MALE REPRODUCTIVE TOXICITY STUDY OF NEFIRACETAM IN RATS

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ABSTRACT — Sprague-Dawley male rats were administered nefiracetam orally at daily doses of 500 and 1500 mg/kg/day for 4 or 9 weeks. Although the copulation index was not affected by nefiracetam treatment, the fertility index was extremely low in the 1500 mg/kg/day group for both treatment periods. This high dose group consistently exhibited decreased testicular weights. Epididymal and prostate weights were also reduced in the 1500 mg/kg/day group after both 4- and 9-week treatments and in the 500 mg/kg/day group after the 9-week treatment. Severe degenerative changes such as degeneration of germ cells, loss of germ cells and atrophy of seminiferous tubules were observed in all rats of the 1500 mg/kg/day groups after both 4 and 9 weeks of treatment. Retention of spermatids in stage IX, X and XI seminiferous tubules was also noted after the 4- and 9-week treatments at 500 mg/kg/day. The testicular sperm head counts were markedly decreased following the 4- and 9-week treatments at 1500 mg/kg/day, and mildly reduced after the 4-weeks treatment at 500 mg/kg/day.

From these results it is concluded that histopathological examination and the testicular sperm head count method are highly useful for detecting testicular toxicity and that testicular lesions caused by nefiracetam can be detected after 4 weeks of exposure.

KEY WORDS : Testis, Rat, Male fertility, Sperm, Reproductive toxicity, Testicular toxicity, Nefiracetam.

INTRODUCTION

Nefiracetam (N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide), having a pyrrolidone (cyclic γ -amino butyric acid, GABA) moiety in its chemical structure, has been shown to enhance learning acquisition and improve experimentally induced amnesia (Sakurai *et al.*, 1989 ; Nabeshima *et al.*, 1990), and is under development as a new cognition-enhancing agent for cerebrovascu-

lar dementia and senile dementia of Alzheimer type. In this study, the reproductive toxicity of nefiracetam in male rats was investigated with 4- or 9-week treatment periods prior to mating.

MATERIALS AND METHODS

Test article : The chemical structure of nefiracetam (N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide) is shown in Fig. 1. Nefiracetam used in this study was synthesized at the Chemical Technology Center, Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). The nefi-

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Fig. 1. Chemical structure of nefiracetam.

racetam preparations were formulated as suspensions in 0.5% sodium carboxymethyl cellulose (CMC) just before use.

Animals: Sprague-Dawley rats (Slc: SD), purchased from Japan SLC, Inc. (Hamamatsu, Japan) were used in this study. Rats were placed in an animal room controlled at a temperature of $23 \pm 2^\circ\text{C}$ and a relative humidity of $55 \pm 15\%$ with 14-hr lighting cycle (06:00–20:00). All animals were allowed free access to commercial rodent chow (MB-1, Funabashi Farm Co., Ltd., Funabashi, Japan) and to tap water.

Experimental design: As shown in Fig. 2, nefiracetam was orally administered to 4 groups of male rats at dose levels of 0, 500 or 1500 mg/kg/day for 4 or 9 weeks before mating with non-treated females. The dose of 500 mg/kg/day was selected as this is near to the highest dose (480 mg/kg/day) applied in an earlier fertility study in rats (Watanabe *et al.*, 1994). The dose of 1500 mg/kg/day was selected because it has already established that nefiracetam causes severe testicular lesions in rats at this dose. Vehicle (0.5% CMC)-treated males served as controls. Each group consisted of 15 males. To compare the effects of 4 or 9 weeks treatment, it was

considered necessary that treated males are necropsied at the same weeks of age, if possible. So, treatment was started at 6 and 11 weeks of age respectively, for the 9- and 4-weeks studies. At 15 weeks of age, males were cohabited with non-treated females (12 weeks of age) for up to 2 weeks. The daily dosing of nefiracetam or vehicle was continued until males were necropsied on the day copulation was confirmed, or 3 to 9 days after commencement of mating. During the experimental period, body weights of male rats were recorded once a week. Copulation and fertility indices were calculated from the results of mating. At necropsy of the males, the right and left testes and epididymes were weighed separately. Weights of prostates were also measured. The left testes were homogenized with water and sonicated for sperm head counting (Meistrich, 1989). Sperm was collected from the left caudal epididymis after mincing in modified Krebs-Ringer bicarbonate solution. Then the motility of sperm was scored as 0, 1 or 2, scores being defined as follows; 0: no motility, 1: a proportion of sperm show motility, 3: almost all sperm move vigorously. The right testes were fixed in Bouin's solution, embedded in paraffin wax and

Fig. 2. Experimental design.

histological sections were cut at 4 μ m and stained with periodic acid-Schiff's solution (PAS).

Statistics : The following statistical tests were selected as appropriate and applied for analysis of the significance of differences found in group means between any treated group and the control groups : Scheffé's test, Dunnett's test, Scheffé type mean rank test, Dunnett type mean rank test and Student's *t*-test.

RESULTS

Data for body weight changes are shown in Tables 1 and 2. In the 1500 mg/kg/day group the body weights were significantly lower than those of the control groups during the entire treatment period of 4 or 9 weeks. Especially in the 4-week 1500 mg/kg/day group case, the body weights were lower than the pre-treatment value at 1 and

2 weeks of treatment.

The results of mating are presented in Table 3. The majority of males in all groups copulated with their partners ; hence, copulation indices were not affected by the nefiracetam treatment. However, there were no or few implants found in females and thus fertility indices and implantation rates were extremely low in the 1500 mg/kg/day group after both treatment periods. On the other hand, fertility indices in the 500 mg/kg/day group were comparable to the control values. There were also no differences in implantation index or fetal mortality between each time period 500 mg/kg/day group and the control group.

Reproductive organ weights of male rats are shown in Table 4. Severely decreased testicular weights were observed in the 1500 mg/kg/day group after both treatment periods. Weights of epididymides and prostates were also decreased

Table 1. Body weights of male rats treated with nefiracetam for 4 weeks.

Weeks of treatment	Dose (mg/kg)		
	0	500	1500
0 Week	323.8 $\pm$ 6.6 ^{a)}	325.3 $\pm$ 6.2	320.2 $\pm$ 6.3
1 Week	359.8 $\pm$ 9.1	356.3 $\pm$ 8.6	264.0 $\pm$ 16.1**
2 Weeks	394.5 $\pm$ 14.5	388.7 $\pm$ 10.2	298.2 $\pm$ 29.5**
3 Weeks	422.3 $\pm$ 16.3	418.7 $\pm$ 12.2	330.3 $\pm$ 19.2**
4 Weeks	434.8 $\pm$ 19.5	429.8 $\pm$ 15.5	348.8 $\pm$ 17.9**

^{a)} : Values represent mean $\pm$ S.D.

** : Significantly different from control ($P < 0.01$).

Table 2. Body weights of male rats treated with nefiracetam for 9 weeks.

Weeks of treatment	Dose (mg/kg)		
	0	500	1500
0 Week	180.0 $\pm$ 6.8 ^{a)}	177.5 $\pm$ 7.5	176.9 $\pm$ 7.4
1 Week	235.3 $\pm$ 10.6	234.2 $\pm$ 9.5	200.1 $\pm$ 18.5**
2 Weeks	291.9 $\pm$ 16.0	292.5 $\pm$ 13.6	251.8 $\pm$ 17.3**
3 Weeks	332.5 $\pm$ 21.5	334.5 $\pm$ 14.4	288.6 $\pm$ 14.6**
4 Weeks	367.5 $\pm$ 25.0	368.8 $\pm$ 17.9	316.2 $\pm$ 13.1**
5 Weeks	392.1 $\pm$ 29.6	392.2 $\pm$ 19.9	332.1 $\pm$ 14.2**
6 Weeks	412.2 $\pm$ 33.6	413.2 $\pm$ 21.8	340.4 $\pm$ 17.4**
7 Weeks	440.3 $\pm$ 38.1	444.3 $\pm$ 25.6	356.6 $\pm$ 16.1**
8 Weeks	460.5 $\pm$ 40.9	465.7 $\pm$ 21.0	363.4 $\pm$ 21.4**
9 Weeks	461.4 $\pm$ 31.1	475.4 $\pm$ 22.8	362.7 $\pm$ 19.6**

^{a)} : Values represent mean $\pm$ S.D.

** : Significantly different from control ($P < 0.01$).

Table 3. Results of mating of nefiracetam-treated males with non-treated females.

Treatment period (Weeks)	4			9		
	0	500	1500	0	500	1500
Dose (mg/kg)						
No. of pairs	15	15	13	13	14	14
No. of copulations	15	15	12	13	12	12
Copulation index (%)	100	100	92.3	100	85.7	85.7
No. of pregnancies	15	14	0	12	11	1
Fertility index (%)	100	93.3	0**	92.3	91.7	8.3**a)
Implantation rate (%)	96.2	97.1	0**	98.3	97.5	7.7**a)
Fetal mortality (%)	5.4	9.2	ND	5.2	6.8	100**a)

** : Significantly different from control ($P < 0.01$).

ND : Not determined, there was no fetus in this group.

a) : Only one placental remnant was found in a dam.

in the 1500 mg/kg/day group after both treatment periods and in the 500 mg/kg/day 9-week treatment group. However the weights of these organs were not affected by 4-weeks treatment of nefiracetam at the dose level of 500 mg/kg/day.

The results of testicular sperm head counting are presented in Table 5. The sperm head counts were seriously decreased in the 1500 mg/kg/day group after both treatment periods. A slight but significant decrease was also noted for the 500 mg/kg/day 4-week treatment group.

The results of sperm motility observation are shown in Table 6. Extremely few spermatozoa were found and all these observed were not motile in the 1500 mg/kg/day group after both treatment periods. On the other hand, motility indices for the 500 mg/kg/day 4- and 9-week treatment cases were comparable to the control values.

Histopathological findings observed in this study are summarized in Tables 7 and 8. Degeneration of germ cells, loss of germ cells and atrophy of seminiferous tubules (Photo. 1B) were observed in all males of the 1500 mg/kg/day group after both treatment periods. Multinuclear giant cells (Photo. 1B) were also noted in the high dose 4- and 9-week treatment cases at high incidence (12 out of 13 animals and 9 out of 14, respectively). At the dose level of 500 mg/kg/day, spermiation was inhibited as characterized by retention of step 19 spermatids in stage IX, X and XI seminiferous tubules (Photos. 1C, 1D), noted in 14 out of 15 males of the 4-week treatment group and in 10 out of 14 males of the 9-week treatment

group. In the epididymis, reduction of sperm and desquamation of epithelial cells were observed frequently in the 1500 mg/kg/day group after both treatment periods.

DISCUSSION

Nefiracetam is now under development as a new cognition-enhancing agent for cerebrovascular dementia and senile dementia of Alzheimer type. The toxicity of nefiracetam has already been studied in various aspects. From the results of these studies, it has been demonstrated that nefiracetam has no mutagenicity (Shimada *et al.*, 1994), no oncogenicity in rats and mice (Kajimura *et al.*, 1994) and no female reproductive toxicity including teratogenicity in rats or rabbits (Watanabe *et al.*, 1994). Concerning male reproductive toxicity, nefiracetam exhibited suppression of spermatogenesis in a 13-week toxicity study in dogs at dose levels of 60 mg/kg or more (Sugawara *et al.*, 1994) and in a 52-week toxicity study in dogs at a dose level of 90 mg/kg (Hooks *et al.*, 1994). However there was no evidence of testicular toxicity in a 13-week repeated dose toxicity study in rats and a reproductive toxicity study in rats at dose levels of 480 mg/kg/day or less.

In the present study the reproductive toxicity of nefiracetam in male rats was investigated with 4- or 9-week treatments prior to mating. The results are summarized in Table 9. Decreases in genital organ weights, testicular sperm head counts and fertility index were observed in the

Table 4. Effects of nefiracetam on reproductive organ weights of male rats.

Treatment period (Weeks)		4			9		
Dose (mg/kg/day)		0	500	1500	0	500	1500
Testis (R)	Mean	1.63	1.62	0.71**	1.68	1.68	0.69**
	S.D.	0.05	0.11	0.06	0.12	0.13	0.08
Testis (L)	Mean	1.68	1.65	0.71**	1.70	1.72	0.70**
	S.D.	0.07	0.09	0.06	0.11	0.14	0.08
Epididymis	Mean	1.02	1.01	0.66**	1.03	0.97*	0.55**
	S.D.	0.06	0.07	0.10	0.06	0.08	0.07
Prostate	Mean	0.47	0.45	0.32**	0.48	0.40*	0.30**
	S.D.	0.09	0.13	0.04	0.08	0.09	0.07

* : Significantly different from control ($P < 0.05$).** : Significantly different from control ($P < 0.01$).**Table 5.** Effects of nefiracetam on testicular sperm head counts in male rats.

Treatment period (Weeks)		4			9		
Dose (mg/kg/day)		0	500	1500	0	500	1500
Sperm head count (number/testis) $\times 10^8$	Mean	2.71	2.13**	0.021**	2.41	2.16	0.015**
	S.D.	0.35	0.36	0.03	0.51	0.48	0.02

** : Significantly different from control ($P < 0.01$).** Table 6.** Effect of nefiracetam on sperm motility in male rats.

Treated Period (Weeks)		4			9		
Dose (mg/kg/day)		0	500	1500	0	500	1500
		1.40	1.87	0**	1.69	1.71	0**

** : Significantly different from control ($P < 0.01$).

1500 mg/kg group after both treatment periods. In addition, histopathological changes in the testis and epididymis were also noted in the 500 and 1500 mg/kg groups. From these results, it can be considered that the reproductive toxicity caused by nefiracetam treatment for 4 weeks is essentially the same as that resulting from 9-weeks treatment. There were some discrepancies since, at the dose level of 500 mg/kg, decreases in epididymal and prostate weights were observed only after the 9-week treatment, whereas the testicular sperm head count was found decreased only after the 4-week treatment. The causes of these discrepancies could not be identified but the differ-

ence in age when treatment was initiated may be one contributory factor.

As a characteristic histopathological finding, retention of step 19 spermatids in stage IX, X and XI tubules was observed in the 500 mg/kg group after both treatment periods. Such a finding has been observed for several testicular toxicants and is considered to be a relatively common change early in lesion development after exposure (Parvinen, 1979 ; Chapin *et al.*, 1984a ; Chapin *et al.*, 1984b ; Chapin *et al.*, 1984c ; Hess *et al.*, 1988 ; Anderson *et al.*, 1989 ; Creasy *et al.*, 1990)

With respect to the sensitivity of each parameter investigated, histopathological examination

Table 7. Histopathological findings in the testis and epididymis from male rats treated with nefiracetam for 4 weeks.

Dose (mg/kg/day)		0			500			1500			
No. of animals		15			15			13			
Grade		—	±	+	++	+++	—	±	+	++	+++
Testis											
Multinuclear giant cells	15	0	0	0	0	0	15	0	0	0	0
Degeneration of germ cells	15	0	0	0	0	0	14	0	1	0	0
Loss of germ cells	15	0	0	0	0	0	14	0	1	0	0
Atrophy of seminiferous tubules	15	0	0	0	0	0	14	1	0	0	0
Retention of step 19 spermatids	15	0	0	0	0	0	1	0	6	7	1
in stage IX, X, XI seminiferous tubules											
Epididymis											
Reduction of sperm	15	0	0	0	0	0	15	0	0	0	0
Desquamation of epithelial cells	15	0	0	0	0	0	15	0	0	0	0

Grades are as follows. —: no change, ±: minimal, +: slight, ++: moderate, +++: severe

Table 8. Histopathological findings in the testis and epididymis from male rats treated with nefiracetam for 9 weeks.

Dose (mg/kg/day)		0			500			1500			
No. of animals		13			14			13			
Grade		—	±	+	++	+++	—	±	+	++	+++
Testis											
Multinuclear giant cells	13	0	0	0	0	0	14	0	0	0	0
Degeneration of germ cells	13	0	0	0	0	0	14	0	0	0	0
Loss of germ cells	13	0	0	0	0	0	14	0	0	0	0
Atrophy of seminiferous tubules	13	0	0	0	0	0	14	0	0	0	0
Retention of step 19 spermatids	13	0	0	0	0	0	4	2	5	3	0
in stage IX, X, XI seminiferous tubules											
Epididymis											
Reduction of sperm	13	0	0	0	0	0	14	0	0	0	0
Desquamation of epithelial cells	13	0	0	0	0	0	14	0	0	0	0

Grades are as follows. —: no change, ±: minimal, +: slight, ++: moderate, +++: severe

Photo. 1. Histopathological findings in the testes from male rats treated with nefiracetam for 4-weeks. (A~C: $\times 50$, D: PAS stain, $\times 100$). A: Control, stage IX (left bottom) and XII (right) seminiferous tubules; B: Multinuclear giant cells, degeneration of germ cells, loss of germ cells and atrophy of seminiferous tubules (1500 mg/kg nefiracetam); C: Retention of step 19 spermatids in a stage XI seminiferous tubule (center, 500 mg/kg nefiracetam); D: Higher magnification of the tubule shown in C. Note the presence of step 19 spermatids (arrowheads).

Table 9. Summary of the effects found in male rats treated with nefiracetam.

Treated period (Weeks)	4			9		
Dose (mg/kg)	0	500	1500	0	500	1500
Body weights	—	—	↓	—	—	↓
Testis weights	—	—	↓	—	—	↓
Epididymis weights	—	—	↓	—	↓	↓
Prostate weights	—	—	↓	—	↓	↓
Testicular sperm head counts	—	↓	↓	—	—	↓
Copulation index	—	—	—	—	—	—
Fertility index	—	—	↓	—	—	↓
Histopathological changes	—	+	+	—	+	+
Sperm motility	—	—	↓	—	—	↓

—: no change

↓: decrease

↑: increase

+: observed

and testicular sperm head counts were considered to be more sensitive than the others since only these two detected the testicular changes caused by nefiracetam at the lower dose level (500 mg/kg/day) and with the shorter treatment period (4-weeks). The testicular sperm head count method has some other advantages; it is simple, easy, rapid and quantitative. In addition, this method is useful to detect testicular toxicity in the study of fertility and early embryonic development to implantation, for the following reason: According to the ICH guideline, it is required to dose males for at least 8 weeks, consisting of a pre-mating period of at least 4-weeks, a mating period of 2 weeks or longer and the first 2 weeks of gestation, because it is advised to delay sacrifice of the males until the outcome of mating is known when the females are sacrificed on days 13–15 of gestation. Since the period of spermatogenesis is shorter than 8 weeks in rats, it is considered that the testicular sperm head count method can detect testicular lesions even if the testicular toxicant attacks the early phase of spermatogenesis. Whilst the present study was not primarily aimed at the mechanisms underlying the testicular toxicity of nefiracetam, the following results obtained in other studies conducted at our laboratory may be of significance: 1) Testicular and serum levels of testosterone were decreased significantly after a single or 1 week repeated dosing with nefiracetam at 1500

mg/kg/day. 2) Testicular sperm head counts were also decreased following a 1-week treatment with nefiracetam at 1500 mg/kg/day. 3) Decreases of testosterone level and testicular sperm head counts caused by the 1-week treatment with nefiracetam were blocked by simultaneous injection of human chorionic gonadotropin (hCG, 1 IU/animal/day). These results suggest that decreased testosterone secretion is at least partially involved in the testicular toxicity of nefiracetam. However, it appears unlikely that decreased testosterone suffices to explain the severe degenerative changes and atrophy of seminiferous tubules, since it has been shown that spermatogenesis can be maintained in the presence of significantly reduced intratesticular testosterone (Rommerts, 1988; Zirkin, 1989). So, there may be some other factors contributing to the development of testicular lesions in nefiracetam-treated animals besides decreased testosterone.

From the results of the present study it is concluded that histopathological examination and the testicular sperm head count method are highly useful for detecting testicular toxicity and that the testicular lesions caused by nefiracetam can be detected after 4 weeks of exposure.

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