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

26) DETECTION OF 1,3-DINITROBENZENE-INDUCED HISTOPATHOLOGICAL CHANGES IN TESTES AND EPIDIDYMIDES OF RATS WITH 2-WEEK DAILY REPEATED DOSING

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ABSTRACT — As part of a collaborative work, male rats were administered 1,3-dinitrobenzene (1,3-DNB) daily at 0, 25 and 50 mg/kg/day from the age of 6 weeks for 4 weeks (4-week exp.), or at 25, 50 and 75 mg/kg/day from the age of 8 weeks for 2 weeks (2-week exp.).

After the end of each administration period, all survivors were sacrificed, and their testes and epididymides were removed, weighed and examined histopathologically. The following results were obtained.

In the 4-week exp.: At 50 mg/kg/day, the weights of testes and epididymides showed decrease with macroscopic atrophy. The testicular spermatogenetic epithelium showed decrease in the number of sperm~spermatocytes, degeneration/necrosis, giant cell formation and vacuolation, reduction in sperm counts also being evident in the ducts of the epididymides.

In the 2-week exp.: At 50 and 75 mg/kg/day, the weights of testes and/or epididymides showed decrease with macroscopic atrophy. Several histopathological changes in the testes and epididymides were essentially the same changes as in the group given 50 mg/kg/day in the 4-week exp., with a clear relation.

These results indicate that a 2-week administration period is sufficient to detect testicular and epididymal histopathological changes induced by 1,3-dinitrobenzene in male rats.

KEY WORDS: 1,3-Dinitrobenzene, Rat, Spermatogenesis, Testis, Toxicity

INTRODUCTION

1,3-dinitrobenzene (1,3-DNB) has been primarily employed as an oxidizing agent and a chemical intermediate in the manufacture of a variety of organic compounds. Major targets of 1,3-DNB toxicity include male reproductive organs, hematopoietic tissues, central nervous system and liver (Beauchamp et al., 1982).

Regarding testicular toxicity it has been demonstrated that 1,3-DNB causes germ cell damage (Brown et al., 1997; Holloway et al., 1990) and germ cell apoptosis (Matsui and Takahashi, 1999; Strandgaard and Miller, 1998) in the rat testes. The Sertoli cell is implicated as the prime target of the toxic action of 1,3-DNB with germ cell damage as a secondary event (Reader et al., 1991; Blackburn et al., 1988). Species differences in susceptibility to 1,3-DNB testicular toxicity (Obasaju et al., 1991) and variation among three isomers of dinitrobenzene (Blackburn et al., 1988) also have been reported.

The present study was conducted as part of a collaborative work of JPMA and NIHS to obtain information on the validity and limitations of 2-week repeated dose studies to detect effects on male reproductive organs in rats.

For comparison two experiments, one of 4-week and the other of 2-week duration, were performed.
MATERIALS AND METHODS

Test article and treatment

1,3-dinitrobenzene (1,3-DNB) and sesame oil were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, USA), respectively. Before each dosing, 1,3-DNB was suspended in sesame oil to prepare 0.25 and 0.5 mg/mL (for the 4-week exp.), and 0.25, 0.5 and 0.75 mg/mL (for the 2-week exp.) suspensions. 1,3-DNB was administered by oral gavage to male rats once daily at a dosing volume rate of 0.1 mL/100 g body weight for 4 weeks or 2 weeks. Actual dosing volumes were adjusted weekly, according to body weights recorded on the most recent day. Control animals received the same dose volume of sesame oil, the vehicle.

Dose levels in present study were selected based on the results of a previous study, “Establishment of sperm examination method in reproductive and developmental studies(II)” (in-house data). 1,3-DNB was thereby administered to 10-week-old male rats at doses of 50 mg/kg/day for up to 4 weeks and 100 mg/kg/day for up to 11 days. As a result, decreased weights and spermatogenic epithelial damage of testes were noted in each dose group with occurrence of death and poorly general condition of animals given 100 mg/kg/day. Taking into account these preliminary results and the use of younger animals in the present study, dose levels of 0, 25 and 50 mg/kg/day for the 4-week exp. and 25, 50 and 75 mg/kg/day for the 2-week exp. were selected.

Experimental animals and husbandry

Forty 5-week-old male Crl: CD (SD) (SPF) rats were purchased from Charles River Japan, Inc. After 5-day quarantine, those that showed favorable weight gains and were in good general condition were used for the study. Four or five male rats were distributed into each group in both studies (4-week and 2-week exp.) in such a way as to make the mean body weights of the groups almost the same. The animals were acclimated prior to initiation of dosing, until they were 6 and 8 weeks old, respectively, for the 4-week and 2-week exp. (Fig. 1). The rats were housed in stainless steel bracket cages (R-IS, Japan CLEA, Inc., one or two to a cage) during the period after grouping and were allowed free access to an autoclaved pellet diet (CE-2; Japan CLEA, Inc.) and tap water sterilized with ultraviolet sterilizer. The animal room was maintained at a temperature of 23±3°C and 45±15% room humidity with 12-hr light/12-hr dark cycle (6:00-18:00) and with not less than 10 changes of air per hr.

Experimental design

The experimental design is illustrated in Fig. 1. The present study comprised two experiments. In the 4-week exp., 1,3-DNB was administered by oral gavage to male rats at 0, 25 and 50 mg/kg/day for 4 weeks. In the 2-week exp., 1,3-DNB was administered by oral gavage to male rats at 25, 50 and 75 mg/kg/day for 2 weeks.

1. Clinical signs, body weights

Rats were observed twice daily for mortality and general conditions, and weighed twice weekly.

2. Macroscopic examination and organ weights

All surviving rats from each group were sacrificed under ether anesthesia and examined macroscopically after the 4-week and 2-week treatments. At necropsy, the right and left testes and epididymides were excised and weighed separately and values relative to body

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weights were calculated.

3. Histopathological examination
Tests and epididymides were fixed in FSA (Formalin-Sucrose-Acetic acid) solution by immersion, dehydrated, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined under a light microscope.

Statistical analysis
Bartlett’s test of variance (Snedecor and Cochran, 1967) was first used to analyze data for body weight and organ weights. Then, one-way layout ANOVA (if the results were homoscedastic) or the Kruskal-Wallis’ test (Hollander and Wolfe, 1973) (if the results were heteroscedastic) were conducted; and if the variation was significant, Dunn’s (Hollander and Wolfe, 1973) (with groups of varying size) or Dunnett’s multiple comparisons (Dunnett, 1955,1964) (with groups of equal size) were performed.

RESULTS

General symptoms and body weights
No animals died during the treatment periods in either the 4-week or 2-week exp. Table 1 shows the mean body weights of each group at the time of necropsy, these for each 1,3-DNB group remaining similar to the control group values.

Organ weights
Table 1 shows organ weights for testes and epididymides.

1. The 4-week experiment
In the group on 25 mg/kg/day, no marked weight changes were found. In the group on 50 mg/kg/day, absolute and relative weights of testes were decreased, but statistical significance were observed only for right absolute epididymides weights.

Macroscopic examination
Necropsy findings for the testes and epididymides after treatment with 1,3-DNB are summarized in Table 2.

1. The 4-week experiment
In the group on 25 mg/kg/day, no marked changes were found. In the group on 50 mg/kg/day, small testes with softening and small epididymides were observed.

2. The 2-week experiment
In the group on 25 mg/kg/day, no marked changes were found. In the groups on 50 and 75 mg/kg/day, small testes with softening and small epididymides were observed dose-dependently.

Histopathological examination
Histopathological findings after treatment with 1,3-DNB are summarized in Table 3. Representative findings are illustrated in Photos 1 to 4.

1. The 4-week experiment
In the group on 25 mg/kg/day, no marked changes were found. In the group on 50 mg/kg/day, several histopathological changes were observed in all animals; decrease in the number of sperm—spermatocytes, multinuclear giant cell formation, vacuolation(probably Sertoli cells) and degeneration/necrosis (spermatids—spermatocytes) in the spermatogenic epithelium of the testes (Photos 1 to 2). Desquamated cells and decrease in the number of sperm in the ducts of the epididymides were also apparent.

2. The 2-week experiment
In the group on 25 mg/kg/day, no marked changes were found. Compared with the 50 mg/kg/day group of the 4-week exp., the same changes were found in the group on 50 mg/kg/day, but the degrees were less. Changes in the group on 75 mg/kg/day were almost the same as those observed in the 50 mg/kg/day group of the 4-week exp.(Photos 3 to 4). Dose-dependence of histopathological findings was noted in the 2-week exp.

DISCUSSION
To obtain information on the validity and limitations of 2-week repeated dose studies to detect effects on male reproductive organs in rats, we conducted the present 4-week and 2-week daily dosing with 1,3-dinitrobenzene. This chemical is known to cause testicular damage evidenced by spermatogenic epithelium atro-
Table 1. Body weight and organ weights for male rats treated orally with 1,3-dinitrobenzene.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>4-week experiment</th>
<th>2-week experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose(mg/kg/day)</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>No. of animals examined</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>346.2 ± 20.1</td>
<td>353.8 ± 12.5</td>
</tr>
</tbody>
</table>

| Absolute organ weights (g) | | | | | | |
| Testis-R | 1.492 ± 0.058 | 1.540 ± 0.102 | 0.775 ± 0.126 | 1.584 ± 0.074 | 1.326 ± 0.260 | 0.712 ± 0.057 |
| Testis-L | 1.520 ± 0.053 | 1.720 ± 0.427 | 0.775 ± 0.132 | 1.586 ± 0.054 | 1.326 ± 0.235 | 0.702 ± 0.053* |
| Epididymis-R | 0.376 ± 0.017 | 0.420 ± 0.051 | 0.325 ± 0.019 | 0.398 ± 0.020 | 0.368 ± 0.033 | 0.312 ± 0.008* |
| Epididymis-L | 0.360 ± 0.028 | 0.405 ± 0.056 | 0.320 ± 0.008 | 0.394 ± 0.024 | 0.378 ± 0.020 | 0.306 ± 0.011 |

| Relative organ weights (g/100 g BW) | | | | | | |
| Testis-R | 0.434 ± 0.036 | 0.438 ± 0.039 | 0.228 ± 0.046** | 0.466 ± 0.029 | 0.380 ± 0.070 | 0.210 ± 0.016** |
| Testis-L | 0.440 ± 0.031 | 0.485 ± 0.116 | 0.228 ± 0.046 | 0.468 ± 0.025 | 0.380 ± 0.064 | 0.204 ± 0.019* |
| Epididymis-R | 0.108 ± 0.008 | 0.120 ± 0.014 | 0.093 ± 0.005 | 0.116 ± 0.015 | 0.104 ± 0.011 | 0.092 ± 0.004 |
| Epididymis-L | 0.104 ± 0.009 | 0.115 ± 0.017 | 0.093 ± 0.005 | 0.116 ± 0.011 | 0.110 ± 0.010 | 0.090 ± 0.007 |

Values represent group mean ± S.D.
Significantly different from the control value (**: p<0.01, *: p<0.05).
Control group: 1,3-dinitrobenzene 0 mg/kg/day.
Statistical method: a) ANOVA, b) Kruskal-Wallis.
R: right, L: left.
Testicular toxicity of 1,3-dinitrobenzene in rats.

Table 2. Necropsy findings in male reproductive organs of rats treated orally with 1,3-dinitrobenzene for 4 or 2 weeks.

<table>
<thead>
<tr>
<th>Site/Finding</th>
<th>Test article Experiment (Dose mg/kg/day)</th>
<th>1,3-dinitrobenzene 4-week</th>
<th>No. of animals*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Testes Size decrease</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Softening</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Epididymides</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*: All animals were sacrificed under ether anesthesia on the day after the last treatment.

Table 3. Histopathological findings in the testes and epididymides for male rats treated orally with 1,3-dinitrobenzene for 4 or 2 weeks.

<table>
<thead>
<tr>
<th>Site/Finding</th>
<th>Test article Experiment (Dose mg/kg/day)</th>
<th>1,3-dinitrobenzene 4-week</th>
<th>No. of animals*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Testes Spermatogenic epithelium</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decrease in the number of sperm ~ spermatocytes</td>
<td>Moderate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multinuclear giant cell formation</td>
<td>Slight</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vacuolation, probably Sertoli cells</td>
<td>Slight</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Degeneration/necrosis (spermatids ~ spermatocytes)</td>
<td>Slight</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Epididymides Duct</td>
<td>Slight</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Desquamated cells</td>
<td>Moderate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*: All animals were sacrificed under ether anesthesia on the day after the last treatment.
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Photo 1. Testis from a control rat showing the normal appearance. HE stain. ×31.2.

Photo 2. Testis from a rat treated with 50 mg/kg/day of 1,3-butanedione for 4 weeks, showing moderate decrease in the number of spermocytes, moderate formation of multinucleate spermatid giant cell, and slight vacuolation in spermatogenic epithelium. HE stain. ×31.2.
Photo 3. Testis from a rat treated with 50 mg/kg/day of 1,3-dinitrobenzene for 2 weeks, showing slight decrease in the number of sperm - spermatocytes and slight vacuolation in spermatogenic epithelium. HE stain. × 31.2.

Photo 4. Testis from a rat treated with 75 mg/kg/day of 1,3-dinitrobenzene for 2 weeks, showing severe decrease in the number of sperm - spermatocytes, slight formation of multinuclear giant cell and slight vacuolation in spermatogenic epithelium. HE stain. × 31.2.
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phy, degeneration and giant cell formation (Hess et al., 1988).

We obtained the following results from the present experiments. In the group on 50 mg/kg/day in the 4-week exp., the weights of testes and epididymides showed decrease with macroscopically evident atrophy. Histopathological changes were also found these, the testicular spermatogenic epithelium showed decrease in the number of sperm ~ spermatocytes, degeneration/necrosis, giant cell formation and vaculation, and the ducts of the epididymides exhibited decreased numbers of sperm.

In the groups on 50 and 75 mg/kg/day in the 2-week exp., the weights of testes and/or epididymides showed decrease with macroscopic atrophy. Histopathologically evident testicular and epididymal changes were almost as pronounced or more severe than in the group on 50 mg/kg/day in the 4-week exp., with dose-dependence.

The data described above indicate that we can detect obvious testicular and epididymal histological lesions with 2-week repeated daily dosing of 1,3-DNB.

Therefore, a 2-week administration period is sufficient for assessment of testicular and epididymal histopathological changes induced by agents like 1,3-dinitrobenzene in male rats.

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REFERENCES


