sacrificed by exsanguination under ether anesthesia. The testes and epididymides were removed and absolute weights were determined.

The testes were preserved in Bouin's solution, and the epididymides in 10% neutral buffered formalin. Both were routinely processed for embedding in paraffin, and sectioning and staining with HE or PAS for light microscopic assessment. For the examination of seminiferous tubules, quantitation of spermatogenic cells using a simplified morphometrical method was performed for stages II-III, V, VII and XII of the spermatogenetic cycle. Five seminiferous tubules per animal were randomly chosen for each stage of the spermatogenetic cycle and the numbers of seminiferous epithelial cells divided into spermatogonia, preleptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, round spermatids, and Sertoli cells were counted. The data were expressed as numbers of spermatogenic cells per Sertoli cell per seminiferous tubule cross section.

In one animal of the treated group sacrificed at Day14, the weights of right testis and epididymis were lower than the left counterparts and those in the control group. On a histopathological examination, unilateral focal atrophy of seminiferous tubules in the testes was observed. Taking the unilateral and focal characteristics as well as reports of the same findings in intact rats into account, it was concluded that the observed lesions were spontaneous. Therefore, we excluded the data for this particular animal from the evaluation of organ weights and morphological characteristics in this study.

### Statistical analysis

The results for both treatment and control groups were analyzed using the Student's t-test (Snedecor, 1959) with the level of significance set at p<0.05.

### RESULTS

#### Body weight and food consumption

The body weights of the rats in the Cp treatment group were significantly lower than those of the controls at 1 and 3 days after treatment. The body weight gain of the Cp treatment group was also significantly lower than that of the controls at 1, 3 and 7 days after treatment. The food consumption of the Cp treatment group was significantly lower than those of the controls at 3 and 7 days after treatment (Table 1).

#### Organ weights

Absolute weights of the testes and epididymides in the Cp treatment group showed no significant differences from the control group values (Table 2).

### Table 1. Body weight and food consumption for male rats after a single oral administration of cyclophosphamide.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control Mean ± S.D. (n)</th>
<th>Cp Mean ± S.D. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days after administration</td>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>Body weight</td>
<td>328.5 ± 15.8 (20)</td>
<td>325.2 ± 16.1 (30)</td>
</tr>
<tr>
<td>Day 1</td>
<td>331.8 ± 17.7 (20)</td>
<td>315.4 ± 17.8** (30)</td>
</tr>
<tr>
<td>Day 3</td>
<td>337.2 ± 19.7 (15)</td>
<td>320.0 ± 18.0** (25)</td>
</tr>
<tr>
<td>Day 7</td>
<td>352.1 ± 25.5 (10)</td>
<td>336.2 ± 22.6 (20)</td>
</tr>
<tr>
<td>Day 14</td>
<td>384.2 ± 41.7 (5)</td>
<td>366.9 ± 21.5 (15)</td>
</tr>
<tr>
<td>Body weight gain</td>
<td>Day 1</td>
<td>Cp</td>
</tr>
<tr>
<td>Day 1</td>
<td>3.4 ± 3.4 (20)</td>
<td>-9.8 ± 5.6** (30)</td>
</tr>
<tr>
<td>Day 3</td>
<td>10.7 ± 5.6 (15)</td>
<td>-6.0 ± 5.9** (25)</td>
</tr>
<tr>
<td>Day 7</td>
<td>27.4 ± 13.2 (10)</td>
<td>11.2 ± 9.6** (20)</td>
</tr>
<tr>
<td>Day 14</td>
<td>60.2 ± 24.7 (5)</td>
<td>43.2 ± 12.2 (15)</td>
</tr>
<tr>
<td>Food consumption</td>
<td>Day 3</td>
<td>Day 1</td>
</tr>
<tr>
<td>Day 3</td>
<td>27.9 ± 3.2 (15)</td>
<td>20.3 ± 2.2** (25)</td>
</tr>
<tr>
<td>Day 7</td>
<td>26.6 ± 2.7 (10)</td>
<td>24.3 ± 2.6** (20)</td>
</tr>
<tr>
<td>Day 14</td>
<td>28.2 ± 3.2 (5)</td>
<td>26.8 ± 2.2 (15)</td>
</tr>
</tbody>
</table>

Cp: Cyclophosphamide,

*, **: Significantly different from the control values at p<0.05 and p<0.01, respectively.

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Routine/traditional histopathological examination

Necrosis of spermatogonia at the base of seminiferous tubules could not be detected in any treated animals. At Day 14, two treated animals showed decrease of zygotone spermatocytes in stage XII detected by careful observation. No drug related changes in the epididymis were observed in any of the treated animals (Table 3).

Quantitative morphometry of seminiferous epithelia

None of the types of spermatogenic cell at stages II-III, V, VII and XII showed any changes at 1 day after the Cp treatment. At Day 3, significant decrease in the number of spermatogonia in stages II-III was seen. At Day 7, significant decreases in the numbers of spermatogonia in stages II-III and V, and preleptotene spermatocytes in stage VII were seen. At Day 14, significant decreases in the numbers of spermatogonia in stage VII, and zygotene spermatocytes in stage XII were evident (Fig. 1).

DISCUSSION

In the present study, we used the simplified quantitative morphometric method of Matsui et al. (1995). This allowed us to detect testicular damage as early as 3 days after the single administration of Cp. At Day 3, decreased numbers of spermatogonia at stages II-III were apparent. At Day 7, decreases in the numbers of spermatogonia in stages II-III and V, and preleptotene spermatocytes in the stage VII were evident. At Day 14, decreased numbers of spermatogonia in stage VII, and zygotene spermatocytes in stage XII were found. By the careful routine/traditional histopathological examination, decrease in the number of zygotene spermatocytes in stages XII - XIII at Day 14 was also detected.

For the analysis of target cell type, we used the method proposed by Ettlin et al. (1984). Based on the

Table 2. Organ weights for male rats after a single oral administration of cyclophosphamide. Unit:(mg)

<table>
<thead>
<tr>
<th>Days after administration</th>
<th>Organs</th>
<th>Group</th>
<th>Right</th>
<th>Left</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1.52 ± 0.05(5)</td>
<td>1.55 ± 0.09(5)</td>
<td>0.33 ± 0.02(5)</td>
<td>0.35 ± 0.03(5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cp</td>
<td>1.54 ± 0.02(5)</td>
<td>1.54 ± 0.06(5)</td>
<td>0.34 ± 0.03(5)</td>
<td>0.34 ± 0.01(5)</td>
</tr>
<tr>
<td>Day3</td>
<td></td>
<td>Control</td>
<td>1.52 ± 0.13(5)</td>
<td>1.42 ± 0.19(5)</td>
<td>0.39 ± 0.03(5)</td>
<td>0.39 ± 0.02(5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cp</td>
<td>1.50 ± 0.08(5)</td>
<td>1.49 ± 0.05(5)</td>
<td>0.35 ± 0.01(5)</td>
<td>0.39 ± 0.05(5)</td>
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<tr>
<td>Day7</td>
<td></td>
<td>Control</td>
<td>1.55 ± 0.16(5)</td>
<td>1.53 ± 0.12(5)</td>
<td>0.38 ± 0.05(5)</td>
<td>0.36 ± 0.03(5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cp</td>
<td>1.52 ± 0.10(5)</td>
<td>1.53 ± 0.12(5)</td>
<td>0.38 ± 0.05(5)</td>
<td>0.36 ± 0.03(5)</td>
</tr>
<tr>
<td>Day14</td>
<td></td>
<td>Control</td>
<td>1.51 ± 0.16(4)</td>
<td>1.53 ± 0.16(4)</td>
<td>0.44 ± 0.04(4)</td>
<td>0.45 ± 0.05(4)</td>
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<tr>
<td></td>
<td></td>
<td>Cp</td>
<td>1.55 ± 0.05(4)</td>
<td>1.56 ± 0.02(4)</td>
<td>0.44 ± 0.01(4)</td>
<td>0.43 ± 0.04(4)</td>
</tr>
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</table>

Mean ± S.D. (n). Cp: Cyclophosphamide,
* , **: Significantly different from the control values at p<0.05 and p<0.01, respectively.

Table 3. Histopathological findings for male rats after a single oral administration of cyclophosphamide.

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<tbody>
<tr>
<td></td>
<td>Testis</td>
<td>Day1</td>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(4)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Decrease of zygotene spermatocytes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1(+)</td>
<td>1(+)</td>
<td>0</td>
<td>0</td>
<td>2(+)</td>
<td>1(+)</td>
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<tr>
<td></td>
<td>Epididymis</td>
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<tr>
<td></td>
<td>Spermatic granuloma/unilateral</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1(+)</td>
<td>1(+)</td>
<td>1(+)</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

Cont.: Control, Cp: Cyclophosphamide,
−: No significant lesions, ±: very slight, +: slight, ++: moderate, +++: severe.
present analysis, we conclude that type A spermatogonia are the target cells most affected initially by Cp.

This is in line with the results of Matsui et al. (1995) who also evaluated the testes of rats 1, 7, 14 and 21 days after single administration of 100 mg/kg of Cp using the simplified quantitative morphometric method. They found significant decreases in the number of spermatogonia in stage V and preleptotene spermatocytes in stage VII at Day 7, and the numbers of preleptotene spermatocytes in stage VII and zygotene spermatocytes in stage VIII at Day 14.

Russell and Russell (1991) evaluated changes in the testes of rats 15 days after two injections of 65 mg/kg of Cp, and reported consistent decreases in the numbers of leptotene, zygotene, and early pachytene spermatocytes. They also concluded that spermatogonia were the cell type affected at the time of injection.

Lu and Meistrich (1979) performed a morphological evaluation at 11 days after a single dose of 200 mg/kg Cp, and determined that cell types A through B spermatogonia were sensitive to Cp in mice. They also suggested that preleptotene spermatocytes were partially destroyed by Cp. Our conclusion about the target cell of Cp is consistent with the reports all three groups of investigators (Matsui et al., 1995; Russell and Russell, 1991; Lu and Meistrich, 1979).

In conclusion, we have detected testicular damage as early as at 3 days after a single administration of Cp using a simplified quantitative morphometric method. We also found decrease in the number of zygotene spermatocytes in stages XII - XIII at 14 days after a single administration of Cp on a careful routine/traditional histopathological examination. These results suggest that the testicular toxicity of Cp can be detected.

Fig. 1. Numbers of spermatogenic cells per sertoli cell in seminiferous tubules of rats that had received a single oral administration of cyclophosphamide.

* p<0.05; ** p<0.01, respectively.

Effects of cyclophosphamide on the male reproductive system in rats.

within two weeks after the first treatment at sufficient doses of Cp in rats.

ACKNOWLEDGMENT

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REFERENCES


