COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS
7) EFFECTS OF RESERPINE IN 2- AND 4-WEEKS STUDIES

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ABSTRACT — To assess the efficacy of different period of treatment for evaluating male reproductive toxicity in rats, reserpine was subcutaneously administered on a daily basis to male Sprague-Dawley rats at dosages of 0.05, 0.1 or 0.2 mg/kg for 2 weeks or at dosages of 0.05 or 0.1 mg/kg for 4 weeks. At the end of the administration period the animals were sacrificed and sperm counts, organ weights and histopathological changes in the reproductive organs were examined. The sperm number in the cauda epididymis and genital organs were not affected by reserpine with either 2- or 4-weeks treatment. In the 4-weeks study, histopathological examination of the testes revealed retention of step 19 spermatids in the seminiferous tubules of stages IX to XII and decreased secretory content of the prostate in the 0.05 and 0.1 mg/kg groups. In the 2-weeks study, although no distinct histopathological changes were observed in the 0.05 mg/kg group, decreased secretory content of the prostate, apoptosis of spermatocytes in the seminiferous tubules of stage VII and cell debris of the epididymis were observed in the 0.1 and 0.2 mg/kg groups. These results suggested that 2-weeks treatment with reserpine is sufficient for detection of testicular toxicity, although higher dosage levels are appropriate than for 4-weeks treatment.

KEY WORDS: Reserpine, Reproductive toxicity, Testis, Sperm, Rat

INTRODUCTION

For the purpose of human safety assessment of new chemicals or compounds, present evaluation of male reproductive organ toxicity in Japan require at least 4-weeks exposure with extensive and detailed histopathological examinations. In contrast, in the United States and the European Union, 2-weeks exposure studies are considered acceptable in the current tripartite-harmonized Guidelines of the International Conference of Harmonization (ICH). To harmonize the above regional differences, an attempt is now being made in Japan to obtain a scientific rationale for adopting the 2-weeks exposure period as a collaborative work of the Ministry of Health and Welfare and the Japan Pharmaceutical Manufacturers Association. The present study was conducted as a part of collaborative work to assess whether the current dosing period of 4 weeks, before the first human trial, can be shortened to 2 weeks, and to determine appropriate parameters for detecting male reproductive toxicity.

Reserpine, a purified alkaloid of Rauwolfia, acts as an antidepressant and is an established medicine for psychosis. The drug is known to have the potential for exerting testicular toxicity in humans and animals (Kishimoto et al., 1995; Bayne et al., 1980; Wollam et al., 1977). One of its action sites is the hypothalamo-hypophyseal system where it inhibits the release of luteinizing hormone-releasing factor (LHRF) and luteinizing hormone (LH) by depleting brain stores of monoamines (Maanen and Smelik, 1968). Exposure to reserpine for 4 or 9 weeks in male rats causes testicular damage characterized by retention of spermatids in the seminiferous tubules (Kishimoto et al., 1995). There have been no reports, however, concerning whether reserpine-induced testicular injury can be detected with
an exposure period shorter than 4 weeks.

The purpose of the present study was to evaluate the
efficacy of 2-weeks exposure in an assay system for
safety assessment of effect in the male genital organs.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, 5 weeks of age, were
purchased from Charles River Japan, Inc. (Hino,
Japan). After 1- to 3-weeks acclimatization, 24 rats
weighting 199-217 g at 6 weeks of age were used for
the 4-week study, and 18 rats weighing 294-321 g at 8
weeks of age for the 2-week study. They were housed 2
animals per cage in metal cages in a barrier room main-
tained with a specific pathogen free environment, a
temperature of 22.4 to 23.3°C, a relative humidity of 51
to 64%, and a 12-hr light-dark cycle. A standard com-
mercial diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo,
Japan) and tap water were available ad libitum. The
animals were classed according to their body weights
and allocated randomly to groups, each of which con-
sisted of 6 rats, using a computerized randomization
program.

Test compound

Reserpine (Apoplon®, Daiichi Pharmaceutical
Co., Ltd., Tokyo, Japan) was diluted to concentra-
tions of 0.005, 0.01 and 0.02% using a vehicle prepared by
dissolving 3 mg of DL-methionine (Wako Pure
Chemical Industries, Ltd., Osaka, Japan) and 70 mg of
propylene glycol (Wako Pure Chemical Industries) in 1
ml of distilled water. Based on the previous study, in
which obvious testicular toxicity was observed at 0.05
mg/kg/day (Kishimoto, 1995), dose levels were set at
0.05 and 0.1 mg/kg/day in the 4-weeks dosing study
and at 0.05, 0.1 and 0.2 mg/kg/day in the 2-weeks dos-
ing study. A group of rats receiving only vehicle served
as the control in each case.

Experimental design

Reserpine was subcutaneously administered to rats
each treatment group once daily for 2 or 4 weeks.
All animals were observed for general signs once a day
during the dosing period. Body weights were recorded
once a week during the dosing period and group mean
body weight values and their standard deviations were
calculated. Food consumption was recorded for each
cage once a week during the dosing period, and the
mean food consumption was calculated. All animals
were killed by exsanguination under deep ether anes-
thesia at the end of the dosing period, and necropsied
immediately. The testes, epididymides, prostate, semi-
nal vesicle and pituitary were weighed. Relative organ
weights were also expressed as percentages of the body
weights on the day of necropsy. For histopathological
evaluation, the right epididymis, prostate, seminal ves-
icle, pituitary, thyroids, adrenals and mammary glands
were fixed in 10% neutral buffered formalin. The testes
were fixed in formalin-sucrose-acetic acid (FSA) solu-
tion. These organs were embedded in paraffin, sec-
tioned at 4 μm and stained with hematoxylin and eosin
(HE). From each testis sections at 3 levels were also
sampled and stained with HE and periodic acid-Schiff
(PAS). The left caudal epididymis was weighed and
then finely minced, and spermatozoa allowed to migrate
into 10 mL of Hanks’ balanced salt solution containing
1% bovine serum albumin warmed to 37°C (sperm solution).
The sperm solution was diluted 40-fold with saline containing 0.5% formalin, and the
number of sperm was counted with a hemacytometer,
and then the number per unit weight of epididymis was
calculated.

Statistical analyses

Body weights, food intake, sperm counts, and
organ weights were analyzed using the following statis-
tical methods; parametric data were analyzed by one-
way ANOVA, and compared with the control group
values by Dunnett’s (Dunnett, 1964) or Scheffe’s test
(Scheffe, 1953). The nonparametric data were analyzed by
Dunnett’s or Scheffe’s method after the Kruskal-
Wallis’s test (Kruskal and Wallis, 1952).

RESULTS

Clinical signs, body weight and food intake

In both 2- and 4-weeks dosing studies, ptosis and
decreased locomotor activity were observed in all rats
of all treatment groups. One animal in the 0.2 mg/kg
group in the 2-week case and one animal in the 0.1
mg/kg group receiving 4-weeks dosing died in the
course of treatment. Body weight gain was signifi-
cantly decreased in the 0.1 and 0.2 mg/kg groups in the
2-week study and in the 0.05 and 0.1 mg/kg groups in the
4-week study, respectively. The degree of decrease in
the 2-week study was higher than that in the 4-week
study. In the 4-week study, body weight gain showed a
tendency for recovery at the end of duration period.
Similarly, food intake was significantly decreased in
the 0.1 and 0.2 mg/kg groups in the 2-weeks dosing
study and in the 0.05 and 0.1 mg/kg groups in the 4-
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week study, respectively. No significant difference was noted in body weight gain or food intake between the 0.05 mg/kg group and the control group in the 2-week study (Fig.1).

Sperm counts

The number of sperm in the caudal epididymis was significantly increased in the 0.05 and 0.1 mg/kg groups in the 4-weeks dosing study. In the 2-weeks dosing study, there was no significant change in the numbers of sperms in any treatment group (Table 1).

Organ weights

Absolute weights of the testes and prostate were decreased in the 0.2 mg/kg groups of the 2-weeks dosing study and those of testes, epididymides and pituitary were also decreased in the 0.1 mg/kg group with the 4-weeks dosing. On the other hand, relative organ weights showed tendencies to increase with both 2- and 4-week dosings. Relative weights of testes with 0.1

![Graphs showing body weight changes](image)

**Fig. 1.** Body weight changes of rats treated with reserpine for 2 or 4 weeks.

| Table 1. Sperm counts for rats treated with reserpine for 2 or 4 weeks. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Dose (mg/kg)  | Control | 0.05 | 0.1 | 0.2 |
| 2-weeks dosing study | (×10⁶) | 1.14 ± 0.21 | 1.08 ± 0.10 | 1.01 ± 0.38 | 1.11 ± 0.14 |
| Sperm count | (×10⁶/caudal epididymis) | 5.85 ± 0.77 | 5.29 ± 0.92 | 5.70 ± 1.24 | 6.67 ± 0.94 |
| 4-weeks dosing study | (×10⁶) | <5> | 1.12 ± 0.19 | 0.92 ± 0.10 |
| Sperm count | (×10⁶/caudal epididymis) | 4.46 ± 0.42 | 5.83 ± 0.58** | 5.44 ± 0.31** |

<5>: Number of animals examined.  
Values are mean ± S.D.  
Statistically significant difference from the control, **: p<0.01.
mg/kg or more in the 2-weeks dosing study and with 0.1 mg/kg group in the 4-weeks dosing study were increased. Relative weights of epididymides in the 0.2 mg/kg group after 2-weeks and with 0.1 mg/kg after 4-weeks were increased. Relative weights of seminal vesicles with 0.05 mg/kg or more and of the pituitary in the 0.2 mg/kg group were increased in the 2-weeks dosing study. However, there were no significant dose-related changes in these organs in the 4-weeks dosing study. (Table 2).

Histopathological findings

Data for histopathological findings are summarized in Table 3.

In the 2-week study, apoptosis of spermatocytes in stage VII seminiferous tubules was detected in 2 of 5 and 6 animals in 0.2 or 0.1 mg/kg dosing group, respectively (Photo 1A). While retention of step 19 spermatids in the seminiferous tubules of stages IX to XII was found in 1 or 2 animals of all groups, these findings were sometimes observed in the control animals and were not dependent on the dosage and therefore appeared to be without significance. In the 0.2 and 0.1 mg/kg groups, cellular debris in the epididymal ducts was found in 2 of 5 or 1 of 6 animals, respectively. Decrease in secretory content of the prostate was noted in 5 animals each of both groups. In the mammary gland, almost all the animals in both high and medium dose groups showed acinar atrophy and ductal proliferation. There was no evidence of effects of 0.05 mg/kg except for reduced prostate secretory in one animal.

In contrast to the testicular pathology in the 2-week study, there were no apoptotic figures in any seminiferous tubules in any animals of the 4-week study. Whereas no significant retention of step 19 spermatids was detected in the 2-week study, 3 of 5 animals in the 0.05 mg/kg group and 4 of 5 animals in the 0.1 mg/kg group showed this change in the 4-week study (Photo 1B). As with in the 0.2 and 0.1 mg/kg groups in the 2-week study, reduction in prostate secretion and mammary changes were detected in almost all animals of the 0.1 and 0.05 mg/kg groups after 4-weeks treatment.

No changes were notable in the pituitary, seminal vesicle, and epididymides.

| Table 2. Organ weights in rats treated with reserpine for 2 or 4 weeks. |
|-----------------------------|-------------------|---------------|----------------|-------------------|
| Dose (mg/kg)                | Control           | 0.05          | 0.1           | 0.2               |
|                             | <6>               | <6>           | <6>           | <5>               |
| 2-weeks dosing study        |                   |               |               |                   |
| Testes                      | (mg)              | 3178 ± 246    | 3117 ± 129    | 2879 ± 252        | 2819 ± 163*       |
|                             | (%)               | 0.92 ± 0.06   | 0.89 ± 0.05   | 1.08 ± 0.07*      | 1.35 ± 0.19**     |
| Prostate                    | (mg)              | 918 ± 140     | 1080 ± 268    | 770 ± 173         | 603 ± 106*        |
|                             | (%)               | 0.27 ± 0.05   | 0.31 ± 0.07   | 0.29 ± 0.05       | 0.29 ± 0.08       |
| Seminal vesicle             | (mg)              | 983 ± 95      | 1740 ± 217**  | 1228 ± 345        | 1188 ± 211        |
|                             | (%)               | 0.29 ± 0.03   | 0.50 ± 0.06** | 0.46 ± 0.11*      | 0.58 ± 0.16**     |
| Epididymides                | (mg)              | 948 ± 94      | 977 ± 114     | 846 ± 150         | 772 ± 95          |
|                             | (%)               | 0.28 ± 0.03   | 0.28 ± 0.03   | 0.31 ± 0.04       | 0.37 ± 0.01**     |
| Pituitary                   | (mg)              | 10 ± 2        | 10 ± 1        | 9 ± 2             | 8 ± 1             |
|                             | (10^-3%)          | 2.88 ± 0.49   | 2.85 ± 0.37   | 3.22 ± 0.53       | 3.96 ± 0.29**     |
| 4-weeks dosing study        |                   |               |               |                   |
| Testes                      | (mg)              | 3140 ± 249    | 3142 ± 128    | 2845 ± 179*       |                   |
|                             | (%)               | 0.86 ± 0.08   | 0.99 ± 0.06   | 1.21 ± 0.16**     |                   |
| Prostate                    | (mg)              | 833 ± 112     | 992 ± 179     | 629 ± 146         |                   |
|                             | (%)               | 0.23 ± 0.04   | 0.31 ± 0.06*  | 0.26 ± 0.04       |                   |
| Seminal vesicle             | (mg)              | 1051 ± 229    | 1446 ± 303*   | 812 ± 232         |                   |
|                             | (%)               | 0.29 ± 0.07   | 0.46 ± 0.11** | 0.34 ± 0.07       |                   |
| Epididymides                | (mg)              | 967 ± 41      | 966 ± 75      | 853 ± 69*         |                   |
|                             | (%)               | 0.26 ± 0.02   | 0.31 ± 0.04   | 0.36 ± 0.05**     |                   |
| Pituitary                   | (mg)              | 11 ± 1        | 10 ± 2        | 8 ± 2**           |                   |
|                             | (10^-3%)          | 3.10 ± 0.30   | 3.12 ± 0.59   | 3.44 ± 0.47       |                   |

< >: Number of animals examined.
Values are mean ± S.D.
Statistically significant difference from the control, *: p<0.05, **: p<0.01.
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DISCUSSION

Injury to the rat male genital system caused by reserpine has been found after 4 weeks or in longer repeated dose toxicity studies (Kishimoto et al., 1995). However, information as to whether genital toxicity of reserpine is detectable in studies shorter than 4 weeks has hitherto been lacking. Our results provide a clear answer to this question. Male genital toxicity of reserpine was detected by detailed histopathological examination with both 2- and 4-weeks treatment periods, although there were no distinct changes in organ weights or sperm counts. In the 4-weeks dosing study, retention of step 19 spermatids in the seminiferous tubules of stages IX to XII and reduction of secretion in the prostate were seen in the 0.05 and 0.1 mg/kg groups. On the other hand, in the 2-weeks dosing study, apoptosis of spermatocytes in the seminiferous epithelium of stage VII was detected in the 0.1 and 0.2 mg/kg groups in addition to cell debris in the epididymis and decreased secretory contents of the prostate. These findings demonstrate that histopathology is the most sensitive among the three methods used in the present study to detect testicular toxicity, supporting the conclusion of the previous collaborative project conducted to clarify the optimal dose period and parameters for detection of male fertility disorders in rats. (Takahashi and Matsui, 1993; Kishimoto et al., 1995; Takayama et al., 1995).

Retention of step 19 spermatids is induced by several testicular toxicants including reserpine, and is considered to be a relatively common change after exposure (Parvinen, 1979; Chapin et al., 1984; Creasy et al.,

<p>| Table 3. Histopathological findings in rats treated with reserpine for 2 or 4 weeks. |
|-------------------------------------------------|-------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Grade</th>
<th>Control</th>
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<tr>
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<td>of spermatocytes</td>
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<td>1</td>
<td>1</td>
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<tr>
<td></td>
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<td>1</td>
<td>0</td>
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<tr>
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<tr>
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<td>Prostatitis</td>
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<td>0</td>
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<tr>
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<td>1</td>
</tr>
<tr>
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<td>Minimal</td>
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<td></td>
<td>Slight</td>
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<td></td>
<td>Ductal proliferation</td>
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<td>4</td>
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<tr>
<td></td>
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<td>4-weeks dosing study</td>
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< >: Number of animals examined.
No remarkable change were evident in the pituitary, adrenals or thyroid glands of any animals.
1990; Kishimoto et al., 1995). Reserpine is known to produce atrophy of the testicular interstitial cells in rats and humans (Dias, 1982; Bayne et al., 1980). It has been suggested to induce functional abnormalities of Sertoli cells through depressed production of testosterone in the interstitial cells and cause retention of step 19 spermatids (Russell et al., 1990). In the present study, no other testicular lesions were seen. However, in a previous investigation of rats given reserpine for 4 weeks, necrosis of germ cells was observed at doses of 0.05 mg/kg or more, in addition to retention of spermatids (Kishimoto et al., 1995). Although the cause of this difference is not clear, it might originate from differences in the ages of the animals employed, those in the study of Kishimoto et al. (1995) being 12 weeks old, in contrast to the 6 weeks in the present case. By this age difference, the effect of reserpine to the sex hormone secretion might be different between in the study of Kishimoto et al. (1995) and in the present study, and cause the discrepancy of histopathological findings mentioned above. As another testicular lesion observed in the present study, apoptosis of spermatocytes in the seminiferous tubules of stage VII was detected only in the 2-week study. The survival of male germ cells is probably dependent on gonadotropins as well as intratesticular androgens induced by LH (Russel et al., 1987). Hypophysectomy increases degeneration of germ cells and induces morphological changes in the testis (Russel and Clermont, 1977). Furthermore, gonadotropins prevent germ cell degener-

ation at specific stages during spermatogenesis (Gosh et al., 1992; Russel et al., 1992). Reserpine is also known to inhibit the release of LHRF and LH by depleting brain stores of monoamines (Maanen and Smelik, 1968; Brown and Fowke, 1972). The available data thus suggest that apoptosis of spermatocytes might be due to a disorder of sex hormone production after reserpine treatment.

In the present study, increased number of rats with retention of step 19 spermatids was observed in the 4-week study, but not after 2-weeks, in clear contrast to the case for apoptosis of germ cells. The reason for this apparent time-dependence is not clear, but attention to body weight changes in the same dosage (0.1 mg/kg) in both studies, suggests that the testicular lesions might be related to systemic toxic effects of reserpine which were strong after 2-weeks treatment, followed by recovery thereafter. Therefore, apoptosis of germ cells might be induced during the period of severe toxicity, and in the following recovery period retention of spermatocytes might appear as a trace of damage to the seminiferous epithelium.

As other toxic effects outside of the testis, decreased fluid secretion by the prostate, an increased number of sperm, and acinar atrophy and ductal development in the mammary gland were observed. Reserpine treatment in rats decreases fluid secretion in the prostate similar to that seen in other exocrine glands (Wen and Wong, 1988) and is known to cause increase in the number of sperm and the viscosity of epididymal

![Photo 1](image)

Photo 1. Histopathological findings for the testes in rats treated with reserpine for 2 (A) or 4 weeks (B). A) Testis from a rat receiving 0.2 mg/kg of reserpine for 2 weeks. Apoptosis of spermatocytes (arrowheads) is evident in a stage VII seminiferous tubule. B) Testis from a rat receiving 0.1 mg/kg of reserpine for 4 weeks. Retention of step 19 spermatids (arrows) is evident in a stage X seminiferous tubule.
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fluid (Wen and Wong, 1988). Accordingly, the number of sperm per unit weight of epididymis was apparently increased because of a decrease in exocrine fluid secretion. The findings in the mammary gland may be considered due to effects of reserpine on the endocrine system such as prolactin release (Lipsett, 1983).

In the present study, testicular toxicity of reserpine was observed after both 2- and 4-weeks repeated dosing. We conclude that a 2-week treatment period of reserpine is appropriate to detect male reproductive toxicity, if we perform a detailed morphological examination and select an appropriate dose.

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