THE ANTISPERMATOGENIC EFFECT AND TOXICITY OF 2, 4-DINITRO-6-*tert*-BUTHYLPHENYL METHANESULFONATE ON SPRAGUE-DAWLEY RAT

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Accepted May 21, 1984

Abstract.....The antispermatogenic effects of 2, 4-dinitro-6-*tert*-butylphenyl methanesulfonate (HE-166) were studied in Sprague-Dawley rats. They were fed 25, 100 and 400 ppm of HE-166 in the basal diet for one year. Laboratory data showed no significant changes except for increases in γ -globulin, alkaline phosphatase, GOT and GPT values in the 100 ppm group. Macroscopically, significant changes were found in the testes in the experimental group, which showed marked atrophy. Histologically, the testes were filled with fibrin exudate in the stroma and there was reduced spermatogonia, cellular debris and giant cells, and even calcification, depending on the dose of HE-166. The anti-fertility effects of HE-166 were also observed by mating rats and checking the pregnancy rate during three generations. These effects might be due to the direct cytotoxic effect of HE-166 on post-meiotic cells as epididymal spermatozoa and testicular sperm and spermatids. As far as tumor incidence was concerned, one case of fibroadenoma of the mammary gland, one case of leiomyosarcoma in the uterus in the 100 ppm group and one case of leiomyoma in the uterus in the 25 ppm group developed at around 8 months, but no other tumors developed.

Key words: 2, 4-dinitro-6-*tert*-butylphenyl methanesulfonate, anti-spermatogenicity, chronic toxicity, Sprague-Dawley rats.

INTRODUCTION

2, 4-dinitro-6-*tert*-butylphenyl methanesulfonate (HE-166) is a herbicide belonging to the alkanesulphonic esters and has an inhibitory effect on the germination of plant seeds. Its direct influence on leaves is slight, resulting in burning.

On the other hand, antispermatogenic and carcinogenic effects of alkylating agents such as alkanesulphonic esters (butenyl-2-enel, 4-dimethanesulfonate and allylmethane

sulfonate), nitrogen mustard and ethyleneimine derivatives have been reported (Roe, 1957; Jackson, 1966, 1970; Cooper, 1969). The antifertility effects may be due to 1) inhibition of stem-cells or spermatogonial development or 2) functional sterility as a manifestation of ability to produce genetic damage in developing spermatids or spermatozoa.

As HE-166 belongs to the alkanesulphonic esters, its antispermatogenic function and toxicity were investigated.

MATERIALS AND METHODS

Animals : One hundred sixty male and 160 female Sprague-Dawley rats of 3-4 weeks of age were purchased from Fuji Animal Co. Ltd. Three females or 2 males were housed together in polycarbonate cage measuring 40×25×17 cm in an air-conditioned animal room at Nara Medical College at 23±1°C. Drinking water was given *ad libitum*

Diet : HE-166 was provided by Kanesho Co. Ltd. It is a light yellow, rod-like, crystalline powder and easily soluble in organic solvents such as benzene or acetone, but insoluble in water (Fig. 1). It was mixed with powdered NMF diet (Oriental Co., Ltd., Tokyo) in a V-type mixer (Kotobuki, Mixwell, V-15 type).

Fig. 1 Chemical composition of 2, 4-dinitro-6-*tert*-butylphenyl methansulfonate (HE-166)

Dose : Intubation was made in 40 female and 40 male rats to determine the LD₅₀. A range-finding test was performed to determine the expected maximum-tolerated dose (around 180 ppm), from which the doses of HE-166 were decided as follows : 400 ppm, 100 ppm, 25 ppm and control. The highest toxic dose (400 ppm) was examined particularly closely in this experiment, in order to study the pathological changes in the genital organs.

Behavior and histopathological examination: General condition and behavior were observed every day. Body weight and food intake were examined twice a week. At the end of the one-year experiment, all rats were necropsied. The blood from the abdominal aorta was tested at the Kinki Laboratory Test Center. The organs were weighed, fixed in 10% formalin and embedded in paraffin for sectioning and hematoxyline-eosin staining. All data were analyzed statistically by the Student t-test.

Antifertility effects of HE-166: Pregnancy rate, litter size and breeding interval were observed to study the functional anti-fertility effects of HE-166 on rats for three generations. All doses of HE-166 (400, 100, 25 ppm) and the control were fed throughout the experiment. Eight males and 16 females were mated 100 days after the start of

the experiment to get the first litters (Fla). Ten days after the Fla litters were weaned, remating was performed among the same parents to get the second litters (Flb). Among 8 males and 16 females of 3 weeks of age segregated from Flb litters, mating was first performed 100 days after their weaning in order to get the first 2nd generation litters (F2a). Ten days after the F2a litters were weaned, remating was performed among the Flb to get the second litters (F2b). In this way, the first and second 3rd generation litters (F3a and F3b) were obtained (FAD, 1959).

RESULTS

1. Survival rate and general behavior

The survival rate, as shown in Fig. 2, was high in the lower dose groups, but all animals of both sexes in the 400 ppm group died within 18 weeks of commencement of feeding. The mortality rate was higher in males in each group than in females. Toxic manifestations were marked in the 400 ppm group, roughness and yellowish changes of the coat appearing within two weeks of the test being started.

Fig. 2 Survival rate of S-D rat fed HE-166

2. Body weight gain (Fig. 3)

Body weight declined in all experimental groups except the 25 ppm group. In particular, rats of both sexes in the 400 ppm group lost significant weight shortly after the experiment started.

3. Food consumption

Average daily food consumption was 20.9 g in the 400 ppm group, 26.2 g in the 100 ppm, 24.7 g in the 25 ppm and 27.2 g in the control. The actual intake of HE-166 was 8.62 mg, 2.62 mg and 0.61 mg respectively.

4. Laboratory tests

The results of laboratory tests are summarized in Table 1. In general, there was no significant difference between the experimental and control groups. But, the titers of

Fig. 3 Growth rate of S-D rat fed HE-166

γ -globulin, alkaline phosphatase, GOT and GPT in the male 100 ppm group increased compared with those of the 25 ppm and control groups.

5. Gross pathological findings

As shown in Table 2, significant changes were found in the testes of the 100 ppm group. In particular, the testes of the 400 ppm group atrophied at 1 month after the experiment started (Photo 1). The organ weight of the lung increased in the male 100 ppm group, probably due to lung abscess.

6. Histopathological findings

As shown in Tables 3 and 4, the affected organs in the experimental groups were the male genital organs, particularly the testes. The seminiferous tubules were affected more severely than the Leydig cells in the higher dose groups. Spermatids decreased or disappeared and the spermatozoa showed giant cells and calcifications (Photos 2, 3). In the 400 ppm group, fibrin exudates were evident in the stroma (Photo 4). In the ovaries of the high dose group, primary follicles disappeared and corpora lutea degenerated (Photo 5). There was hypersecretion of liquid in the follicles, associated with the increase in the granular cell layer. Prostates showed exfoliation of the epithelial cells. Uteri were infiltrated by round cells, and squamous cell metaplasia was found in 4 females in the 25 ppm group. In the liver, pseudo-bile ducts proliferated and round cells infiltrated the Glisson's sheath in 1 male in the 100 ppm group and 2 females in the 25 ppm group. Abscess and pneumonia were seen in 5 males and 2 females in the experimental groups. Round cell infiltration was also noted in the interstitial tissue of the kidney of males in the experimental and control groups, but not in the females in the control group. Protein casts were occasionally seen in the renal tubules of both sexes, including controls. Infiltration of lymphocytes into the pancreas was also seen in females in the lower dose groups. Some adrenals evidenced vacuole formation in the zona glomerulosa. Slight congestion of the pituitary gland was observed, but there were no other remarkable findings.

Table 1 Laboratory Tests of S-D Rats fed HE-166 for One Year

Dose	No. of	Tot.	Albumin	Globulin			Glucose	Kunk	ALK	GOT	GPT	Tot.	LDH	RBC	Hb	Ht	WBC	
ppm	Rats	Prot.	%	α_1	α_2	β	γ	pho.	(unit)	(unit)	(unit)	mg/dl	(unit)	$\times 10^4/\text{cmm}$	Sahl	g/dl	cmm	
100	6	7.1 ± 0.5	40.3 ± 7.5	15.7 ± 3.9	8.6 ± 2.0	24.1 ± 6.3	11.3* ± 3.3	125.7 ± 30.7	4.0* ± 2.8	54.3* ± 11.0	498.7* ± 69.7	97.7* ± 37.6	94.6 ± 1.4	1950 < ± 39.9	103.8 ± 4.3	16.5 ± 0.6	48.0 ± 5.7	10266.7 ± 3512.2
25	13	7.5 ± 0.4	40.5 ± 8.0	23.7 ± 6.2	14.3 ± 6.9	21.2 ± 3.7	10.3* ± 8.2	126.6 ± 4.5	1.8 ± 0.7	29.6 ± 5.6	266.1 ± 102.3	84.6* ± 10.3	135.2 ± 15.4	1950 < ± 25.7	107.7 ± 4.4	17.2 ± 0.7	49.7 ± 3.3	12200.2 ± 2570.3
Cont.	10	6.4 ± 0.4	46.7 ± 3.0	18.0 ± 3.9	15.2 ± 7.7	22.4 ± 2.6	6.5 ± 3.6	127.7 ± 17.4	1.7 ± 1.4	34.3 ± 13.4	225.7 ± 81.8	51.0 ± 11.6	87.7 ± 11.2	1950 < ± 72.5	97.5 ± 4.2	15.6 ± 0.7	46.5 ± 1.6	13600.0 ± 4755.7
100	12	7.9 ± 0.3	53.0 ± 1.2	12.9 ± 2.0	9.6 ± 3.6	16.9 ± 1.5	7.4 ± 1.4	127.5 ± 31.1	1.0 ± 0.2	67.3* ± 32.7	240.1 ± 101.9	78.7 ± 18.6	107.2 ± 10.9	1950 < ± 72.4	91.3 ± 9.1	14.6 ± 1.4	44.2 ± 4.7	8916.7 ± 1610.8
25	15	8.1 ± 0.5	46.7 ± 9.6	16.1 ± 4.8	12.1 ± 5.8	16.9 ± 4.8	8.5 ± 2.5	115.0 ± 19.3	2.0 ± 1.1	37.4 ± 17.7	325.7** ± 130.3	77.5 ± 39.1	113.3 ± 16.5	1950 < ± 70.9	94.8 ± 10.2	15.1 ± 1.6	41.1 ± 7.0	12733.3 ± 7355.5
Cont.	15	7.4 ± 0.6	50.7 ± 5.7	17.9 ± 2.7	11.1 ± 4.7	16.9 ± 1.4	3.4 ± 1.1	98.4 ± 10.2	1.8 ± 0.2	24.0 ± 9.2	255.0 ± 97.6	68.8 ± 28.4	99.8 ± 11.7	1950 < ± 79.1	89.2 ± 6.9	14.3 ± 1.0	39.6 ± 2.1	8508.0 ± 2282.4

* Significant different from control ($p < 0.01$)** Significant different from control ($p < 0.05$)Values expressed mean $\pm$ SD

Table 2 Average Organ Weight of S-D Rats fed HE-166 for One Year

Dose ppm	No. of Rats	Final B. W. g	Heart mg	Lung mg	Liver mg	Spleen mg	Panc. mg	Adrenal		Kidney		Thymus mg	Brain mg	Pit. mg	Test./Ovary		Uterus mg
								R	L	R	L				R	L	
Male	100	462 ±19	1448 ±287	7120 ±921	16651 ±943	502 ±39	851 ±313	30 ±4	22 ±4	1919 ±102	1823 ±85	172 ±0	1691 ±221	9 ±5	1163* ±120	1195* ±55	—
	25	625 ±25	1594 ±201	3826 ±984	19265 ±3759	709 ±140	827 ±100	34 ±8	24 ±9	1718 ±110	2066 ±120	186 ±60	1638 ±309	9 ±2	1730 ±57	1686 ±78	—
	Cont.	562 ±84	1431 ±272	2651 ±899	15766 ±3190	690 ±133	665 ±157	32 ±11	21 ±4	1812 ±58	1825 ±89	227 ±103	1689 ±142	12 ±5	1870 ±125	1802 ±135	—
Female	100	314 ±22	1129 ±111	2178 ±145	12938 ±1593	683 ±161	735 ±191	44 ±13	42 ±12	1179 ±113	1171 ±125	148 ±79	1732 ±174	7 ±2	62 ±12	65 ±10	1027 ±377
	25	374 ±48	1183 ±79	2429 ±721	14288 ±6788	573 ±94	635 ±195	40 ±15	38 ±11	1180 ±58	1176 ±85	253 ±86	1732 ±77	13 ±4	69 ±11	87 ±14	938 ±182
	Cont.	340 ±20	1241 ±145	2123 ±479	12061 ±2749	566 ±96	710 ±155	40 ±10	40 ±10	1080 ±120	1073 ±75	328 ±43	1661 ±159	8 ±1	81 ±10	89 ±16	1088 ±315

* Significant different from control ($p < 0.01$)
Values expressed mean $\pm$ SD

Antinpermatogenic effect of HE-166 in rats

Table 3 Histopathological Findings of S-D Rats fed HE-166 for One Year

Organ	Pathological Change HE-166 in Diet (ppm)	Male				Female			
		400	100	25	Cont.	400	100	25	Cont.
Brain		—	—	—	—	—	—	—	—
Liver	Degeneration of hepatic cell	—	—	—	2	—	—	1	1
	Round cell infiltration	—	1	—	—	—	—	2	—
	Hyperplasia of cholangial duct	4	4	2	—	2	2	4	—
	Congestion	1	—	—	—	—	—	—	—
Heart	Degeneration	—	—	1	2	1	—	2	—
	Granulation tissue	—	—	2	—	1	1	1	—
Spleen	Hemosiderosis	1	2	2	2	5	3	5	1
	Follicular hyperplasia	—	1	—	—	1	1	—	1
Lung	Abscess	1	3	1	—	—	—	2	—
	Cell infiltration	1	3	3	1	3	—	3	—
	Bronchitis	—	1	—	1	—	3	2	—
	Bronchiectasis	3	1	2	—	1	3	4	1
	Lymphoid hyperplasia	2	1	4	1	2	6	4	1
Kidney	Cellularity of glomerulus	—	1	—	1	—	—	1	—
	Interstitial nephritis	—	1	2	1	2	—	4	—
	Degeneration of tubules	—	2	1	2	—	2	1	1
	Protein cast	—	1	2	1	—	1	1	—
Pancreas	Inflammation	—	—	—	—	—	1	4	1
	Enlargement of islets	1	—	2	—	—	—	1	1
Adrenal	Congestion	—	—	1	—	1	1	—	—
	Vacuolization	—	1	—	—	2	2	1	2
	Infiltration	—	—	—	—	—	—	1	—
Thyroid	Hyperkeratosis of follicular cells	—	—	1	—	1	1	1	—
Uterus	Distension of uterine glands	—	—	—	—	1	3	3	2
	Hyperplasia of columnal cells	—	—	—	—	1	1	1	2
	Squamous metaplasia	—	—	—	—	—	—	5	—
	Cell infiltration	—	—	—	—	1	1	4	1
Prostate	Exfoliation of epithelial cells	—	1	1	—	—	—	—	—
	Cell infiltration	—	—	—	—	—	—	—	—
Tumor		—	—	—	—	—	2	1	—
No. of rats examined		5	6	6	6	5	8	10	6

Table 4 Histopathological Findings of Reproductive Organ of S-D Rats fed HE-166 for One Year and Sacrificed at Interval

Dose (ppm)	100	25	Cont.	400*
Number of Male Rats Period	6 1 yr.	6 1 yr.	6 1 yr.	5 4 M
Fibrin exudation	2	2	0	5
Increase of Leydig cells	0	0	0	2
Edema	1	1	0	1
Degeneration	1	1	1	5
Giant cell formation	0	0	0	2
Calcification	0	0	0	3
Decrease of spermatogonia	2	1	0	5
Reduction of spermatogenic layer	4	2	0	5
Increase of Sertoli cells	2	2	0	1
Exfoliation of cells	3	0	1	5
Aspermatogenesis	2	0	0	5
Number of Female Rats	8	10	6	5
Stromal change	0	2	0	0
Abnormal lutein	2	2	0	3
Decrease of corpora Lutea	3	2	0	2
Growth of granulosa cells	2	1	1	4
Cyst formation	2	2	1	1
Loss of primary follicle	1	0	0	2

* Rats sacrificed or died at interval

Table 5 Reproduction of S-D Rats fed HE-166

HE-166	Generation	No. of Female	1st Litter		2nd Litter	
			Pregnancy rate	Litter size	Pregnancy rate	Litter size .
400 ppm	I	7	0%	0	0%	0
100 ppm	I	8	50%	9.3±1.5†	0%	0
25 ppm	I	11	46%	5.6±2.3	11%	11
	II	8	13%	1.0	0%	0
Control	I	11	64%	9.0±3.9	0%	8.8±3.5
	II	14	57%	9.9±3.7	50%	8.2±2.1
	III	2	100%	9.0±1.0	100%	8.5±3.5

† : Values expressed mean±SD

Antispermatic effect of HE-166 in rats

Photo 1 Gross finding of atrophied testes in the 400 ppm group sacrificed at 1 month after the experiment started.

Photo 2 Histology of degenerated testis in a male rat of the 400 ppm group. H-E stain $\times 40$.

Photo 3 Histology of giant cells of testis in a male rat of the 400 ppm group. H-E stain $\times$ 150.

Photo 4 Histology of degenerated testis with calcification and fibrin exudate in a male rat of the 25 ppm group. H-E stain $\times$ 150.

Photo 5 Histology of degenerated follicle of ovary in a female rat of the 400 ppm group. H-E stain $\times 150$.

Photo 6 Leiomyosarcoma in the uterus in a female rat of the 100 ppm group. H-E stain $\times 150$.

7. Tumor incidence

One case of the fibroadenoma of mammary gland and one case of leiomyosarcoma in the uterus in the 100 ppm group (Photo 6) and one case of leiomyoma in the uterus in the 25 ppm group developed at around 8 months. However, no other tumors developed among females in the dosage groups, and none at all developed among males in the dosage group or either sex in the control group, throughout the duration of the experiment.

The antifertility effects of HE-166 :

Table 5 shows the pregnancy rate and average litter sizes for three generations. The pregnancy rate in the 100 ppm and 400 ppm groups was nil for F1b rats. The pregnancy rate in the 25 ppm group was 11% for F1b, but 0% for F2b, while that in the control group was 55%, 50% and 100% for F1b, F2b and F3b rats respectively. In this experiment, no F3b rats were obtained in the experimental groups and it was impossible to perform comparative necropsies.

The day of the first parturition was delayed more in the experimental groups than in the control.

DISCUSSION

Many kinds of chemicals are known to have antispermatic effects on many species (Jackson, 1970 ; Patanelli, 1978). The discovery of the sterlant action of the alkylating chemical TEM by Bock et al. (1957) prompted a search for effective male sterilants for mammals and insects. The single monoesters of methanesulfonate also produced temporary sterility in rodents. Among them, methylmethanesulfonate (MMS) resulted in involving all post-meiotic cells as spermatids and spermatozoa with small doses. Methylene dimethanesulfonate (MDS) also produced rapidly transient sterility, and Fox et al. (1965) observed the direct effect of MDS on mature spermatozoa in the distal part of the epididymis. Ethylene dimethanesulfonate (EDS), which is an ester of ethylene glycol and methanesulfonic acid, is less toxic than the other ester groups. According to Jackson (1966), administration of a single dose of 50 mg/kg of EDS produced sterility and a single dose of 75 mg/kg by intraperitoneal injection led to the disappearance of mature sperm from the seminiferous tubules, with giant cell formation and reduction of spermatocytes. Cooper et al. (1969) also reported marked loss in weight of prostate and seminal vesicles by a single injection of EDS. It was suggested that the diesters of methanesulfonic acid provided sterlant effects by direct cytotoxic actions to the spermatogonia. HE-166 seems to have marked direct cytotoxic effects on the testes as shown in the other alkanesulfonic esters. Feeding of 400 ppm, even 100 ppm, caused a marked loss in testis weight. Histology revealed aspermatogenesis, giant cells and calcification in the seminiferous tubules with fibrin exudate in the stroma. These pathological changes in the testis correlated well with the dose of HE-166 and sterlant action over three generations. As shown in the results, the pregnancy rate decreased when the rats were fed the higher dose of HE-166. There

was about three weeks delay in reproduction in the experimental group compared with that of the control. Unless the HE-166 affected sexual activity which was noted in other methanesulfonate (Jackson, 1970), the effects of HE-166 on the testes were supposed to be direct cytotoxic effects on the postmeiotic cells as epididymal spermatozoa and testicular sperm and spermatids, which was also seen in testis exposed to EMS or MMS (Jackson, 1970., Takahashi., 1973). On the other hand, effects of androgen secretion by Leydig cells are said to derive from a feedback mechanism between the hypothalamus and the genital organs, which influenced meiotic cells and consequently, suppressed pituitary gonadotropin leading to antispermatic effects (Johnson, 1964). In the male 400 ppm group, Leydig cells in the testes increased. From these results, the antifertility effects of HE-166 may be influenced by the gonadotropin. It is reported that increased basophile cells are seen in the pituitary gland due to the hyperactivity of these cells by way of gonadotropin. In this case, however, histology showed no increase of basophile cells in the pituitary gland. Therefore, the antifertility effects of HE-166 may not be due to the feedback mechanism.

In addition to the direct cytotoxic effect on the testis, there are possibilities of toxic damage to the ovary by HE-166, because 2 out of 5 rats of the 400 ppm lost the primary follicles at around 4 months histologically. But, few rats were used here and further study is needed in this respect.

In regard to the toxicity of HE-166, the titer of γ -globulin rose slightly and liver functional tests revealed an increase in titers of ALP, GOT and GPT in the 100 ppm group. However, most laboratory values remained within the normal range. It was reported that alkylating agents such as triethylenmetamine, busulphan and isopropyl methanesulfonate induced marked depression of white cell counts due to the bone marrow aplasia (Jackson et al., 1961). In this experiment, however, red cell and white cell counts remained within the normal range and no marked bone marrow aplasia was found histologically. In the liver, the only histological change was a proliferation of bile ducts. Cullberg et al. (1965) reported a large increase in ALP value even in untreated aged rats. Therefore, it is unlikely that liver functional damage was caused by HE-166. At the highest dose both sexes lost weight significantly shortly after the beginning of the experiment. Body weight loss was reported from long-term administration of alkanesulphonic esters such as busulphan (Jackson et al. 1961). Histologically, lung abscess was found in the experimental groups. Kimbroth (1966) also described how daily treatment (100 mg/kg orally) of an alkylating agent such as hexamethyl phosphoramide produced multiple lung abscesses in rats. But laboratory animals were often apt to be infected and abscess incidence in this experiment was low. No marked changes in other organs were observed. From these results, the toxicity of HE-166 was judged to be not so high. In particular, the rats of the 100 ppm and 400 ppm groups died during the experiment, but in the 25 ppm groups they showed no pathological or biochemical changes. Therefore, the maximum-no-effect level of HE-166 should be around 25 ppm.

As far as tumor incidence is concerned, one case of mammary fibroadenoma and

leiomyosarcoma in the uterus developed in the 100 ppm and one case of leiomyosarcoma in the uterus in the 25 ppm group at 8 months. A variety of alkylating agents such as nitrogen mustards and ethyleneamine derivatives were reported to be carcinogenic (Alexander, 1952). In regard sulphonic esters, Roe et al. (1957) observed a high incidence of papilloma in the skin of mice induced by 1, 4-dimethanesulfonyl-2 butane with croton oil and also the development of malignant tumors on administration of 1, 4-dimethanesulfonyl-2-butene. EMS has been shown to increase the incidences of lung adenoma, renal adenoma and thymoma in RF mice given drinking water containing 30 mg/kg of MMS over their full life. The possibility of carcinogenesis of HE-166 can not be ruled out. But, it is well known that many kinds of spontaneous tumors occurred in Sprague-Dawley and Wistar rats (Tsubura et al., 1980.). At the same time, the period of experiment was one year and a life-span experiment needs to be done to clarify the carcinogenesis of HE-166.

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