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A TIME-COURSE CHARACTERIZATION OF MALE REPRODUCTIVE TOXICITY IN RATS TREATED WITH METHYL METHANESULPHONATE (MMS)

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ABSTRACT — Methyl methanesulphonate (MMS), a potent alkylating agent and testicular toxicant, was orally administered to rats for 5 days at doses of 20, 30, and 40 mg/kg. During the recovery period of 5 weeks, males were evaluated for multiple endpoints such as organ weights, fertility, and sperm parameters. The 5-week recovery periods are designated as follows: Day 1 (1 day after final treatment); Week 1, Week 2, Week 3, Week 4, and Week 5 (first, second, third, fourth, and fifth week after final treatment). A clear time-course of dominant lethals was observed. The peak severities of the dominant lethals were observed in Week 2. It was judged that the most sensitive cellular targets for the dominant lethals are late spermatids. Sperm examination revealed a clear time-course and dose-dependent changes in the frequency of sperm morphological abnormalities. The peak severities of the sperm morphological alterations in cauda epididymis were observed in Week 4. Sensitive cellular stages for the induction of sperm morphological abnormalities were judged to be late spermatocytes and early spermatids. The most frequently observed type of morphologically abnormal spermatozoa was tailless sperm, followed by no-hook head sperm. Although the initial cause for both sperm morphological alterations and dominant lethals was suggested to be genetic insult to the germ cells, there were no obvious relationships observed between these two findings.

KEY WORDS: Time-course study, Sperm morphology, Dominant lethal, Reproductive toxicity, MMS, Rats

INTRODUCTION

Once toxicity is found in a toxicology study, it is important to characterize the profile of toxicity, identify the cellular target and clarify the mechanism of action. For the evaluation of toxicity to male fertility, ICH guideline (Step 4 29 November 1995, amended in November 2000) recommends histopathology of testis as the most sensitive method for the detection of effects on spermatogenesis; sperm analysis including sperm count, motility, and morphology as an optional procedure for confirmation or better characterization of the observed toxicology; and mating with female for the evaluation of functional effects on male fertility that cannot be detected by histopathological examination. A time-course observation of multiple endpoints, after a short-term exposure to testicular toxicants, is a useful measure for the identification of cellular targets and may also provide insight into the possible mechanisms of toxicological action for male fertility (Linder *et al.*, 1992; Perreault *et al.*, 1989).

Methyl methanesulphonate (MMS) is a potent alkylating agent which is known as a testicular toxicant, causing decrease of germ cells, exfoliation of germ cells, and vacuolar degeneration of Sertoli cells after oral treatment of 40 mg/kg for 2 weeks in rats

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(Ozawa *et al.*, 2000) and abnormalities in testicular sperm head (Cassidy *et al.*, 1983). MMS is also known to induce dominant lethals after a single intraperitoneal injection of 40 mg/kg in rats (Ashby *et al.*, 1996) and mice (Ehling and Neuhauser-Klaus, 1990). In mice, MMS is known to induce morphologically abnormal head sperm (Wyrobek and Bruce, 1975) and chromosome aberrations in fertilized eggs after treatment to males (Matsuda *et al.*, 1989). It was anticipated that MMS would cause morphologically abnormal spermatozoa in epididymis together with the dominant lethals after oral treatment to male rats.

In the present study, in order to investigate the effect of MMS on the morphological alterations of the spermatozoa in epididymis, to identify cellular targets for each toxicological finding, and to clarify the relationship between sperm morphological alterations and dominant lethals, a time-course evaluation was performed with a 5-day treatment, followed by a 5-week recovery period. Rats, the most commonly used animals for toxicity evaluation, were selected for the study.

MATERIALS AND METHODS

Animals

All animal experiments were carried out in accordance with the Guide to the Care and Use of Experimental animals of the Toxicology Research Lab., Kissei Pharmaceutical Co., Ltd. Crj:CD(SD), SPF rats, 7- to 8-week-old, 250 males and 150 females, were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were kept in a barrier system where the temperature (22.8 to 24.6°C), the relative humidity (40.9 to 57.0%), the ventilation (11.13 times/hr) and illumination cycle (12 hr light/12 hr dark) were maintained. The male animals were housed in a stainless mesh individual cage (sized 260 × 230 × 180 mm) throughout the quarantine, acclimation and study periods. The female animals were housed in a stainless mesh group cage (sized 260 × 380 × 180 mm, housed up to 4 animals per cage) until mating to the males. The animals were allowed free access to tap water and pellet diet (CE-2, CLEA Japan, Inc., Tokyo, Japan) throughout the quarantine, acclimation and study periods. The quarantine and acclimation period was set at 17 days and all the animals were 10 weeks old at the start of male treatment. No signs in clinical observations or negative findings in body weights were detected during the quarantine and acclimation period.

Chemical

Methyl methanesulphonate (MMS, 99.9%, Aldrich Chemical Co., Inc., Milwaukee, WI, USA) was dissolved in distilled water (JP grade, Otsuka Pharmaceutical Industries, Tokushima, Japan). MMS was prepared freshly each day.

Experimental design

Male animals were allocated to 4 groups, including the control group, and each group consisted of 55 male animals. Male rats were treated with MMS (20, 30, 40 mg/kg, p.o., 5 mL/kg body weight) once daily for 5 consecutive days. The route of administration and dosages was selected based on our previous study (Ozawa *et al.*, 2000), in which testicular toxicity was observed after 2 to 4 weeks treatment. The highest dose of 40 mg/kg was also consistent with Ashby's study (1996), in which dominant lethal effects were induced after a single i.p. dosage. A 5-day treatment period and 5-week recovery period were selected based on our preliminary study, in which significantly increased sperm abnormalities were observed on Day 30 after oral administration of MMS for 5 days at 40 mg/kg (data not shown). Control males received distilled water in the same regimen as MMS-treated groups. On the next day after final administration (Day 1), and first, second, third, and fourth weeks after final administration (Week 1, Week 2, Week 3, and Week 4), 5 out of 10 males from each dose group were sacrificed for sperm examination and the others were used for mating. Mating was not performed in the fifth week after final administration (Week 5). The experimental design is shown in Fig. 1.

Necropsy

All the male animals were sacrificed by inhalation of carbon dioxide, examined for gross abnormalities and weighed for testes and epididymides individually. The right cauda epididymis from the males unused for mating was employed for sperm examination, such as motility examination, sperm morphological examination and caudal epididymal sperm count. The right caput epididymis was used for sperm morphological examination.

Mating

Males designated for mating were paired to untreated virgin pro-estrus females overnight on a one-to-one basis. Plugs observed in the following morning were considered to be Day 0 of gestation. Males that failed to copulate were allowed to pair up to 3 consec-

utive nights in the same manner. On Day 13 of gestation, all the dams were sacrificed by inhalation of carbon dioxide, caesarean sectioned, and examined for gross abnormalities. The numbers of implants, corpora lutea, live embryos, and dead embryos were counted.

Sperm examination

The right cauda epididymis was dissected, weighed and placed in the medium (37°C, Hank's solution containing 0.5% BSA) to allow dispersion of sperm from cauda epididymis. After several minutes, the sperm suspension was placed on a slide glass that had been warmed to 37°C and more than 100 spermatozoa were manually counted to estimate the percentage of motile sperm.

The caudal epididymis was then placed in 5 mL medium, minced, incubated for more than 1 hr at 37°C, filtrated for debris removal and diluted roughly 50 times for sperm counting. The sperm suspension was manually counted under microscopy for the calculation of number of sperm per weight of caudal epididymis.

Sperm morphological samples were prepared as follows. Sperm suspension prepared for sperm motility was used for the morphological examination of cauda sperm. The distal portion of caput epididymis was dissected, placed in the medium (37°C, Hank's solution containing 0.5% BSA), allowed for dispersion for a few hours and used for the morphological examination of caput sperm. A small amount of cauda and caput sperm suspension was stained with 0.5% eosin Y, smeared onto a slide glass, air-dried overnight, and fixed in methanol. Two hundred spermatozoa in each sample were examined microscopically for head, neck and tail morphological abnormalities. For the classification of sperm morphological abnormalities, Yoshizaki's method (Yoshizaki *et al.*, 1999) was slightly modified and the sperm were classified as follows: 1) tailless sperm, 2) no-hook head sperm, 3) amorphous head sperm, 4) sperm with neck abnormalities, 5) sperm with tail abnormalities, and 6) other abnormal sperm.

Statistical analysis

Data were statistically analyzed as follows: All quantitative data were tested for equal variance. If equal variance was found, one-way analysis of variance (Snedecor and Cochran, 1967) was used. If not, the Kruskal-Wallis procedure for nonparametric analysis (Hollander and Wolfe, 1973) was used. When significant inter-group differences were found, the Dunnett multiple comparison test (Dunnett, 1955 and 1964) or the Dunnett rank test (Hollander and Wolfe, 1973) was applied. For the analysis of copulation index and fertility index, the chi square test (Fisher, 1922) was employed. For the analysis of implantation index and post-implantation loss, the Wilcoxon (Ichihara, 1996) test was used. Probabilities less than 5% were considered statistically significant.

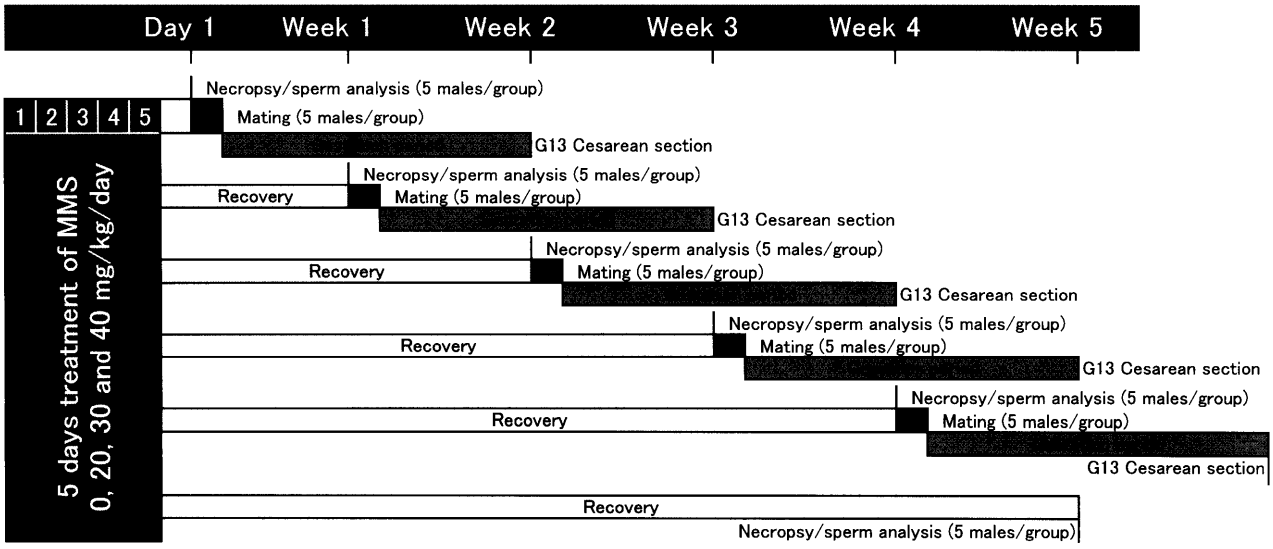

Fig. 1. Experimental design.

RESULTS

Clinical signs and body weights

No clinical signs related to MMS treatment were observed in male animals. There was a slight but statistically significant decrease in body weight at 40 mg/kg on the final day of treatment. In the recovery period, body weights in male animals were almost comparable to control.

Fertility

Results of matings are shown in Table 1. Successful copulation was observed in almost all the male animals. Fertility indices were dose-dependently decreased in a chorological manner. The most severe effect was observed in Week 2 and none of the males in the 30 and 40 mg/kg group succeeded in impregnating females. There was no detectable effect on the fertility index in Week 4.

Results of caesarean sections are shown in Table 2. In the caesarean section findings, decreases in the number of implants, number of live embryos, and implantation index were observed in a clear chorological manner. An increase in the post-implantation loss

Table 1. Results of matings.

Recovery period \ Dose	Control	MMS (mg/kg)		
		20	30	40
Day 1				
No. of paired	5	5	5	5
No. of copulation	5	5	5	5
Copulation index[a)]	100	100	100	100
No. of impregnating males	5	5	5	4
Fertility index[b)]	100	100	100	80
Week 1				
No. of paired	5	5	5	5
No. of copulation	5	5	5	5
Copulation index[a)]	100	100	100	100
No. of impregnating males	5	5	3	2
Fertility index[b)]	100	100	60	40
Week 2				
No. of paired	5	5	5	5
No. of copulation	5	5	5	5
Copulation index[a)]	100	100	100	100
No. of impregnating males	5	3	0	0
Fertility index[b)]	100	60	0**	0**
Week 3				
No. of paired	5	5	5	5
No. of copulation	5	3	5	5
Copulation index[a)]	100	60	100	100
No. of impregnating males	5	2	3	4
Fertility index[b)]	100	66.7	60	80
Week 4				
No. of paired	5	5	5	5
No. of copulation	5	5	5	5
Copulation index[a)]	100	100	100	100
No. of impregnating males	5	5	5	5
Fertility index[b)]	100	100	100	100

[a)]: (No. of copulations / No. of matings) × 100.
[b)]: (No. of impregnating males / No. of copulations) × 100.
**: Significant difference from control ($p<0.01$).

Table 2. Cesarean section data.

Recovery period \ Dose	No. of dams	No. of corpora lutea (Mean ± S.D.)	No. of implants (Mean ± S.D.) [%, Mean ± S.D.][a)]	No. of live embryos (Mean ± S.D.)	No. of dead embryos [%, Mean ± S.D.][b)]
Day 1		82	79	64	15
Control	5	(16.4 ± 0.55)	(15.8 ± 0.84) [96.4 ± 5.31]	(12.8 ± 5.54)	[19.7 ± 34.1]
		78	69	40	29
20 mg/kg	5	(15.6 ± 1.14)	(13.8 ± 3.96) [88.8 ± 25.13]	(8.0 ± 3.32)	[42.5 ± 14.8]
		72	55	16	39
30 mg/kg	5	(14.4 ± 3.44)	(11.0 ± 6.63) [70.0 ± 34.05]	(3.2 ± 5.02) **	[62.6 ± 46.9]
		57	44	6	38
40 mg/kg	4	(14.3 ± 1.71)	(11.0 ± 2.45) [77.6 ± 16.05] *	(1.5 ± 1.29) **	[87.4 ± 10.3] *
Week 1		87	85	83	2
Control	5	(17.4 ± 0.89)	(17.0 ± 1.41) [97.6 ± 3.32]	(16.6 ± 1.95)	[2.6 ± 3.6]
		74	29	0	29
20 mg/kg	5	(14.8 ± 2.39)	(5.8 ± 2.59) ** [40.8 ± 21.89] *	(0.0) **	[100.0] **
		35	8	0	8
30 mg/kg	3	(11.7 ± 2.31) **	(2.7 ± 1.53) ** [21.7 ± 9.93] *	(0.0) *	[100.0] *
		24	8	0	8
40 mg/kg	2	(12.0 ± 1.41) *	(4.0 ± 1.41) ** [34.3 ± 15.84]	(0.0) *	[100.0]
Week 2		80	78	76	3
Control	5	(16.0 ± 2.55)	(15.6 ± 2.07) [97.9 ± 4.70]	(15.2 ± 2.17)	[3.7 ± 5.4]
		54	4	0	4
20 mg/kg	3	(18.0 ± 3.00)	(1.3 ± 0.58) [7.3 ± 2.01] *	(0.0)	[100.0] *
30 mg/kg	0	–	–	–	–
40 mg/kg	0	–	–	–	–
Week 3		79	77	75	2
Control	5	(15.8 ± 1.30)	(15.4 ± 1.52) [97.4 ± 3.59]	(15.0 ± 1.58)	[2.5 ± 5.6]
		30	20	11	9
20 mg/kg	2	(15.0 ± 2.83)	(10.0 ± 8.49) [62.5 ± 44.76]	(5.5 ± 7.78)	[65.7 ± 48.6]
		45	29	26	3
30 mg/kg	3	(15.0 ± 1.73)	(9.7 ± 7.09) [62.3 ± 42.31]	(8.7 ± 7.09)	[21.8 ± 24.5]
		56	32	22	10
40 mg/kg	4	(14.0 ± 0.82)	(8.0 ± 2.45) [57.6 ± 19.72] *	(5.5 ± 3.42) *	[35.1 ± 32.2] *
Week 4		74	72	65	7
Control	5	(14.8 ± 1.48)	(14.4 ± 1.34) [97.4 ± 3.59]	(13.0 ± 1.41)	[9.5 ± 8.9]
		86	85	81	4
20 mg/kg	5	(17.2 ± 0.84)	(17.0 ± 1.00) [98.8 ± 2.64]	(16.2 ± 1.48) *	[4.6 ± 7.6]
		81	77	66	11
30 mg/kg	5	(16.2 ± 1.64)	(15.4 ± 2.88) [94.7 ± 11.94]	(13.2 ± 2.59)	[13.3 ± 14.7]
		81	76	66	10
40 mg/kg	5	(16.2 ± 1.79)	(15.2 ± 2.05) [93.9 ± 9.00]	(13.2 ± 1.64)	[12.6 ± 9.6]

[ ]: Values in parentheses represent average percent per litter.
a): Implantation index = (No. of implants / No. of corpora lutea) × 100.
b): Post-implantation loss = (No. of dead embryos / No. of implants) × 100.
*: Significant difference from control ($p<0.05$). **: Significant difference from control ($p<0.01$).

was also observed in a chorological manner. The earliest effect on the caesarean section data was detected on Day 1. Severe effects were observed in Week 1 and Week 2, in which there were no live embryos in the MMS treatment groups. Recoveries from the effects were observed beginning in Week 3 and there were no detrimental effects detectable in Week 4. The decrease in the number of corpora lutea at 30 and 40 mg/kg in Week 1 might be related to the early embryonic deaths observed in this study (Morishige and Rothchild, 1974).

Organ weights

Organ weights are shown in Table 3. Significantly decreased absolute epididymis weights were observed at 30 mg/kg in Week 4 and 40 mg/kg in Week 1 and Week 5. No statistical difference from control was observed in the relative epididymis weights throughout the study period. There were no clear changes observed in the testis weights.

Sperm examination

Sperm examination data are shown in Table 4. The number of sperm in cauda epididymis was signifi-

Table 3. Organ weights.

Recovery period \ Dose	No. of animals	Final Body weight (g)	Testes		Epididymes	
			Absolute	Relative	Absolute	Relative
Day 1						
Control	10	440.8 ± 21.6	3.33 ± 0.20	7.56 ± 0.39	0.98 ± 0.09	2.22 ± 0.15
20 mg/kg	10	433.9 ± 18.6	3.19 ± 0.16	7.36 ± 0.48	0.96 ± 0.07	2.21 ± 0.11
30 mg/kg	10	432.2 ± 20.9	3.12 ± 0.45	7.23 ± 1.03	0.91 ± 0.14	2.10 ± 0.32
40 mg/kg	10	428.4 ± 20.0	3.20 ± 0.19	7.47 ± 0.34	0.91 ± 0.06	2.12 ± 0.14
Week 1						
Control	10	461.7 ± 43.7	3.32 ± 0.33	7.27 ± 1.05	1.10 ± 0.08	2.40 ± 0.27
20 mg/kg	10	451.8 ± 41.2	3.26 ± 0.27	7.26 ± 0.70	1.05 ± 0.08	2.33 ± 0.28
30 mg/kg	10	451.7 ± 31.1	3.33 ± 0.18	7.39 ± 0.57	1.07 ± 0.08	2.38 ± 0.20
40 mg/kg	10	449.3 ± 37.7	3.19 ± 0.31	7.11 ± 0.47	0.98 ± 0.11 *	2.19 ± 0.21
Week 2						
Control	10	499.1 ± 27.6	3.24 ± 0.35	6.51 ± 0.68	1.18 ± 0.10	2.37 ± 0.16
20 mg/kg	10	494.0 ± 32.2	3.39 ± 0.29	6.87 ± 0.64	1.26 ± 0.07	2.55 ± 0.27
30 mg/kg	10	498.5 ± 34.9	3.35 ± 0.27	6.72 ± 0.41	1.22 ± 0.11	2.44 ± 0.18
40 mg/kg	10	491.6 ± 32.8	3.37 ± 0.20	6.86 ± 0.49	1.15 ± 0.10	2.35 ± 0.18
Week 3						
Control	10	534.7 ± 27.6	3.44 ± 0.29	6.45 ± 0.58	1.19 ± 0.14	2.22 ± 0.23
20 mg/kg	10	534.6 ± 32.4	3.42 ± 0.21	6.41 ± 0.29	1.23 ± 0.09	2.31 ± 0.15
30 mg/kg	10	532.1 ± 32.7	3.41 ± 0.11	6.41 ± 0.32	1.17 ± 0.06	2.20 ± 0.17
40 mg/kg	10	522.4 ± 28.3	3.38 ± 0.24	6.47 ± 0.49	1.15 ± 0.09	2.20 ± 0.19
Week 4						
Control	10	545.2 ± 30.0	3.46 ± 0.22	6.35 ± 0.50	1.26 ± 0.09	2.31 ± 0.24
20 mg/kg	10	530.6 ± 28.7	3.49 ± 0.20	6.59 ± 0.43	1.25 ± 0.08	2.36 ± 0.15
30 mg/kg	10	536.6 ± 37.2	3.26 ± 0.18	6.11 ± 0.59	1.15 ± 0.09 *	2.16 ± 0.26
40 mg/kg	10	526.1 ± 41.4	3.32 ± 0.30	6.35 ± 0.81	1.19 ± 0.08	2.28 ± 0.29
Week 5						
Control	5	516.5 ± 30.8	3.55 ± 0.19	6.89 ± 0.57	1.28 ± 0.06	2.47 ± 0.11
20 mg/kg	5	521.8 ± 13.8	3.38 ± 0.19	6.48 ± 0.49	1.24 ± 0.07	2.38 ± 0.16
30 mg/kg	5	522.0 ± 43.8	3.45 ± 0.23	6.66 ± 0.88	1.21 ± 0.04	2.33 ± 0.20
40 mg/kg	5	511.5 ± 31.1	3.35 ± 0.18	6.58 ± 0.76	1.11 ± 0.05 **	2.18 ± 0.22

Data shown as mean ± S.D..
*: Significant difference from control ($p<0.05$).
**: Significant difference from control ($p<0.01$).

cantly decreased at 40 mg/kg in Week 1. Slightly decreased sperm counts were consistently observed at 40 mg/kg throughout the study period. Sperm motility was not affected by MMS treatment. Significantly increased frequencies of morphologically abnormal sperm in cauda epididymis were observed at 30 and 40 mg/kg from Week 3 to Week 5. These changes were observed in a dependent manner. There was also a chronological change in the frequency of morphologically abnormal sperm. The frequency firstly increased in Week 3, peaked in Week 4, and slightly recovered in Week 5. In caput epididymis, a chronological change was only observed at 40 mg/kg. The frequency of morphologically abnormal sperm in caput epididymis increased in Week 2 and Week 3. The peak severity of the effect was observed in Week 3, in which the difference from the control was statistically significant. Recovery was observed in Week 4 and later.

The content of morphologically abnormal sperm in cauda epididymis is shown in Table 5. The most fre-

Table 4. Sperm examination data.

Recovery period \ Dose	No. of animals	No. of sperm[a)]	Sperm motility[b)]	Morphologically abnormal sperm[c)]	
				caput epididymis	cauda epididymis
Day 1					
Control	5	723.3 ± 85.6	83.2 ± 7.2	2.3 ± 1.8	1.9 ± 1.8
20 mg/kg	5	654.3 ± 84.0	87.9 ± 1.8	3.3 ± 2.2	2.7 ± 2.1
30 mg/kg	5	771.2 ± 177.9	84.7 ± 7.0	2.2 ± 2.0	3.8 ± 2.4
40 mg/kg	5	588.3 ± 44.4	87.4 ± 4.3	4.2 ± 4.9	2.4 ± 1.9
Week 1					
Control	5	833.8 ± 97.7	82.7 ± 8.5	3.1 ± 4.0	2.9 ± 2.0
20 mg/kg	5	896.3 ± 175.7	85.1 ± 2.5	3.8 ± 2.6	1.8 ± 0.9
30 mg/kg	5	718.9 ± 101.8	85.1 ± 5.0	5.2 ± 4.3	3.1 ± 0.4
40 mg/kg	5	577.5 ± 161.5 *	83.9 ± 5.7	5.1 ± 2.4	2.7 ± 1.5
Week 2					
Control	5	741.2 ± 99.0	86.1 ± 5.4	4.3 ± 6.0	3.1 ± 1.1
20 mg/kg	5	783.4 ± 73.7	87.6 ± 1.6	3.4 ± 1.6	3.4 ± 2.2
30 mg/kg	5	787.4 ± 75.3	77.9 ± 20.9	2.2 ± 1.0	2.5 ± 1.2
40 mg/kg	5	631.1 ± 137.6	84.7 ± 6.1	12.8 ± 15.4	4.4 ± 3.8
Week 3					
Control	5	800.4 ± 126.0	88.3 ± 3.7	4.3 ± 4.6	1.7 ± 1.4
20 mg/kg	5	792.5 ± 86.9	89.3 ± 5.2	2.7 ± 0.8	3.4 ± 1.6
30 mg/kg	5	765.0 ± 112.3	87.7 ± 2.1	3.9 ± 2.4	5.3 ± 1.5 *
40 mg/kg	5	741.6 ± 153.1	73.0 ± 16.0	23.5 ± 16.9 *	8.8 ± 2.8 **
Week 4					
Control	5	816.4 ± 70.0	86.9 ± 3.6	1.1 ± 0.5	1.8 ± 1.4
20 mg/kg	5	881.3 ± 29.9	82.0 ± 4.4	3.5 ± 3.3	2.5 ± 1.6
30 mg/kg	5	758.4 ± 100.0	84.3 ± 5.9	3.1 ± 1.9	11.4 ± 4.6 *
40 mg/kg	5	731.6 ± 237.1	65.2 ± 37.1	3.7 ± 4.4	26.4 ± 37.0 **
Week 5					
Control	5	894.7 ± 154.0	85.7 ± 5.1	1.6 ± 0.7	2.6 ± 2.3
20 mg/kg	5	770.2 ± 251.5	79.3 ± 9.2	2.8 ± 2.1	5.1 ± 2.7
30 mg/kg	5	838.1 ± 101.7	67.9 ± 27.8	8.5 ± 12.7	15.2 ± 10.3 *
40 mg/kg	5	639.8 ± 153.3	72.0 ± 13.7	3.3 ± 3.0	16.2 ± 10.0 *

a): Data shown as mean number of sperm($\times 10^6$) per 1 g epididymis ± S.D..
b): Data shown as percent motile sperm ± S.D..
c): Data shown as percent morphologically abnormal sperm ± S.D..
*: Significant difference from control ($p<0.05$).
**: Significant difference from control ($p<0.01$).

quently observed abnormalities in cauda epididymis were tailless sperm followed by no-hook head sperm regardless of dose and week observed. There were significantly increased frequencies of tailless sperm (from Week 3 to Week 5 at 40 mg/kg and in Week 4 at 30 mg/kg) and no-hook head sperm (in Week 4 and Week 5 at 30 and 40 mg/kg). Frequencies of other morphological abnormalities, such as amorphous head sperm or sperm with tail or neck abnormalities, were almost comparable to corresponding control.

DISCUSSION

MMS is a potent alkylating agent reported as a testicular toxicant (Ozawa *et al.*, 2000), causing abnormalities in testicular sperm head (Cassidy *et al.*, 1983) and dominant lethals (Ashby *et al.*, 1996) in rat studies. MMS is also known to cause dominant lethals (Ehling and Neuhauser-Klaus, 1990), and chromosomal aberrations in fertilized eggs in mice after treatment to males (Matsuda *et al.*, 1989).

Fig. 2 indicates the time-course of exposure of

Table 5. Content of morphologically abnormal sperm in caudal epididymis.

Recovery period \ Dose	No. of animals	Tailless	No hook head	Amorphous head	Neck abnormalities	Tail abnormalities	Other abnormalities
Day 1							
Control	5	1.0 ± 0.9	0.7 ± 1.1	0.1 ± 0.2	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0
20 mg/kg	5	1.8 ± 1.8	0.7 ± 0.4	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.0
30 mg/kg	5	2.4 ± 1.6	1.1 ± 1.0	0.1 ± 0.2	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.0
40 mg/kg	5	1.1 ± 0.9	1.1 ± 1.3	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.0
Week 1							
Control	5	1.3 ± 0.8	1.3 ± 1.3	0.0 ± 0.0	0.3 ± 0.4	0.0 ± 0.0	0.1 ± 0.2
20 mg/kg	5	1.2 ± 0.6	0.4 ± 0.2	0.0 ± 0.0	0.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
30 mg/kg	5	2.0 ± 1.2	0.8 ± 0.8	0.1 ± 0.2	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.0
40 mg/kg	5	1.9 ± 1.5	0.3 ± 0.3	0.2 ± 0.3	0.3 ± 0.3	0.1 ± 0.2	0.0 ± 0.0
Week 2							
Control	5	1.9 ± 0.8	0.8 ± 0.4	0.2 ± 0.4	0.2 ± 0.3	0.1 ± 0.2	0.0 ± 0.0
20 mg/kg	5	2.7 ± 2.2	0.4 ± 0.5	0.3 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
30 mg/kg	5	1.8 ± 1.0	0.1 ± 0.2	0.3 ± 0.4	0.3 ± 0.4	0.2 ± 0.3	0.0 ± 0.0
40 mg/kg	5	2.9 ± 2.2	0.8 ± 1.0	0.0 ± 0.0	0.5 ± 0.6	0.4 ± 0.7	0.0 ± 0.0
Week 3							
Control	5	1.2 ± 0.9	0.4 ± 0.7	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
20 mg/kg	5	2.3 ± 1.0	0.5 ± 0.5	0.3 ± 0.7	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
30 mg/kg	5	3.0 ± 1.3	1.7 ± 1.6	0.1 ± 0.2	0.5 ± 0.4	0.2 ± 0.3	0.0 ± 0.0
40 mg/kg	5	6.2 ± 2.9 **	2.1 ± 1.1	0.2 ± 0.3	0.2 ± 0.3	0.1 ± 0.2	0.0 ± 0.0
Week 4							
Control	5	1.2 ± 1.0	0.3 ± 0.3	0.0 ± 0.0	0.1 ± 0.2	0.2 ± 0.3	0.0 ± 0.0
20 mg/kg	5	1.8 ± 1.1	0.6 ± 0.7	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
30 mg/kg	5	6.0 ± 2.2 *	3.9 ± 3.2 *	0.3 ± 0.3	1.1 ± 0.4 *	0.3 ± 0.4	0.0 ± 0.0
40 mg/kg	5	17.1 ± 28.3 *	5.8 ± 3.8 **	2.5 ± 4.8	1.4 ± 2.1	0.8 ± 1.2	0.0 ± 0.0
Week 5							
Control	5	1.6 ± 1.9	0.7 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	0.0 ± 0.0
20 mg/kg	5	3.7 ± 2.9	1.0 ± 0.6	0.2 ± 0.3	0.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
30 mg/kg	5	8.1 ± 8.9	4.5 ± 4.7 *	0.1 ± 0.2	1.6 ± 1.6	1.9 ± 1.6	0.0 ± 0.0
40 mg/kg	5	9.1 ± 6.7 *	6.7 ± 3.9 *	0.1 ± 0.2	1.5 ± 2.5	1.3 ± 1.5	0.0 ± 0.0

Data shown as mean±S.D.

*: Significant difference from control ($p<0.05$).

**: Significant difference from control ($p<0.01$).

Time-course characterization of MMS-induced male reproductive toxicity in rats.

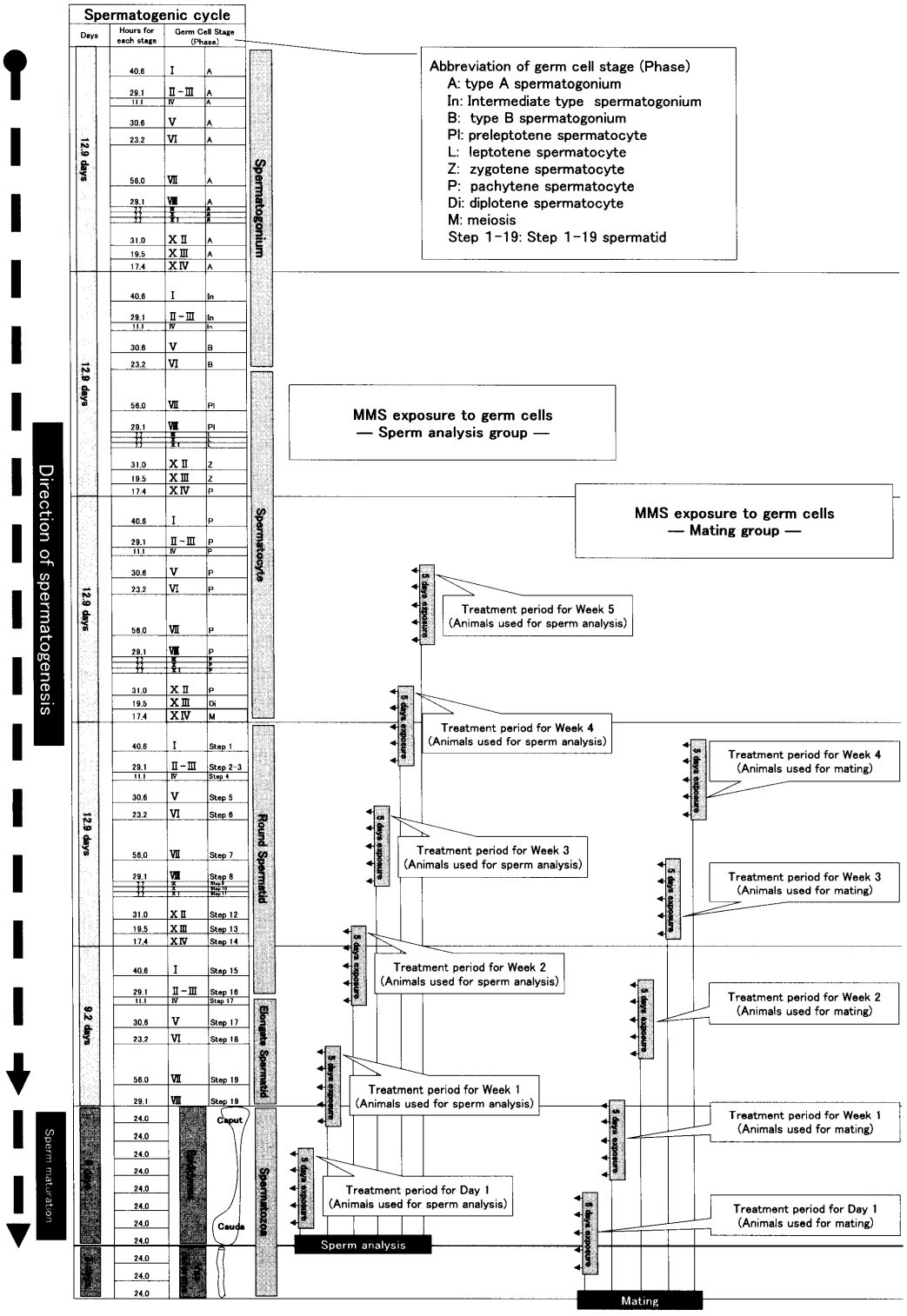

Fig. 2. Time-course of exposure.

MMS to spermatogenic cells in this study. In this figure, total transit time of spermatozoa from the initial caput segment to vas deferens was set at 11 days. Breakdown of the time is as follows: 8 days for the transit of epididymal spermatozoa from the initial caput segment to the caudal epididymis in rats (Sujarit and Pholpramool, 1985), and 3 days for caudal spermatozoa to move into the vas deferens (adopted from the data in mice, Sega and Owens, 1978).

In the present study, increase in dominant lethals was observed from Day 1 to Week 3. In the chronological observation of the dominant lethals, the most severe effects were observed in Week 2, in which none of the males succeeded in impregnating females at 30 and 40 mg/kg. Slight recoveries from these effects were observed beginning in Week 3. There were no effects detectable in Week 4. From these chronological changes, taking the 5-day treatment period and each part of the recovery period into account, the most sensitive cellular stages for the induction of dominant lethals were judged to be late spermatids (Week 2), followed by spermatozoa in the epididymis (Week 1). Early spermatid stage germ cells (Week 4) were judged to be insensitive to the induction of dominant lethals. Ashby *et al.* (1996) have indicated in their time-course study that the strongest dominant lethal induction was observed between 15 and 21 days after a single administration of MMS in rats. From the result of their study, the most sensitive germ cell stage for the induction of dominant lethals was judged to be late spermatids when the total transit time in epididymes and vas deferens was set as 11 days. This consistency in the most sensitive cellular stages in both Ashby's study and the present study assures the cellular targets of MMS for dominant lethal induction. It is widely known that dominant lethals are caused by chromosomal damage which occurred in the gamete. Matsuda *et al.* (1989) demonstrated induction of chromosomal aberrations in fertilized eggs in mice after treatment of males with MMS. Namely, they have indicated high frequency of chromosomal aberrations in fertilized eggs when the inseminated spermatozoa had been exposed to MMS in the stages from mid spermatid to early spermatozoa. On the other hand, they hardly observed the chromosomal aberrations when the inseminated spermatozoa had been exposed to MMS in the stages from late spermatocyte to very early spermatid. In the present study, the most sensitive cellular stages for the induction of dominant lethals were judged to be late spermatids, followed by spermatozoa in the epididymis. The early spermatids were judged to be insensitive to the dominant lethals. These agreements on the cellular sensitivity for the induction of chromosomal aberrations in mice and the induction of dominant lethals in our study suggest the possible involvement of chromosomal aberrations as the main cause of the dominant lethals after MMS treatment. Failed impregnations observed at 30 and 40 mg/kg in Week 2 might be interpreted as the result of early embryonic eliminations before or at the early stage of implantation due to possible chromosomal aberrations which occurred in the fertilized eggs.

None of the sperm examination parameters, such as the number of sperm in the cauda epididymis, sperm motility and sperm morphology, was successful in showing the relationship between sperm examination parameters and dominant lethals in Week 2, in which the effect on dominant lethals was the most severe. Studies with alkylating agents, such as ethyl methanesulphonate (Takagi *et al.*, 2000) and cyclophosphamide (Higuchi *et al.*, 2001), have also failed to detect the relationship between dominant lethals and sperm examinations. These negative consistencies among the studies with alkylating agents indicate that it may be difficult to detect sperm conditions that are potentially dominant lethal. Thus, it can be suggested that employing a fertility study is important for the profiling of comprehensive toxicity for this class of compounds such as alkylating agents.

In the present study, there were dose-dependent and chronological changes in the sperm morphological examinations. Namely, frequencies of morphologically abnormal spermatozoa derived from cauda epididymis increased beginning in Week 3, peaked in Week 4, and somewhat recovered in Week 5. Morphologically abnormal spermatozoa in caput epididymis started to increase in Week 2, peaked in Week 3, and recovered in Week 4 and later at 40 mg/kg. There was a one-week lag in the chronological peak in the frequency of morphologically abnormal spermatozoa between caput and cauda epididymis. This phenomenon seems in agreement with the report of Sujarit and Pholpramool (1985), in which they have indicated 8 days for the transportation of spermatozoa from caput to cauda epididymis. In this sense, decreased morphologically abnormal spermatozoa in caput epididymis in Week 4 suggests the likelihood of recovery in the frequency of morphologically abnormal spermatozoa in cauda epididymis in Week 5 and later. Taking the duration of both treatment and recovery period into account, the most sensitive spermatogenic cell stages for the induction of morphologically abnormal spermatozoa were judged

to be early spermatid, followed by mid spermatid. One of the causes of the induction of morphologically abnormal spermatozoa is alteration in testicular DNA that in turn disrupts the differentiation of spermatozoa (Bruce and Heddle, 1979). Inoue *et al.* (1993) reported that UDS (unscheduled DNA synthesis, an indirect measure of DNA lesions) after MMS treatment takes place in the germ cell stages from leptotene to mid spermatid in mice. They also suggested that lesions in sperm DNA increase in severity throughout the movement and maturation in the reproductive tract. In this connection, the cause of the morphologically abnormal sperm in the present study is suggested to be initiated from the possible DNA lesions that occurred in the spermatocyte stage, and it consequently inhibited the spermiation process and caused the development of morphologically abnormal sperm after a certain period of time. In the recovery process in the frequency of abnormal spermatozoa in the caput epididymis in Week 4 and later, and in the caudal epididymis in Week 5, intervention of the particular deleting function for genetically damaged cells during meiosis might be involved. Further investigations are required.

In the present study, tailless sperm was the most frequently observed morphological abnormality in caudal epididymis. There are several compounds that have already been identified as cellular targets for the induction of sperm morphological abnormalities, such as ethylene glycol monomethyl ether (EGME) for spermatocytes (Anderson *et al.*, 1987); boric acid for mid spermatid (Linder *et al.*, 1990); dibromo acetic acid (DBAA) for late and/or elongating spermatid (Linder *et al.*, 1994). Although the terminology employed in each study was different, substantially the same finding of "tailless sperm" has been consistently observed as a major finding among the studies, such as EGME, separated heads (Anderson *et al.*, 1987); boric acid, decapitation and no head (Linder *et al.*, 1990; Yoshizaki *et al.*, 1999); DBAA, isolated heads (Tsuchiya *et al.*, 2000). Since the tailless sperm have been detected in the sperm morphological examination regardless of the cellular target, it can be suggested that the tailless sperm counting is a robust measure in the sperm morphological examination for the detection of spermatotoxicity.

In summary, in the present study, we have successfully characterized the morphological alteration of epididymal spermatozoa together with dominant lethals in a time-course manner after the oral treatment of rats with MMS. The most frequently observed type of morphologically abnormal spermatozoa was tailless sperm, followed by no-hook head sperm. The most sensitive cellular stages for the induction of sperm morphological abnormalities were judged to be late spermatocytes and early spermatids, and for the dominant lethals these were judged to be late spermatids. Although the initial cause for both sperm morphological alterations and dominant lethals was suggested to be genetic insult to the germ cells, there were no obvious relationships observed between these two findings.

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