COLLABORATIVE WORK TO EVALUATE TOXICITY ON MALE REPRODUCTIVE ORGANS BY REPEATED DOSE STUDIES IN RATS 20) TESTICULAR TOXICITY OF NITROFURAZONE AFTER 2 AND 4 WEEKS

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ABSTRACT — Nitrofurazone (NF) has been previously demonstrated to induce testicular toxicity with 4 weeks of oral administration in rats. In the present study, rats were administered NF to assess whether testicular toxicity becomes evident with a 2-week administration period. Male Sprague-Dawley rats were administered oral doses of NF at 50 mg/kg for 2 or 4 weeks. Another group was administered NF at 100 mg/kg for 2 weeks. The control animals received the vehicle (0.5% methylcellulose) for 4 weeks. Organ weights of the testis and epididymis were significantly decreased in all NF-administered animals, and seminiferous tubules were severely atrophied, due to a total absence of spermatids and degeneration and desquamation of spermatocytes. In the epididymis, decreased numbers of spermatozoa were evident in the ducts. In rats that were administered NF at 50 mg/kg, the changes in the epididymis in the 2-week group were less prominent than those in the 4-week group. In the testis, however, the changes were similar in both groups. Thus it was demonstrated that NF-induced testicular toxicity comparable to that observed after 4 weeks of administration is also detectable after 2 weeks.

KEY WORDS: Nitrofurazone, Rat, Testicular toxicity

INTRODUCTION

The National Institute of Health Sciences and the Japan Pharmaceutical Manufacturers Association have organized a joint study to assess whether a 2-week treatment is sufficient to detect male reproductice organ toxicity of drugs. Drug-induced testicular toxicity has previously been shown to be detectable after 4 weeks. The present study was conducted as part of this collaborative study.

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone; NF) is a broad-spectrum antibiotic (Chamberlain, 1976) which also has antiprotozoal and antiparasitic activities (Reynolds, 1982), and is widely used in both human and veterinary medicine (Reynolds, 1982).

Lethal doses of NF produce relatively mild hepatic and renal changes (Dodd, 1946), while much lower doses are sufficient to cause well-recognized testicular lesions in rodents. Seminiferous tubule degeneration was seen in the testis of Sprague-Dawley rats following daily doses ranging between 25 and 64 mg/kg for 4 weeks, with a concomitant decrease in organ weights (Hageniäs et al., 1978; Nishimura et al., 1995), and in Fischer rats after 2 weeks of feeding diets containing 630, 1250, 2500, 5000, or 10,000 ppm NF (Kari et al., 1989). However, no study has so far been conducted to compare the testicular changes after 2 and 4 weeks of treatment under the same experimental conditions.

In the present study, in accordance with the joint study objectives, it was assessed whether toxic changes comparable to those observed after administration of NF for 4 weeks are also detectable after 2 weeks.

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MATERIALS AND METHODS

This study was carried out with the approval of the Committee of Laboratory Animal Experimentation, Safety Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd.

Materials

NF was purchased from Wako Chemicals, Co. (Osaka, Japan), Lot No. WTR1862, purity 99.6%, and stored protected from light at room temperature until use. Dosing solutions were prepared by suspending NF at 10 mg/mL (for 50 mg/kg groups) or 20 mg/mL (for the 100 mg/kg group) in a 0.5% methylcellulose solution, and stored at room temperature protected from light.

Animals

SPF Sprague-Dawley male rats were purchased from Charles River, Japan Inc. (Kanagawa, Japan) at the age of 5 weeks. The animals were acclimated until aged 6 (for 4-week groups) or 8 weeks (for 2-week groups) old, when administration was started. Ten rats were allocated to each group. The animals were housed 2 or 3 to a hanging aluminium cage in a room maintained at 23°C ±2°C, with a relative humidity of 55% ±10%, 15 ventilations/hr, and lighted for 13 hr from 8:00 a.m. to 9:00 p.m. The animals were provided with a commercially available diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water, both ad libitum.

Dosing procedures

It has been previously demonstrated that the testicular toxicity of NF is observable after 4 weeks at 50 mg/kg (Nishimura et al., 1995). In the present study, therefore, the dose was set at 50 mg/kg for 2- and 4-week groups. Another group was administered NF at 100 mg/kg for 2 weeks since the dose was considered tolerable for 2 but not 4 weeks. The animals were given the designated doses once a day for 2 or 4 weeks by gavage using a Teflon stomach tube. Control animals received a 0.5% methylcellulose solution in the same manner for 4 weeks. The dose volume of each animal was 5 mL/kg, calculated using the most recently recorded body weight.

The animals were observed for general conditions and mortality at least once a day before the initiation of dosing and at least twice a day during the dosing period.

Body weights were recorded on the first day of treatment, weekly thereafter before dosing, and immediately prior to the terminal sacrifice. The body weights before sacrifice were used for relative organ weight calculations.

Necropsy and organ weights

Animals were sacrificed by exsanguination under ether anesthesia, and visceral organs were examined macroscopically. The testis and epididymis were collected. Organ weights were recorded before (testis) and after (epididymis) fixation in Bouin’s solution.

Histopathology

All the testes and epididymides were processed routinely for paraffin sections. The testicular sections were stained with H&E and PAS, and for the epididymis, with H&E.

The extents of seminiferous tubule atrophy and of decrease in spermatozoa in the epididymal duct were categorized into two grades according to the following criteria.

1. Seminiferous tubule atrophy

   Grade 1: Spermatids were totally absent but spermatocytes were abundant within the tubules (Photo 1B).
   Grade 2: In addition to the absence of spermatids, late stage (late pachytene, diplotene) spermatocytes were also absent, or greatly reduced in number. (Photo 1C).

2. Decreased spermatozoa in the epididymal duct

   Grade 1: Spermatozoa were decreased in the caput, but abundant in the cauda.
   Grade 2: Spermatozoa were decreased in both the caput and the cauda.

Statistical analysis

Body weight and organ weights were analyzed statistically by the Bartlett’s test of homogeneity of variance (Bartlett, 1937). Where variances were homogenous, the control versus treatment mean comparison was performed by the Dunnnett’s multiple comparison (Dunnett, 1964). If otherwise, the Dunnett type rank test was carried out.

RESULTS

General conditions and morbidity

Several animals dosed NF at 100 mg/kg for 2 weeks showed salivation and decreased spontaneous locomotive activity, and adopted a prone position. Three of them were found dead on days 8, 12 and 14. 
Testicular toxicity of nitrofurazone after 2 and 4 weeks.

**Photo 1.** Photomicrographs of the testis.
(A) Control testis, with spermatogenesis actively taking place.
(B) Grade 1 seminiferous tubule atrophy. There is a total absence of spermatids. Some multinucleated cells (arrows) and degenerated cells (arrowheads) are apparent.
(C) Grade 2 seminiferous tubule atrophy. In addition to the absence of spermatids, late stage (late pachytene, diplotene) spermatocytes are also absent, or greatly reduced in number.
respectively. Convulsive seizures, which have been induced in rats and mice given multiple doses of NF (Kari et al., 1989), were also observed in one of the animals which died. One animal dosed NF at 50 mg/kg for 4 weeks died accidentally at dosing.

Other animals dosed NF at 50 mg/kg for 2 or 4 weeks showed no clinical symptoms.

Body weight

Data for mean body weights are summarized in Fig. 1.

In rats given 100 mg/kg, body weight gain was negative (-3% at the end of the dosing period). At 50 mg/kg, body weight gains were lower than in controls after both 2 and 4 weeks, final body weights being 93.6% and 90.9% of the control value, respectively.

Gross pathology

All animals dosed 100 mg/kg had small and soft testes. Similarly, this was the case for all animals given 50 mg/kg, except one rat dosed for 2 weeks. Other abdominal and thoracic organs were macroscopically normal in all animals.

Organ weights

Organ weights are summarized in Fig. 2.

1. Testis

In all NF-treated groups the absolute weights of the testis were less than half the control value, and the relative weights were significantly lower.

2. Epididymis

Absolute and relative weights were significantly lower than the control values in all animals treated with NF.

Histopathology

Histopathological findings are summarized in Table 1.

1. Testis

All NF-treated animals had atrophy of the seminiferous tubules, characterized by the total absence of spermatids. Multinucleated giant cells, with nuclei resembling those of spermatocytes, and degenerated or desquamated cells were often seen (Photo 1B). All of the 100 mg/kg group animals showed grade 2 atrophy (Photo 1C). At 50 mg/kg, no clear histopathological difference was evident between the 2- and 4-week groups.

Fig. 1. Mean body weights.
The mean body weight of each group. Animals were dosed from 6 weeks (4-week groups) or 8 weeks (2-week groups) of age, and necropsied at the same time at the age of 10 weeks.

* and **: significantly different from the control value, *p<0.05, **p<0.01.

Fig. 2. Mean organ weights.
Mean absolute weights of the testis (a) and the epididymis (b). **: significantly different from the control value, p<0.01.
Testicular toxicity of nitrofurazone after 2 and 4 weeks.

2. Epididymis

All of the treated animals had reduced numbers of spermatozoa and increased numbers of degenerated or desquamated cells in the epididymal duct lumen. However, while all the animals administered NF for 2 weeks (50 and 100 mg/kg) showed grade 1 decrease in spermatozoa, all of the 4-week animals showed grade 2 decrease (50 mg/kg).

DISCUSSION

NF arrests spermatogenesis, and degeneration and atrophy of the seminiferous tubules have been described in rodents such as mice and rats (Nishimura et al., 1995; Kari et al., 1989; IARC Monographs, 1990). NF is mutagenic to a variety of bacteria, but not toward nitroreductase-deficient strains; however, mutagenicity is observed upon addition of exogenous enzymes (Baars et al., 1980; Olive and Durand, 1978; Rosenkrantz and Speck, 1976). Thus nitroreduction is thought to be necessary for NF's mutagenicity to bacteria. On the other hand, little is known of the mechanisms of NF-induced toxicity in mammals. However, its metabolism, including nitroreduction, has also been implicated in manifestation of its toxicity. NF metabolites have been shown to bind to cellular DNA, RNA and proteins (Tatsumi et al., 1977). There are data indicating that reductive activity in the testis is comparable to that of the liver (McCalla et al., 1971). Since some reductive processes including nitroreduction proceed at a faster rate under a low oxygen concentration, the somewhat low oxygen tension in the testis (Free et al., 1976; Klotz et al., 1996) may contribute to its greater susceptibility to NF compared with other organs.

In the present study, rats were administered NF for 2 or 4 weeks to assess whether the testicular changes are detectable after 2 weeks. There was basically no difference in findings between the 2- and 4-week groups. The changes include decreased weights of the testis and epididymis, and seminiferous tubule atrophy due to loss of spermatids and to degeneration and desquamation of spermatocytes. These findings are in agreement with those previously reported (Nishimura et al., 1995; Kari et al., 1989; IARC Monographs, 1990), and show that the NF-induced effects on the testis similar to those seen after 4 weeks by oral gavage are clearly observable after 2 weeks of treatment.

In the testis, the degree of seminiferous tubule atrophy in animals administered NF at 50 mg/kg seemed independent of the dosing period, 2 or 4 weeks, though it did depend on the dosage. Atrophy due to NF was characterized by the absence of spermatids, and degeneration and desquamation of spermatocytes. The similar results between the two groups imply that the changes leading to atrophy occurred early in the dosing period. Indeed, NF-induced testicular toxicity appears

<table>
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<tr>
<th>Table 1. Summary of histopahtology.</th>
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<tr>
<td>Dose (mg/kg/day)</td>
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<td>Dosing period (w)</td>
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<tr>
<td>Number of Animals Examined*</td>
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<tr>
<td>Seminiferous tubule/atrophy</td>
</tr>
<tr>
<td>Grade 1(a)</td>
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<tr>
<td>Grade 2(b)</td>
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<td>/ multinuclear giant cells and/or degenerated/ desquamated cells</td>
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<tr>
<td>Epididymis</td>
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<td>Duct/ lumina/ decrease in spermatozoa</td>
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<tr>
<td>Grade 1(c)</td>
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<tr>
<td>Grade 2(d)</td>
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<td>Duct/ lumina/ increase in degenerated cells</td>
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*: terminal sacrifice.

Keys:
(a) grade 1: Spermatids were totally absent but spermatocytes were abundant within the tubules.
(b) grade 2: In addition to the absence of spermatids, late stage (late pachytene, diplotene) spermatocytes were also absent, or greatly reduced in number.
(c) grade 1: Spermatozoa were decreased in the caput, but abundant in the cauda.
(d) grade 2: Spermatozoa were decreased in both the caput and the cauda.
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as early as 2 or 3 days after treatment (Hagenäs et al., 1978; Uematsu, 1966). In addition to an early onset, these initial changes must have progressed rapidly to the "terminal" stage seen at 50 mg/kg before the end of the 2-week period, leading to similar results in the 2- and 4-week groups.

The extent of seminiferous tubule atrophy was dose-dependent; in contrast to the loss of spermatids as seen in the 50 mg/kg groups, NF at 100 mg/kg additionally affected late stage spermatocytes. A higher dose would produce an increased amount of reactive metabolites, which are known to bind to cellular macromolecules. Drug metabolic activities in the testis have been poorly characterized. There is, however, some evidence of reductive metabolism in Sertoli cells (Foster et al., 1986; Foster et al., 1987; Brown and Miller, 1991). Reactive metabolites produced in Sertoli cells would bind to macromolecules within these cells, interfering with their functions; this would consequently affect germ cells, which are supported by the Sertoli cells. Alternatively, some metabolites may reach adjacent germ cells to cause direct damage. The germ cells, therefore, may be affected both directly and indirectly. However, it is conceivable that the indirect mechanism plays the more important role, since not all germ cell types reacted equally to NF, even though a similar amount of metabolites from the Sertoli cells would have reached them. The affected cells, including spermatocytes and spermatids, were comparatively differentiated, with a greater degree of dependency on Sertoli cells for vital substances such as nutrients. A higher dose will probably result in greater damage to Sertoli cells, affect their supportive functions to a greater extent, and, in turn, affecting more types of germ cells, leading to severer atrophy of the tubules.

In the epididymis, the degree of the decrease in spermatozoa was dependent on the dosing period. Spermatozoa released from the seminiferous tubules enter the epididymis from the caput (the head) and move gradually to the cauda (tail) where they are stored for some time. In the animals dosed NF for 2 weeks, the decrease in spermatozoa, reflecting spermatogenic arrest, was restricted to the caput, while it was seen in both the caput and cauda in the animals treated for 4 weeks. The results are thus in line with expectation.

The present study was conducted to assess whether testicular toxicity of NF is detectable with a 2-week dosing period. After both 2 and 4 weeks, seminiferous tubule atrophy was seen in the testis due to the absence of spermatids and to degeneration or desquamation of spermatocytes. These changes were reflected in the epididymis as a decrease in ductal spermatozoa. The results demonstrate that NF-induced testicular toxicity is indeed detectable within 2 weeks.

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