COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS 16) EFFECTS OF SHORT-TERM ADMINISTRATION OF CARMOFUR ON SPERMATOGENESIS

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ABSTRACT — Since morphological changes in the testes were observed in a 4 week repeated dose toxicity study of carmofur in rats, male Sprague-Dawley rats were administered the compound orally at 200 mg/kg by gavage for 11 days to assess the possibility of detecting abnormalities in spermatogenesis within a 2 week period. For comparison two studies were performed, treatment groups of fifteen and fourteen rats being given carmofur for 28 and 11 days, respectively.

There were seven and nine rats that died or were killed in a moribund condition with 28 and 11 days treatment, respectively. Histopathologically, vacuolar degeneration of Sertoli cells, degeneration of elongated spermatids, exfoliation of round spermatids and multinucleated giant cell formation were observed in the testes of all animals and cell debris in ductus epididymidis was noted in almost all rats in the 28 days study. Exfoliation of round spermatids and multinucleated giant cell formation were detectable in two of five rats in the 11 days study, and vacuolar degeneration of Sertoli cells was also evident in four, though the changes were slight. No abnormalities were observed in the epididymides in the 11 days study.

These results suggest that effects of carmofur on spermatogenesis can be detected with a treatment period of less than 2 weeks using histopathological examination of the testes. Therefore a 2-week repeated dose toxicity study has validity for detection of effects of carmofur on the male reproductive organs in the rats.

KEY WORDS: Carmofur, Rat, Testis, Sertoli cell, Epididymis

INTRODUCTION

Before first administration of drug candidates to humans, results of repeated dose toxicity studies must be evaluated. However, the period for such repeated dose studies was not harmonized in ICH (ICH-M3 step IV guideline, 1997), Japan and the EU/US recommending 4 weeks and 2 weeks, respectively. This was because there were not enough data in Japan indicating that 2 weeks studies are appropriate to detect effects on male reproduction parameters (ICH-M3 step IV guideline, 1997). Therefore, the JPMA and NIH have jointly 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 carmofur for 2 weeks and compared the results with those of a 4-weeks administration.

Many chemotherapeutic agents show adverse effects on the testis of rodents (Meistrich et al., 1982). Carmofur is a pyrimidine derivative chemotherapeutic agent, classified as an antimetabolite, which is absorbed quickly at the intestine, subsequently releasing fluorouracil (5-FU) slowly. This is reported to be responsible for its anticancer activities (Hoshi et al., 1976). Morphological changes in the testis have
already been observed in a 4-week repeated dose toxicity study of carmofur in rats (Ishimura et al., 1979).

MATERIALS AND METHODS

Chemicals

The chemical structure of carmofur (5-fluoro-N-hexyl-1,2,3,4-tetrahydro-2,4-dioxo-1-pyrimidinecarboxamide) is shown in Fig. 1. The compound was obtained from Mitsui Pharmaceuticals Inc. (Tokyo, Japan) and suspended in a 0.5% methylcellulose solution.

Animals

A total of 40 male Sprague-Dawley rats of SPF origin (Jcl:SD) were purchased from Clea Japan Inc. (Tokyo). At the initiation of the administration, the rats were 6 weeks old, and their body weights ranged from 165 to 203 g. During the experiment, the animals were housed in stainless steel cages with wire meshed floors in an animal room with a controlled temperature of 23 ± 2°C, a humidity of 50 ± 10%, ventilation at 15 times an hr and lighting from 6:00 a.m. to 6:00 p.m. The animals were allowed free access to solid chow (LABO MR stock, Nihon Nosan Kogyo Inc., Yokohama) and local tap water (Mobara, Chiba).

![Chemical structure of carmofur](image)

**Fig. 1.** Chemical structure of carmofur (5-fluoro-N-hexyl-1,2,3,4-tetrahydro-2,4-dioxo-1-pyrimidinecarboxamide).

Experimental design

The experimental design is shown in Fig. 2. For each study, twenty rats were divided into two groups. In the 28 days study, the control and treatment groups consisted of five and fifteen rats, respectively and in the 11 days study, six and fourteen, respectively. The animals were treated with carmofur orally at 200 mg/kg/day by gavage in a volume of 4 mL/kg. Control animals were given the vehicle (4 mL/kg of 0.5% methylcellulose solution).

General observation

The first day of administration was defined as Day 0 for each study. Mortality was monitored daily during the treatment period. Body weights were recorded on Days 0, 4, 11, 18 and 28.

Organ weights and histopathology

At the termination of treatment, organs including the testes and epididymides were weighed to allow calculation of organ weight / body weight ratios, and fixed in FSA fixative, which is composed of 37% formalin, 5% sucrose solution, acetic acid at a volume ratio of 5:15:0.8, for histological examination.

Statistical analysis

Mean values and standard deviations were calculated for body and organ weight data. The significance of differences between control and treatment groups in each study was analyzed and evaluated at 5% or 1% levels of probability. Analysis of variance for mean values of each group was performed using the F-test (Yoshimura, 1987). In case of equal variance, the Student’s t-test was applied, and if this was not the case the Welch-test was used (Yoshimura, 1987).
RESULTS

General observation

There were seven and nine rats that died or were killed in a moribund condition in the 28 days and 11 days studies, respectively. In the 28 days study, 2, 2, 2 and 1 rats were involved on days 5, 7, 8, and 27, respectively. In the 11 days study, there were 1, 1, 1, 3, 1, 1 and 1 on days 2, 4, 7, 8, 9, 10 and 11 respectively. Consequently, eight rats treated for 28 days and five rats treated for 11 days survived to the termination.

Table 1 summarizes data for body weight change of rats treated for 28 or 11 days. Statistically significant decreases in body weight gain were observed in both the 28 and 11 days studies with compound administration.

Organ weights and histopathology

Absolute and relative organ weight data are summarized in Table 2. Decreases in absolute weights of the testes and epididymides and increases in their relative organ weights were found in both 28 and 11 days study.

Histopathological findings for the testes and epididymides are summarized in Table 3. In the testes, vacuolar degeneration of Sertoli cells, degeneration of elongated spermatids, exfoliation of round spermatids and multinucleated giant cell formation in seminiferous tubules were recognized in all surviving animals treated for 28 days, and severe changes were observed in two to three rats (Photos 1-4). Exfoliation of round spermatids and multinucleated giant cell formation were detectable in two of five rats in the 11 days study, and vacuolar degeneration of Sertoli cells was apparent in four, though the changes were slight (Photos 5, 6). In the epididymides, cell debris in ducts was recognized in almost all animals treated for 28 days, but not in these treated for 11 days (Photos 7, 8).

Table 1. Body weight changes of male rats treated with carmofur for 28 or 11 days.

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>Control</th>
<th>200</th>
<th>Control</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dosing period (days)</td>
<td>28</td>
<td>28</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Day 0</td>
<td>5; 173 ± 2.7\textsuperscript{a)}</td>
<td>8; 173 ± 5.2</td>
<td>6; 194 ± 5.3</td>
<td>5; 187 ± 4.0#</td>
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<tr>
<td>4</td>
<td>5; 208 ± 4.5</td>
<td>8; 172 ± 12.7\textsuperscript{**}</td>
<td>6; 223 ± 5.1</td>
<td>5; 173 ± 22.4#</td>
</tr>
<tr>
<td>11</td>
<td>5; 263 ± 9.0</td>
<td>8; 183 ± 28.2\textsuperscript{**}</td>
<td>6; 278 ± 10.5</td>
<td>5; 155 ± 42.4#</td>
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<tr>
<td>18</td>
<td>5; 314 ± 7.8</td>
<td>8; 205 ± 38.6\textsuperscript{**}</td>
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<tr>
<td>28</td>
<td>5; 374 ± 11.3</td>
<td>8; 224 ± 39.5\textsuperscript{**}</td>
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</table>

\textsuperscript{a)}Number of animals; mean ± S.D.

Significantly different from the control (28days)(t-F test), \textsuperscript{**}; 1% level of probability.

Significantly different from the control (11days)(t-F test), \#; 5%, \#; 1% level of probability.

Table 2. Absolute and relative organ weights for male rats treated with carmofur for 28 or 11 days.

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>Control</th>
<th>200</th>
<th>Control</th>
<th>200</th>
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<tbody>
<tr>
<td>Dosing period (days)</td>
<td>28</td>
<td>28</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>No. of animals</td>
<td>5</td>
<td>8</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>374.4 ± 11.3\textsuperscript{a)}</td>
<td>223.9 ± 39.5\textsuperscript{**}</td>
<td>277.5 ± 10.5</td>
<td>154.7 ± 42.4#</td>
</tr>
<tr>
<td>Organ weights</td>
<td></td>
<td></td>
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<tr>
<td>Testes (g)</td>
<td>3.48 ± 0.15</td>
<td>2.17 ± 0.59\textsuperscript{**}</td>
<td>2.86 ± 0.12</td>
<td>2.39 ± 0.29#</td>
</tr>
<tr>
<td>(g/100 g body weight)</td>
<td>0.93 ± 0.04</td>
<td>0.96 ± 0.16</td>
<td>1.03 ± 0.05</td>
<td>1.61 ± 0.32#</td>
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<tr>
<td>Epididymides (g)</td>
<td>0.93 ± 0.11</td>
<td>0.67 ± 0.13\textsuperscript{**}</td>
<td>0.44 ± 0.08</td>
<td>0.31 ± 0.08#</td>
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<td>(g/100 g body weight)</td>
<td>0.25 ± 0.03</td>
<td>0.30 ± 0.04#</td>
<td>0.16 ± 0.02</td>
<td>0.21 ± 0.03#</td>
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\textsuperscript{a)}mean ± S.D.

Significantly different from the control (28days)(t-F test), *; 5%, **; 1% level of probability.

Significantly different from the control (11days)(t-F test), #; 5%, ##; 1% level of probability.
Table 3. Histopathological findings for male rats treated with carmofur for 28 or 11 days.

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>Control</th>
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<tr>
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<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
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<thead>
<tr>
<th>Findings</th>
<th>5&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>2</th>
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<th>1</th>
<th>4</th>
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<tr>
<td>Testes</td>
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<tr>
<td>Vacuolar degeneration of Sertoli cells</td>
<td>5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
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<tr>
<td>Degeneration of elongated spermatids</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
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<td>0</td>
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<tr>
<td>Exfoliation of round spermatids</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>3</td>
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<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
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<tr>
<td>Multinucleated giant cell formation</td>
<td>5</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
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<td>Epididymides</td>
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<tr>
<td>Cell debris in ducts</td>
<td>5</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>5</td>
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<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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</table>

<sup>a</sup>Number of animals.

Grade: — no change, + slight, ++ moderate, +++ severe.

Photo 1. Seminiferous tubules of a control rat after four weeks vehicle treatment.
HE stain. ×200.
Effects of short-term administration of carmofur on spermatogenesis.

Photo 2. Seminiferous tubules of a rat treated with 200 mg/kg/day of carmofur for four weeks. Note the severe vacuolar degeneration of Sertoli cells and degeneration of elongate spermatids. HE stain. ×200.

Photo 3. Seminiferous tubules of a rat treated with 200 mg/kg/day of carmofur for four weeks. Note the severe exfoliation of round spermatids. HE stain. ×200.
Photo 4. Seminiferous tubules of a rat treated with 200 mg/kg/day of carmofur for four weeks. Note the severe multinucleated giant cell formation. HE stain. ×200.

Photo 5. Seminiferous tubules of a rat treated with 200 mg/kg/day of carmofur for two weeks. Note the slight vacuolar degeneration of Sertoli cells. HE stain. ×200.
Effects of short-term administration of carmofur on spermatogenesis.

**Photo 6.** Seminiferous tubules of a rat treated with 200 mg/kg/day of carmofur for two weeks. Note the slight multinucleated giant cell formation. HE stain. ×200.

**Photo 7.** Ductus epididymidis of a control rat after four weeks vehicle treatment. HE stain. ×100.
DISCUSSION

Morphological changes in the testes like those found here were observed in an earlier 4 week repeated dose toxicity study of carmofur in rats (Ishimura et al., 1979). Histopathologically, disorder of spermatogenesis and necrosis of germinal cells were observed in rats treated with 200 mg/kg/day of carmofur, a dose causing appreciable mortality.

In a first study we tried to compare the effects of 4 weeks treatment starting from six weeks of age with those of 2 weeks treatment from eight weeks. However, we failed to assess differences, because all animals died within a week after beginning the administration in the latter group. Therefore, we designed the present schedule with 4 and 2 weeks treatment from six weeks old with 14 or 15 rats in the treatment groups, hoping thereby to be able to examine histopathologically more than five, because about half the rats treated with 200 mg/kg/day might be die. Administration beyond day 11 in the 2 weeks study proved impossible because of mortality in excess of expectation. The mean (±S.D.) body weights on day 0 of the animals which died, at 195 (±6.5)g, on the bases of which the administration volume was calculated, was higher by about 10% in the 11 days as compared with the 28 days case, 177 (± 5.4)g. This could be the reason for greater adverse effects of carmofur on the general conditions of rats in the 11 days study.

A decrease in body weight was observed in rats treated with carmofur for 11 days. Decreases in absolute weights but increases in relative weights in the testes and epididymides were observed in the 28 days study. Increases in relative weights of those organs were considered to be due to markedly low body weights. The weights of the testes and epididymides treated for 11 days showed similar changes to those in the 28 days study.

Histopathologically, vacuolar degeneration of Sertoli cells, degeneration of elongated spermatids, exfoliation of round spermatids and multinucleated giant cell formation were observed in the testes. These changes were observed in all surviving animals treated for 28 days, but were less prominent in the 11 days study. Degeneration of elongated spermatids was not observed in the 11 days study, but exfoliation of round spermatids and multinucleated giant cell formation were apparent in two rats. The finding along with the

Photo 8. Ductus epididymidis of a rat treated with 200 mg/kg/day of carmofur for four weeks. Note the marked cell debris in ducts. HE stain. ×100.
Effects of short-term administration of carmofur on spermatogenesis.

Vacuolar degeneration of Sertoli cells in 4 animals suggest that carmofur toxicity forward male reproductive organs can be detected with 2 weeks treatment in the same way with 4 weeks, using careful histopathological examination of the testes.

Testicular toxicity of 5-fluorouracil (5-FU), a pyrimidine derivative antimitabolite, has been reported (Miyazaki et al., 1974). 5-FU does not cause spermatogonial damage but arrests spermatid development and result in abnormally shaped spermatids (Russell, 1991), and vacuolar degeneration of Sertoli cells (Meistrich et al., 1982). Similar changes were observed in this study using carmofur with histological change in the testes mainly observed in Sertoli cells and not in spermatogonia. This suggests that the target is mainly the Sertoli cell.

Based on objective evidence, it can be concluded that toxic effects of carmofur on spermatogenesis can be detected by treatment for less than 2 weeks using histopathological examination of the testes.

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REFERENCES


